Brome Mosaic Virus RNA Replication Proteins 1a and 2a Form a Complex In Vitro

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Brome mosaic virus (BMV) is a positive-strand RNA virus that encodes two RNA replication proteins, the helicase-like 1a and the polymerase-like 2a. 1a and 2a share extensive sequence similarities with proteins encoded by many other members of the alphavirus-like superfamily. While further purifying enzymatically active RNA-dependent RNA polymerase from plants infected by BMV, we observed that 1a, 2a, and the polymerase activity all cofractionated through multiple independent purification steps. Moreover, using immunoprecipitation, we found that BMV 1a and 2a proteins synthesized in rabbit reticulocyte lysates or insect cells can form a specific complex in vitro. Complex formation was more efficient when 1a and 2a were cotranslated than when they were mixed after independent synthesis. In an antibody-independent assay, in vitro-translated 1a protein was also found to bind to 2a protein fixed on a nylon membrane. A three-amino-acid insertion in 1a that blocks BMV RNA replication in vivo also blocked in vitro interaction with 2a, while another two-amino-acid insertion that renders the 1a protein temperature sensitive for RNA replication interacted in vitro with 2a at 24°C but not at 32°C. These results and previous genetic data suggest that the 1a-2a interaction observed in vitro is required for BMV RNA replication and may have direct implications for other members of the alphavirus-like superfamily.

Viruses with single-stranded, messenger-sense RNA genomes are a taxonomically diverse group that include important pathogens of both animals and plants. These viruses can be broadly categorized into two or possibly three superfamilies, the picornavirus-like, alphavirus-like, and carmovirus-like viruses, based on similarities in replication strategy and the amino acid sequences of proteins required for RNA replication (for reviews, see references 15, 24, and 38).

Brome mosaic virus (BMV), which infects grasses of the family Graminae, has been used as a model system for studies of RNA replication and gene expression in the alphavirus-like viruses (for reviews, see references 1 and 28). The BMV genome is composed of three RNA species of 3.2 kb (RNA1), 2.9 kb (RNA2), and 2.1 kb (RNA3) that are packaged into separate virions. RNA-dependent transcription from an intercistronic promoter within the negative strand of RNA3 results in subgenomic RNA4, which encodes the 23-kDa capsid protein (12, 30). RNA3 also encodes the 32-kDa 3a protein, which is required for BMV spread from primary infection sites (4, 8). RNAs 1 and 2 are monocistronic and, respectively, encode the 1a (109-kDa) and 2a (94-kDa) polypeptides.

RNA1 and RNA2 encode *trans*-acting functions required for RNA replication and, in barley protoplasts, can replicate independently of RNA3 (13, 23). Consistent with these observations, the 1a and 2a proteins are both present in RNA-dependent RNA polymerase (RdRp) preparations from plants infected by BMV (19, 35) or the related cucumber mosaic virus (18).

The amino acid sequences of the 1a and 2a proteins also suggest several functions for viral RNA synthesis. 1a has homology to viral and cellular helicases (16, 26) and to the Sindbis virus nsP1 protein, which has methyltransferase and

possibly guanylyltransferase activities that may be involved in capping of viral RNAs (2, 31, 32, 37). Mutational analyses have shown that both the putative helicase and capping domains of 1a are required for in vivo RNA replication (26). The 2a protein contains a central portion that has sequence homology to viral RNA polymerases (5, 17, 21). This polymerase-like domain contains the Gly-Asp-Asp motif and other motifs found in picornavirus and bacteriophage Q β proteins required for elongation of initiated RNA (7, 36). Mutational analyses have shown that the polymerase-like domain is required for bromovirus RNA replication (25, 40).

Genetic and biochemical data suggest that some form of effective interaction between the 1a and 2a proteins is required for RNA replication (1). Allison et al. (3) found that in vivo replication of BMV RNA3 is supported by RNA1 and RNA2 from either BMV or the closely related bromovirus cowpea chlorotic mottle virus, but not by heterologous combinations of RNA1 and RNA2. Compatible function in such heterologous combinations can be partially restored by constructing suitable BMV-cowpea chlorotic mottle virus 2a protein hybrids (39). More recently, one of us has shown that antiserum to protein 1a can immunoprecipitate 1a, 2a, and approximately five host proteins from a BMV RdRp preparation (34), suggesting that at least some of the 1a and 2a proteins in such preparations are physically associated, either directly or through plant cell factors.

To gain further insight into BMV RNA replication and the RdRp complex, we biochemically investigated the possibility of interaction between the 1a and 2a proteins. In this report, we show that the BMV 1a and 2a proteins strictly cofractionate throughout purification of enzymatically active RdRp fractions. Moreover, using immunoprecipitation and probing membrane-bound polypeptides with labeled proteins (far Western assay), we find that wild-type (wt) 1a and 2a interact in vitro with specificity and in the absence of any

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plant cell factors. By contrast, a three-amino-acid 1a insertion mutation that blocks RNA replication in vivo also blocks interaction with 2a in vitro. Thus, the interaction of replication proteins 1a and 2a is a highly specific process that is likely to be a necessary step in BMV RNA replication.

