Immunological Analysis of Brome Mosaic Virus Replicase

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SUMMARY

Antisera specific for the non-structural proteins 1a and 2a of brome mosaic virus (BMV) were prepared by the gene fusion method. Plasmids were constructed which expressed parts of 1a and 2a in Escherichia coli as fusion proteins with Protein A. After induction of fusion protein synthesis in E. coli, the fusion proteins accumulated in insoluble fractions. Antisera raised against these proteins (anti-1a and anti-2a sera) reacted specifically with the respective proteins translated in vitro and in vivo. These antisera were used to investigate the involvement of the 1a and 2a proteins in the BMV replicase preparation which initiated complementary strand synthesis de novo on BMV RNA. Immunoblot analysis using these antisera revealed the presence of 1a and 2a in the BMV replicase fraction. The replicase activity was inhibited by the addition of anti-1a serum, but not anti-2a serum. Further addition of Protein A-Sepharose to remove each immunocomplex gave similar results. These results suggested the involvement of at least the 1a protein in our BMV replicase preparation.

INTRODUCTION

Brome mosaic virus (BMV) is a positive-stranded RNA virus which infects Gramineae. Its genome is divided between three separately encapsidated components designated RNA 1 (3-2 kb), RNA 2 (2-9 kb) and RNA 3 (2-1 kb). BMV also encapsidates a subgenomic RNA designated RNA 4 (0-9 kb) (Ahlquist et al., 1981, 1984). BMV RNAs 1 and 2 each encode a single protein, of Mr 109K (1a) and 94K (2a) respectively, while RNA 3 encodes two proteins of 35K (3a) and 20K (coat protein). RNA 4 is an mRNA for the coat protein (Ahlquist et al., 1981, 1984). The function of each of these proteins, except for the coat protein, is still not clear.

The inoculation of barley protoplasts with BMV RNAs 1 and 2 alone gives rise to viral RNA replication (French et al., 1986). This indicates involvement of the translation products of these RNAs, namely the 1a and 2a proteins, in BMV RNA replication. However, direct involvement of these proteins in the BMV replicase complex has not been verified. The BMV replicase fraction, which was shown to initiate complementary strand synthesis de novo on BMV RNA had been extracted and partially purified from BMV-infected barley leaves (Bujarski et al., 1982; Miller & Hall, 1983; Maekawa & Furusawa, 1984; Horikoshi et al., 1987). In this paper, we report the preparation of antisera specific for the 1a and 2a proteins and the investigation of the reactivities of these antisera towards the BMV replicase.

METHODS

Virus and RNA purification. BMV (ATCC66) was obtained from Dr C. Hiruki, University of Alberta, Edmonton, Canada. The virus was propagated and purified, and BMV RNA was isolated and purified, as described by Okuno & Furusawa (1979).

Bacterial strains and plasmids. Escherichia coli N99clt which carries the wild-type λ repressor, N4830-1 which carries the temperature-sensitive λ cI857 repressor and the Protein A gene fusion vector pRIT2T (Nilsson et al.,
overnight at 4 °C, immunocomplexes were adsorbed to Protein A-Sepharose CL-6B (Pharmacia) by incubation
nitrocellulose sheet as described by Towbin thus obtained were inserted into the pEBRI3n. To adjust the reading frames for Protein A and the cDNA, the plasmid was modified as follows. Plasmid pEBRI3n was digested between the Protein A gene and the cDNA with BamHI, filled in using the Klenow fragment of DNA polymerase I, and self-ligated. Consequently, 4 bp were inserted between the Protein A gene and the cDNA, resulting in a one base shift of the cDNA frame (pEBRI3n + 1). Plasmid pEBRI3n + 1 was further modified with XmaI and the Klenow fragment, as described above (pEBRI3n + 2). All plasmids derived from pRIT2T were grown in E. coli N99cl+.

Plasmid pB2 #794 was modified as in the case of pB1 #4021 to generate a series of three plasmids. The resultant plasmids were named pEBRII3n, pEBRII3n + 1 and pEBRII3n + 2.