MATERIALS AND METHODS

Plasmids. pB1TP3, pB2TP5, and pB3TP8, containing cDNA copies of RNAs 1, 2, and 3, respectively, were constructed by Janda et al. (20).

Purification of active RdRp. Total cellular membranes from BMV-infected barley plants were isolated as previously described (34). RdRp activity was solubilized by resuspending the membranes in buffer A (34) containing 0.75% Triton X-100 (18). After stirring for 30 min, the insoluble material was removed by centrifugation at $100,000 \times g$ for 30 min. The pellet, which contained no RdRp activity, was discarded, while the RdRp-containing supernatant was fractionated through a column of Sephacryl 400 resin (2.5-cm diameter by 35-cm length; Sigma). The fractions were assayed for RdRp activity as previously described (34). The active fractions were pooled and applied to a DEAE-Biogel A column (1.6-cm diameter by 6-cm length; Bio-Rad) and subsequently eluted with a gradient of 0 to 0.5 M KCl.

In vitro transcription and translation. In vitro transcription was performed with bacteriophage T7 RNA polymerase (New England Biolabs) as previously described (11) except that the cap analog was omitted. In vitro translation with rabbit reticulocyte lysates (Novagen) was performed in 30- μ l volumes using the manufacturer's protocol except that the incubation temperature was 28°C unless indicated otherwise. After synthesis, the translations were frozen in dry ice before storage at -70°C. Before use in immunoprecipitation, the reticulocyte lysate reaction was treated with 0.5 μ g of RNase A for 10 min at room temperature.

Antisera. Antisera against the BMV 1a, 2a, and 3a proteins were a generous gift from John Miglietta and Paul Kaesberg. These antisera had been generated against polypeptides produced in *Escherichia coli* CAG1699 (C600 *galK lon-100 rec-56 srl::Tn10*) (33). Briefly, a complete cDNA of each of these BMV genes was cloned behind the bacteriophage lambda PR promoter in a plasmid also carrying the temperature-sensitive lambda c1857 repressor gene. Expression of the BMV polypeptides was induced by growth of the recombinant *E. coli* at 42°C for 90 min to inactivate the temperature-sensitive lambda repressor. The expressed polypeptides were size fractionated on preparative sodium dodecyl sulfate (SDS)-polyacrylamide gels, and the appropriate bands were excised. After elution by diffusion, the polypeptides were emulsified with Freund's complete adjuvant and injected into female New Zealand White rabbits. The rabbits were boosted with antigen 3 weeks after the initial inoculation, and after 3 more weeks, antisera were collected.

Immunoprecipitation assays. All immunoprecipitation reactions were performed at least twice, and most were performed many more times. Representative autoradiographs are shown in the figures. The protocol previously published in Kao et al. (22) was modified to analyze the interaction between BMV proteins. Polypeptides translated in the reticulocyte extracts and labeled with [³⁵S]methionine (Amersham) were diluted in L buffer (50 mM Tris-Cl [pH 7.4], 50 mM KCl, 15 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol) and incubated on ice for 10 min. Protein A-expressing *Staphylococcus aureus* (Sigma) was then added to absorb molecules that nonspecifically bind to protein A. After

a 10-min incubation, the *S. aureus* cells were pelleted by centrifugation at $9,000 \times g$ for 3 min, and the supernatant was aliquoted to a tube containing antiserum (usually at 1% of total volume) and incubated at 4°C for 2 h. Then protein A-Sepharose (Sigma) was added to bind immunoglobulins, and the slurry was mixed continuously on a rocking platform for 3 h at 4°C. Afterward, the beads were pelleted by a 1-min centrifugation at $2,000 \times g$, and the supernatant was removed by aspiration. The Sepharose beads were washed with 0.5 ml of L buffer, then washed with a 0.5-ml solution of 0.5 M LiCl-20 mM Tris-Cl (pH 7.4), and finally washed again with L buffer. The immunoprecipitates were dissolved in sample buffer (62.5 mM Tris-Cl [pH 6.8], 2% SDS, 10% glycerol, 0.715 M β -mercaptoethanol, 0.04% bromophenol blue), heated at 95°C for 3 min, and electrophoresed in SDS-polyacrylamide gels.

Electrophoresis. SDS-polyacrylamide gels were made by the protocol of Laemmli (27) with a ratio of 30 to 0.8 of acrylamide to bisacrylamide. After electrophoresis, the gel was fixed in 20% methanol-20% acetic acid for 15 min, rehydrated in deionized water for 30 min, and soaked in 1 M sodium salicylate for 30 min before the gel was dried and fluorographed.

Western immunoblots. After electrophoresis, protein extracts were electrophoretically transferred onto Immobilon-P membranes (Millipore) at 50 V for 3 h in transfer buffer (191 mM glycine, 25 mM Tris-Cl [pH 8.3], 20% methanol). The membrane was blocked with TBS-T buffer (20 mM Tris-Cl [pH 7.4], 137 mM NaCl, 0.1% Tween 20) amended with 10% nonfat powdered milk. Then antiserum was added to a final dilution of 1:3,000 in a solution of TBS-T and 2% powdered milk. After a 3-h incubation at room temperature, the filter was washed twice for 5 min each with TBS-T, once for 10 min with 1 M LiCl, and three times for 5 min each with TBS-T. A secondary antibody (at 1:3,000 dilution), conjugated to horseradish peroxidase, was used with ECL chemiluminescent detection reagents (Amersham) to visualize the antigens.

Far Western blots. Far Western assays were performed by a protocol modified from that of Lee et al. (29). Protein blots were prepared as described above up to the step before antibody addition. The membrane was soaked in K buffer (L buffer amended with 0.5-mg/ml bovine serum albumin and 0.05% polyvinylpyrrolidone) for 30 min before incubation for 14 to 16 h at 4°C with fresh K buffer and 100 μ l of in vitro-translated radiolabeled 1a. The filter was then washed five times with K buffer, once with 0.5 M LiCl, and twice with L buffer before autoradiography.

Expression of 2a in baculovirus-infected Sf21 cells. The BMV 2a open reading frame was cloned into the polyhedrin locus of the baculovirus *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) transplacement vector pEV55 (Genetics Institute, Cambridge, Mass.) and then introduced into the complete baculovirus genome via recombination between the polyhedrin loci of the transplacement plasmid and a wt baculovirus DNA in cotransfected Sf21 cells, yielding a 2a-expressing baculovirus clone designated vAcB2RH1 (18a). Total protein extracts for far Western and immunoprecipitation assays were made from cells infected by vAcB2RH1 or wt AcMNPV and lysed with STEE buffer (0.25 M sucrose, 10 mM Tris-Cl [pH 8], 1 mM EDTA, 1 mM EGTA) containing 0.2% Nonidet P-40. A highly 2a-enriched fraction (see Fig. 4A, lane 9) used for immunoprecipitation assays was then made from the vAcB2RH1-infected total protein extract by pelleting the insoluble fraction containing 2a at $3,000 \times g$. After the pellet

FIG. 1. Cofractionation of 1a protein, 2a protein, and RdRp activity. Detergent-solubilized RdRp from BMV-infected barley cells was fractionated first on a Sephacryl 400 (S-400) column (A and B) and then on a DEAE-Biogel A column (C and D). In the representative experiments shown, peak fractions 17 to 22 from the S-400 column were pooled and loaded on the DEAE-Biogel A column. Portions of the resulting fractions from both columns were examined by Western blotting with a mixture of anti-1a and anti-2a sera (A and C) and were analyzed for RdRp activity (B and D) by measuring [α - 32 P]CTP incorporation into RNA newly synthesized from added BMV virion RNA templates (34). The positions of 1a and 2a are indicated between panels A and C.

was washed in STEE amended with 0.3% sodium deoxycholate and 0.2% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), the pellet was dissolved in STEE amended with 0.3% sodium sarcosyl.

RESULTS

Association of 1a and 2a proteins during purification of active BMV RdRp extract. While using Western blotting to monitor the distribution of 1a and 2a proteins during purification of detergent-solubilized BMV RdRp activity from infected barley leaves, we consistently observed the cofractionation of 1a and 2a (Fig. 1). The solubilized RdRp, after differential centrifugation steps, was fractionated on a Sephacryl 400 (S-400) size exclusion column, and the resultant fractions were tested for the ability to synthesize BMV negative-strand RNA from input virion RNAs (Fig. 1B). Parallel chromatography of marker proteins of known molecular weight showed that the peak of RdRp activity eluted at a position corresponding to a molecular mass greater than 2×10^6 Da. The most active fractions from the S-400 column (fractions 17 to 22 of Fig. 1B) were pooled and loaded on a column of DEAE-Biogel A, a weak anion-exchange resin. Proteins were eluted from the DEAE column with a KCl gradient of 0 to 500 mM, with the RdRp activity eluting at approximately 250 mM KCl (fractions 14 to 18, Fig. 1D). In the enzymatically active final DEAE fractions, approximately 15 to 20 distinct protein bands, including 1a and 2a, were visualized by silver staining after electrophoresis (data not shown).