Expression of plasmids and purification of the fusion proteins. E. coli N4830-1 was transformed with each of the six plasmids produced as described above. Fusion protein synthesis was induced by temperature shift (Zabeau & Stanley, 1982). After induction for 1.5 h, cells were harvested, resuspended and then lysed by sonication. The lysates were separated into soluble and insoluble fractions by centrifugation at 20000 g for 20 min. The soluble fractions were applied to IgG-Sepharose 6 Fast Flow (Pharmacia) columns and the fusion proteins were purified according to the manufacturer’s instructions. Two μg samples of the eluted proteins were subjected to SDS–PAGE in a 12-5% gel as described by Laemmli (1970) and the gel was stained with Coomassie Brilliant Blue R-250. The insoluble fractions from 0-5 ml of medium were also analysed by SDS–PAGE.

The insoluble fractions from pEBRI3n + 1 and pEBRII3n + 1, rich in the fusion proteins because of the correct reading frames, were then subjected to preparative SDS–PAGE and the fusion proteins were recovered by electro-elution after staining with 1 m-KCl (Nelles & Bamburg, 1976).

Preparation of antiserum. The electro-eluted fusion proteins were used as antigens. Each antigen was mixed with an equal volume of complete Freund’s adjuvant and portions containing approx. 100 μg of the fusion protein were injected subcutaneously into Japanese white rabbits. The rabbits were reimmunized with the fusion proteins mixed with an equal volume of incomplete Freund’s adjuvant every 2 weeks, and antisera were collected 10 to 12 days after the fourth injection. These antisera were designated the anti-la and anti-2a sera as appropriate.

In vitro translation and immunoprecipitation. BMV RNA was translated in vitro in a rabbit reticulocyte lysate (Promega) in the presence of [3H]leucine. The lysates were diluted 20-fold with IP buffer (50 mM-Tris–HCl pH 7-5, 150 mM-NaCl, 2 mM-EDTA and 1% NP40), and normal serum was added at a 1:50 dilution. After incubation overnight at 4 °C, immunocomplexes were adsorbed to Protein A-Sepharose CL-6B (Pharmacia) by incubation for 1 h at 4 °C with gentle rocking. After centrifugation, anti-la, anti-2a or normal sera at dilutions of 1:50 were added to the supernatants. Immunocomplexes were collected as above, washed three times with IP buffer, once with IP buffer without NP40, released by boiling in Laemmli sample buffer and analysed by SDS–PAGE in a 12.5% gel. Radioactive proteins were detected by fluorography with En3Hance (New England Nuclear).

Immunoblotting. Samples were subjected to SDS–PAGE in a 10% gel and proteins were transferred to a nitrocellulose sheet as described by Towbin et al. (1979). After transfer, proteins were immunodetected with the Bio-Rad Immun-Blot (goat anti-rabbit-horseradish peroxidase) assay kit using the anti-la or anti-2a sera at a 1:400 dilution.

Preparation of BMV replicase. The BMV replicase fraction was prepared as described by Horikoshi et al. (1987). Briefly, crude membrane fractions (31000 g pellet) were extracted from BMV- or mock-infected barley leaves. After solubilization, BMV replicase was first purified by DEAE-Bio-Gel A (Bio-Rad) and then further by phosphocellulose (P11, Whatman) column chromatography. These were designated the DEAE and PC fractions, respectively.

BMV replicase assay. The standard assay mixture was essentially as described by Horikoshi et al. (1987). The mixture contained 1 μg actinomycin D, 2-5 μg BMV RNA, 25 μg each of ATP, GTP and CTP, 1 μCi [3H]UTP (430 μCi/mmol), 65 units RNasin (Promega) and 20 μl enzyme fraction to give a final volume of 65 μl. The mixture was incubated for 60 min at 30 °C, and incorporation of radioactivity into the acid-insoluble fraction was determined as described by Okuno & Furusawa (1979).