The elution profiles of the BMV 1a and 2a polypeptides in the fractions from the S-400 and DEAE columns were

FIG. 2. Immunoprecipitation of BMV in vitro translation products. Autoradiographs of SDS-polyacrylamide gels from representative experiments are shown. (A) Tests for cross-reaction of anti-1a serum and anti-2a serum with the 1a and 2a proteins. Proteins 1a (lanes 1 to 3) and 2a (lanes 4 to 6) were translated in separate reticulocyte lysate reactions programmed with in vitro transcripts synthesized by T7 RNA polymerase from cDNA clones of BMV RNA1 or RNA2, respectively. For each protein, a 1- μ l portion was electrophoresed directly (lanes 1 and 4), while 7- μ l portions were immunoprecipitated with antiserum (Ab) against 1a (lanes 3 and 5) or against 2a (lanes 2 and 6) before electrophoresis. (B) Immunoprecipitation of a 1a and 2a complex. Reticulocyte lysate translation was programmed with total BMV virion RNA, and a portion of the products was reserved for direct electrophoreses (lane 1). The remainder of each translation reaction was mixed with protein A-expressing *S. aureus* and incubated, and the cells were pelleted to remove any proteins nonspecifically binding to protein A. The supernatant was then aliquoted into four tubes and immunoprecipitated with anti-3a serum (lane 2), anti-1a serum (lane 3), or anti-2a serum (lanes 4 and 5) before electrophoresis. As indicated by the Δ , the sample in lane 5 was incubated for 5 min at 45°C before addition of antiserum.

determined by Western blotting with polyclonal antisera to both 1a and 2a (Fig. 1A and C). Throughout the fractions from both columns, the presence and relative abundance of 1a were paralleled by 2a. The abundance of 1a and 2a in the eluants from both columns (Fig. 1A and C) also correlated well with the level of RdRp activity in each fraction (Fig. 1B and D). Trailing fraction 24 from the S-400 column showed a slight alteration in 1a:2a ratio and the presence of a low-molecular-weight, antibody-reactive band that may be a degradation product of 1a or 2a. However, this and all other active fractions from both columns synthesized the same spectrum of full-length negative-strand RNA products from added BMV virion RNA templates, just as seen in previous BMV RdRp assays (34).

In vitro-translated 1a and 2a proteins form a complex. The strict cofractionation of BMV 1a, 2a, and RdRp activity in the above experiments confirms and extends previous observations (34), suggesting that an interaction between 1a and 2a is an integral feature of BMV RNA synthesis. To explore this relation further, we used immunoprecipitation to test whether 1a and 2a proteins translated in rabbit reticulocyte lysates could interact with each other in vitro. As a control to these experiments, we first determined the level of 1a protein immunoprecipitated by anti-2a serum in the absence of 2a, and vice versa (Fig. 2A). In both cases,

the level of cross-reaction was at the limit of experimental detection and was at the same low level observed in immunoprecipitations by control antiserum from nonimmunized rabbits or anti-3a serum.

Total BMV virion RNA was translated in rabbit reticulocyte lysates, and the labeled proteins (Fig. 2B, lane 1) were subjected to immunoprecipitation. Under these conditions, anti-1a serum not only precipitated 1a, but also significant amounts of 2a (Fig. 2B, lane 3). Similarly, anti-2a serum coprecipitated significant levels of 1a (Fig. 2B, lane 4). Using a digital radioactive imaging detector (Betagen), analytical comparison of the 1a:2a ratio in pre- and postimmunoprecipitation samples from several experiments indicated that 20 to 50% of the total 1a protein in such cotranslation reactions was routinely precipitated with 2a protein. By contrast, the level of the BMV 3a protein precipitated by anti-1a or anti-2a serum was trivial, as was the precipitation of 1a or 2a by anti-3a serum (Fig. 2B).

The interaction between 1a and 2a was resistant to 500 mM LiCl, which was routinely used to wash the immunoprecipitates, and to at least 250 mM KCl, which was sometimes used in the dilution buffer added before immunoprecipitation. The degree of 1a-2a coprecipitation was also unaffected by the addition of either RNase or DNase (data not shown). However, when the translation products were heated for 5 min at 45°C before the addition of anti-2a serum, the amount of 1a that was coprecipitated with 2a was significantly reduced (Fig. 2B, lanes 4, 5). The amount of the observed 1a-2a complex was also decreased if the translation products were repeatedly frozen and thawed before immunoprecipitation or if they were diluted in buffer containing 500 mM KCl.

Proteins 1a and 2a that had been cotranslated from *in vitro* transcripts made from biologically active BMV cDNA clones (20) were also able to form a specific coprecipitable complex (Fig. 3, lanes 10 to 12). Translation of *in vitro* transcripts rather than virion RNAs usually resulted in increased amounts of incomplete 1a and 2a translation products that reacted with anti-1a or anti-2a serum (compare Fig. 2A and 3 with 2B). Under these circumstances, accurate assessment of 2a coprecipitation by anti-1a serum may be impeded by the electrophoretic comigration of incomplete 1a translation products with 2a. Accordingly, we performed the immunoprecipitation assays with anti-2a serum and looked for the coprecipitation of the higher-molecular-weight 1a protein. Translation of transcripts from either RNA1 or RNA2 cDNA clones also produced a lower-molecular-weight polypeptide that may be derived from vector rather than BMV cDNA sequences (see band noted by asterisk in Fig. 3, lanes 1 to 3 and 10). While the origin of this polypeptide is uncertain, its failure to be precipitated with anti-1a, anti-2a, or anti-3a antisera further demonstrates the specificity of the immunoprecipitation reactions (Fig. 2A and 3).

To determine whether cotranslation of 1a and 2a was necessary to produce an immunoprecipitable complex, we programmed separate reticulocyte lysate reactions with either *in vitro*-synthesized RNA1 or RNA2 (Fig. 3, lanes 1 and 2). The translation reactions were then treated with RNase A to stop further translation, and aliquots of 1a and 2a were mixed together, incubated at 28°C for various times, and immunoprecipitated (Fig. 3, lanes 3 to 9). When independently translated 1a and 2a were incubated together for increasing times (0 to 30 min) before immunoprecipitation with anti-2a serum, increasing amounts of 1a protein coprecipitated with 2a (Fig. 3, lanes 5 to 9). Further incubation

FIG. 3. Independently synthesized 1a and 2a form an immunoprecipitable complex upon mixing. Proteins 1a (lane 1) and 2a (lane 2) were translated in separate reticulocyte lysate reactions programmed with *in vitro* transcripts from cDNA clones of BMV RNA1 or RNA2, respectively. Equal volumes of RNase A-treated 1a and 2a translation products were then mixed together (lane 3), and aliquots were incubated at 28°C for 0 (lane 5), 2 (lane 6), 10 (lane 7), 20 (lane 8), or 30 (lanes 4 and 9) min. The reaction mixtures were then immunoprecipitated with anti-2a serum (lanes 5 to 9) or with the control anti-3a serum (lane 4). For comparison, 1a and 2a were cotranslated from a mixture of the RNA1 and RNA2 *in vitro* transcripts and electrophoresed directly (lane 10) or after immunoprecipitation with anti-3a serum (lane 11) or anti-2a serum (lane 12). An autoradiograph of the final SDS-polyacrylamide gel is shown. The asterisk at left marks an approximately 48-kDa band discussed in the text. As can be seen by comparing lanes 3 and 5, and also lanes 10 and 12, a slight retardation of 1a and 2a migration consistently resulted from immunoprecipitation, probably due to the LiCl salt washes used (see Materials and Methods). Ab, antibody.

beyond 30 min did not significantly increase the level of 1a coprecipitation (data not shown). As observed for cotranslation, the 1a-2a complex resulting from posttranslation mixing was still labile to incubation at 45°C (data not shown) and was not detected when antiserum to the BMV 3a protein was used as a control (Fig. 3, lanes 4 and 11). However, in each of several experiments, the degree of 1a-2a coprecipitation after mixing was consistently lower than when 1a and 2a were cotranslated (compare lanes 9 and 12 of Fig. 3).

1a binds to 2a protein synthesized in insect cells. Since separately translated 1a and 2a can form a specific complex after coinubation, we tested for possible interaction between *in vitro*-translated 1a protein and 2a protein partially purified from overexpressing cells. The 2a protein had been synthesized in Sf21 insect cells by substituting the complete, unmodified 2a open reading frame for the polyhedrin gene of the baculovirus AcMNPV. The AcMNPV-expressed 2a protein (designated Ac2a) migrated in SDS-polyacrylamide gels with mobility identical to that of natural 2a in RdRp preparations from infected barley (Fig. 4A) or *in vitro*-translated 2a (data not shown) and reacted specifically with the anti-2a serum in Western blot analyses (Fig. 4A, lane 6). Partially purified Ac2a (Fig. 4A, lane 9) was mixed with *in vitro*-translated 1a protein, incubated at 28°C for 30 min, and immunoprecipitated with anti-2a serum. With increasing amounts of unlabeled Ac2a, increasing amounts of the radiolabeled 1a were coprecipitated by anti-2a serum (Fig. 4B, lanes 3 to 6). As shown in Fig. 4B, 1a was not precipitated under otherwise identical conditions if Ac2a was replaced by a total protein extract from Sf21 cells infected with wt AcMNPV (Acwt, lane 2), if the reaction

FIG. 4. (A) Expression of 2a protein in Sf21 insect cells. Total lysates of Sf21 cells infected with a 2a protein-expressing AcMNPV vector (Ac2a, lanes 2 and 6), Sf21 cells infected with wt AcMNPV expressing the natural polyhedrin (ph) protein (Acwt, lanes 3 and 7), or uninfected Sf21 cells (lanes 4 and 8) were electrophoresed on an SDS-8% polyacrylamide gel and either stained with Coomassie brilliant blue R250 (lanes 1 to 4) or analyzed by Western blotting with anti-2a antiserum (lanes 5 to 8). The positions of the polyhedrin protein (ph) and the 2a protein are indicated. Molecular mass markers of the sizes indicated are at the left of the autoradiograph (lane 1). A sample from an active RdRp preparation purified through the DEAE-Biogel A chromatography (Fig. 1C and D) was used as a standard in lane 5. A Coomassie brilliant blue-stained sample of the partially purified Ac2a used for the immunoprecipitation assay shown in panel B is shown in lane 9. This Ac2a extract was fractionated as described in Materials and Methods from a total protein extract of 2a-expressing Sf21 cells. (B) Binding of in vitro-translated 1a to 2a protein produced in insect cells. 1a protein was translated in reticulocyte lysates (lane 1) and portions of the [³⁵S]methionine-labeled 1a translation products were incubated for 30 min at 28°C with 1 (lane 3), 5 (lane 4), 10 (lanes 5, 7, and 8), or 20 (lane 6) arbitrary units of unlabeled, partially purified Ac2a protein. Immunoprecipitation was then performed with anti-2a serum (lanes 2 to 7) or anti-3a serum (lane 8), and the samples were electrophoresed and autoradiographed. As a control (lane 2), the 1a protein was incubated with a total protein extract from Sf21 cells infected with wt AcMNPV before immunoprecipitation with anti-2a serum. As indicated by the Δ , the sample in lane 7 was heated for 5 min at 45°C before the addition of anti-2a serum. The asterisk marks a band discussed in the text (see Fig. 3).

mixtures were heated to 45°C immediately before immunoprecipitation (lane 7) or if anti-3a serum was substituted for anti-2a serum (lane 8).

Antibody-independent detection of 1a-2a protein interaction. To examine whether 1a and 2a could interact in the absence of immunoprecipitation, we electrophoresed unlabeled total protein extracts from Sf21 cells, including one extract from cells expressing Ac2a, on a polyacrylamide-SDS gel (Fig. 5A). The proteins were blotted onto a nylon membrane, and the blot was probed with [³⁵S]methionine-labeled 1a protein that had been translated in reticulocyte lysates and treated with RNase A (Fig. 5B). The 1a polypeptide bound to Ac2a with much higher specificity than to any other polypeptide on the membrane (Fig. 5B, lane 4). Some 1a did bind to the highly prevalent polyhedrin protein present in extracts from cells infected with wt baculovirus (lanes 2 of Fig. 5A and B). However, quantitation with a Betagen radioactive imaging detector showed that the strength of this polyhedrin signal was threefold lower than the signal of 1a interaction with Ac2a, even though polyhedrin was present at more than a threefold molar excess to Ac2a. When a similar membrane was probed with labeled 3a protein, no specific binding to Ac2a was observed (data not shown).

Effect of 1a mutations on 1a-2a interaction. PK16 and PK19 are engineered insertion mutants of BMV RNA1 (26). The PK16 insertion, consisting of three amino acids (Gly-Pro-Thr) inserted after 1a residue 464, lies within the short region between the methyltransferaselike and helicaselike domains. In transfected protoplasts, the PK16 insertion totally blocks BMV RNA replication (26). The insertion in PK19, consisting of two amino acids (Gly-Ser) inserted after 1a residue

670, lies 12 residues N terminal to the highly conserved nucleotide-binding site consensus within the helicaselike domain. In vivo, the PK19 mutation renders 1a temperature sensitive for BMV RNA replication, so that at 24°C replication is nearly wt, while at 35°C all viral RNA synthesis is blocked (26).

We compared the binding of 2a by reticulocyte-synthe-

FIG. 5. Antibody-independent assay for 1a-2a interaction by a far Western assay. Total protein lysates from Sf21 cells infected with wt AcMNPV (Acwt, lane 2), uninfected Sf21 cells (lane 3), or Sf21 cells infected with a 2a protein-expressing AcMNPV vector (Ac2a, lane 4) were electrophoresed and either stained with Coomassie brilliant blue (A) or transferred to a membrane and incubated with radiolabeled, in vitro-translated 1a protein to generate an autoradiograph (B). Lane 1 contains molecular mass markers with the sizes indicated at left. ph indicates the position of polyhedrin in lane 2 of both panels.

FIG. 6. Effect of 1a mutations on interaction with 2a. (A) Reticulocyte lysates were programmed with in vitro transcripts from biologically active cDNA clones of wt RNA1 (lanes 1, 4, 7, 8, and 11), the PK19 RNA1 mutant (lanes 2, 5, 9, and 12), or the PK16 RNA1 mutant (lanes 3, 6, 10, and 13), and translation was performed at either 24°C (lanes 1 to 3 and 7 to 10) or 32°C (lanes 4 to 6 and 11 to 13). A 1- μ l portion of each [35 S]methionine-labeled sample was then electrophoresed directly (lanes 1 to 6). Another 7- μ l aliquot was first incubated for 30 min at 24°C with unlabeled, partially purified Ac2a protein (see Fig. 4A) and immunoprecipitated with anti-2a serum (lanes 8 to 13) or anti-3a serum (lane 7). The samples were then electrophoresed on an SDS-10% polyacrylamide gel and autoradiographed. The asterisk at left denotes an approximately 48-kDa non-BMV band discussed in the legend to Fig. 3. (B) Reticulocyte lysates were programmed with in vitro transcripts of wt RNA2 plus either wt RNA1 (lanes 1, 3, 5, and 7) or the PK19 RNA1 mutant (lanes 2, 4, 6, and 8), and the reactions were incubated at either 24°C (lanes 1, 2, 5, and 6) or 32°C (lanes 3, 4, 7, and 8). Autoradiographs of the radiolabeled in vitro translation products before immunoprecipitation (lanes 1 to 4) or after immunoprecipitation with anti-2a serum (lanes 5 to 8) are shown. Ab, antibody.

sized wt 1a and the PK16 and PK19 1a mutants after each had been translated at 24 or 32°C, which are temperatures at which we could obtain both efficient in vitro translation and detectable binding between wt 1a and 2a. Each of the [35 S]methionine-labeled 1a polypeptides (Fig. 6A, lanes 1 to 6), was mixed with an unlabeled Ac2a extract from 2a-expressing Sf21 cells (Fig. 4A, lane 9) and was incubated for 30 min at 24°C before immunoprecipitation (Fig. 6A, lanes 7 to 13). When synthesized at 24°C, anti-2a serum coprecipitated wt 1a protein with Ac2a as before, but the PK16 mutant 1a protein was not precipitated. By contrast, the PK19 mutant 1a protein, which could support RNA replication in vivo at 24°C, was coprecipitated with the unlabeled Ac2a by anti-2a serum, although to a lesser extent than wt 1a protein. Thus, there was a good correlation between the ability of the

1a protein derivatives to support BMV RNA synthesis in vivo at 24°C and their ability to bind 2a in vitro at the same temperature.

When synthesized at 32°C, the temperature-sensitive PK19 mutant of protein 1a was no longer detectably coprecipitated with Ac2a by anti-2a serum (Fig. 6A, lane 12). Interpretation of this result was complicated, however, by a concomitant reduction in the coprecipitation of wt 1a protein when it was synthesized at 32°C rather than 24°C (Fig. 6A, lanes 8 and 11). Because cotranslation facilitated 1a-2a interaction, we also compared binding to 2a by wt 1a and the PK19 1a mutant after their cotranslation with 2a at 24 and 32°C (Fig. 6B). Overall, the cotranslation results were similar to those obtained with added Ac2a: wt 1a was coprecipitated with 2a after translation at 24°C or, at a reduced level, after translation at 32°C (Fig. 6B, lanes 5 and 7). The PK19 1a mutant was coprecipitated at an intermediate level when 1a and 2a were synthesized at 24°C, but was not detectably coprecipitated when the proteins were synthesized at 32°C (Fig. 6B, lanes 6 and 8).

DISCUSSION

In this report, we showed that the BMV RNA replication proteins 1a and 2a specifically interact in vitro to form a complex. 1a-2a complexes formed with reticulocyte-translated and baculovirus-expressed proteins were detected only with highly selective antisera directed against 1a or 2a, but not with unrelated antisera, including one against the BMV 3a protein. The specificity of the observed 1a-2a interaction was further demonstrated by the fact that other labeled polypeptides present in the various reactions, including BMV 3a, did not coprecipitate with 1a and 2a. As an independent check, the ability of 1a protein to interact with 2a protein was also verified in an antibody-independent far Western assay.

As outlined in the Introduction, a variety of genetic results suggest that the in vitro 1a-2a interaction observed here reflects an interaction that occurs in vivo and is functionally important for at least some steps in BMV RNA replication. Additional results presented in this report further strengthen this view. BMV RdRp activity, 1a, and 2a were found to strictly cofractionate through multiple independent steps in the purification of BMV RdRp activity from BMV-infected plants. Furthermore, the ability of the two 1a mutants to bind 2a in vitro was correlated with their ability to support RNA replication in vitro (Fig. 6). The PK16 1a mutation, which blocks RNA replication in vivo, abolishes 2a binding in vitro. By contrast, the PK19 1a mutant, which supports in vivo RNA replication at 24°C, retained a reduced, but significant ability to interact with 2a at this permissive temperature. Consistent with its temperature-sensitive behavior in vivo, PK19 also fails to detectably interact with 2a at 32°C, although wt 1a also showed reduced interaction at this temperature.

Nature of the 1a-2a association. The association of 1a and 2a is noncovalent. The complex is dissociated by incubation with 0.1% SDS, by a 5-min incubation at 45°C, or by prolonged incubation in buffers containing greater than 0.5 M KCl. However, the complex is sufficiently stable to withstand brief washing with 0.5 M LiCl, which had been used to remove any nonspecifically trapped polypeptides (22, 29). Even after the immunoprecipitates were washed with 0.5 M LiCl, approximately 20 to 50% of the total wt 1a protein present in the translation reaction remained in a coprecipitable complex with 2a.

The interaction between 1a and 2a reported here might be direct or mediated by some adapter molecule. However, the interaction is not likely to be mediated by nucleic acid since nuclease digestions did not affect 1a-2a interaction in the immunoprecipitation assay. Furthermore, in the far Western experiment (Fig. 5), the *in vitro*-translated 1a protein was treated with RNase before incubation with the filter on which Ac2a and other proteins were immobilized. While some other type of cellular factor might be involved in 1a-2a binding, no plant factors were present in either the immunoprecipitation or far Western experiments. Thus, if any cellular factor acts as a required adapter between 1a and 2a, it must be conserved between monocotyledonous plant cells and mammalian reticulocytes.

Cotranslated 1a and 2a formed a complex more readily than when the two proteins were translated separately and mixed together. Perhaps the domain(s) required for 1a-2a interaction is more accessible during the synthesis of the polypeptides. Nevertheless, it appears likely that the same domain(s) is required for complex formation during cotranslation and during incubation of separately translated proteins. Conditions which affected complex formation (e.g., 45°C, salt concentration) of the cotranslated 1a and 2a also affect the complex formation of the separately translated 1a and 2a. Also, in the mixing experiments, no complex was observed if the protein extract did not contain Ac2a, or if antisera which did not recognize 1a and 2a were used.

In the immunoprecipitation assays, we always observed more of the antigenic polypeptide than the coprecipitated polypeptide, suggesting that the antibody-antigen complex is more stable than the 1a-2a complex. It is thus difficult to extrapolate the initial stoichiometry of the complex from these experiments, since specific complexes may be dissociated by the stringent washing conditions used.

When detergent-solubilized BMV RdRp was fractionated on Sephacryl 400 (Fig. 1) or Superose 6 size exclusion columns, proteins 1a and 2a coeluted with each other and the RdRp activity at a position corresponding to an estimated molecular mass of greater than 2×10^6 Da (33a). This large apparent size may reflect a high stoichiometry of the viral replication proteins in the RdRp complex, or the association of additional cellular factors, or both. Previous genetic results suggest that BMV RNA synthesis is more sensitive to reductions in 1a protein synthesis than 2a protein synthesis (1, 26). Consistent with this observation, the relative intensities of the 1a and 2a bands in Western blot analyses of the RdRp (Fig. 1A and C) and preliminary data on 1a and 2a levels in RdRp preparations stained with silver (33a) suggest that an excess of the 1a protein is present relative to the 2a protein.

Relation of 1a and 2a to proteins of other viruses. All members of the alphaviruslike superfamily encode proteins with domains related to the putative methyltransferase, helicase, and polymerase domains of BMV 1a and 2a. However, the linkage of these domains can differ between viruses. Tobacco mosaic virus, for example, encodes two overlapping nonstructural polypeptides that are needed for RNA replication (14). The larger of these polypeptides (p183) contains domains closely related in sequence to the complete ensemble of putative methyltransferase, helicase, and polymerase domains present in 1a and 2a (17). As previously suggested (1, 40), a noncovalent complex of BMV 1a and 2a may be a functional analog of tobacco mosaic virus p183 that allows all three conserved domains to function together in a coordinated fashion to mediate specific initiation, elongation, and capping steps in RNA synthesis. The

observation that 1a and 2a form a complex provides experimental support for this hypothesis.

Like the bromoviruses, many other alphaviruslike viruses express their putative polymerase domain within a protein that is physically distinct from the protein(s) encoding the putative methyltransferase and helicase domains. Examples include the animal alphaviruses (2, 38), rubella virus (9), and a number of plant viruses such as the cucumoviruses, almoviruses, hordeiviruses, etc. (10). The results presented here for BMV 1a and 2a suggest that the RNA replication proteins of each of these other viruses also interact. Indeed, Barton et al. (6) were able to precipitate all viral nonstructural polypeptides as a single complex from Sindbis virus-infected and Semliki Forest virus-infected cells.

While the *in vitro*-translated BMV 1a and 2a proteins were competent for specific interaction, they were not competent for the synthesis of negative-strand RNA from exogenously added BMV RNA templates. In addition, no such activity was observed when extracts from uninfected plants were added or when Ac2a was used in place of *in vitro* translated 2a (34a). These results were expected since, to date, it has not been possible to reconstitute RdRp activity from individual components. Presumably, other requirements, such as a proper complement and assembly of host factors (1), must also be met for RdRp activity.

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