RESULTS

Construction of plasmids encoding the fusion proteins

In our laboratory, cDNA clones complementary to approx. 1200 bases from the 3’ termini of BMV RNAs 1 and 2 have already been obtained. Each cDNA encodes approx. 300 amino acids from the carboxy termini of each of the la and 2a proteins. We inserted each cDNA fragment into the Protein A gene fusion vector pRIT2T and constructed a series of three plasmids with
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Fig. 1. SDS-PAGE of the fusion proteins in the insoluble fractions from induced E. coli. Proteins were extracted from E. coli carrying pEBRI3n (lane 1), pEBRI3n + 1 (lane 2), pEBRI3n + 2 (lane 3), pRIT2T (lane 4), pEBRII3n (lane 5), pEBRII3n + 1 (lane 6) or pEBRII3n + 2 (lane 7). The positions of the Mr standards are shown on the left. The arrowheads indicate fusion proteins with the expected Mr values.

The fusion proteins in soluble fractions of induced E. coli lysates were purified by IgG-Sepharose column chromatography. The fusion proteins from E. coli carrying pEBRI3n, pEBRI3n + 2, pEBRII3n or pEBRII3n + 2 had almost identical electrophoretic mobilities as the Protein A from E. coli carrying pRIT2T (data not shown). It was therefore suggested that these plasmids were out-of-frame. No fusion proteins except some non-specific proteins, were observed in the fractions from E. coli carrying pEBRI3n + 1 or pEBRII3n + 1 (data not shown).

Insoluble fractions (20,000 g pellets) were then analysed by SDS-PAGE in a 10% gel (Fig. 1). Specific bands of 58K and 59K were observed in the insoluble fractions from E. coli carrying pEBRI3n + 1 and pEBRII3n + 1, respectively. These bands had the Mr values expected for the true fusion proteins, that is the deduced amino acid sequences gave Mr values for Protein A of 31K and for the virus-encoded components of approx. 30K, adding up to a total Mr for the fusion proteins of approx. 61K.

Characterization of antisera

Rabbits were immunized with the electro-eluted fusion proteins. After the fourth injection, antisera (anti-la and anti-2a sera) were collected. To investigate their reactivities and specificities the in vitro translation products of BMV RNA were immunoprecipitated with them. BMV RNA was translated in vitro into four proteins (the la, 2a, 3a and coat proteins) (Fig. 2, lane 1). Among these proteins, the anti-la and anti-2a sera preferentially precipitated the la and 2a proteins, respectively (Fig. 2, lanes 2 and 3). The result with normal serum (Fig. 2, lane 4) suggested that the precipitation of other proteins with the anti-la and anti-2a sera was non-specific. Consequently, the anti-la and anti-2a sera raised against the fusion proteins were shown to react respectively with the la and 2a proteins translated in vitro.
Fig. 2. Immunoprecipitation of the products of BMV RNA translated in vitro in a rabbit reticulocyte lysate. Total products of BMV RNA (lane 1), precipitates with anti-1a serum (lane 2), anti-2a serum (lane 3) and normal serum (lane 4) were analysed by SDS–PAGE followed by fluorography. Translation products of BMV RNA are indicated on the left.

Fig. 3. Immunoblot analysis of the in vivo translation products of BMV RNA with the anti-1a serum (lanes 1 and 2) or anti-2a serum (lanes 3 and 4). Lanes 1 and 4, crude membrane fraction from mock-infected barley leaves; lanes 2 and 3, crude membrane fraction from BMV-infected barley leaves. The positions of 1a and 2a proteins translated in vitro are shown on the left.

The immunoblotting procedure was used to investigate the reactivity and specificity of each antiserum with the in vivo translation products of BMV RNA in the crude membrane fraction from BMV-infected barley leaves. When the anti-1a serum was used (Fig. 3, lanes 1 and 2), a specific band of approx. 105K was detected in the fraction from BMV-infected leaves, in addition to some non-specific bands which were also detected in the fraction from mock-infected leaves. When the anti-2a serum was used (Fig. 3, lanes 3 and 4), a specific band of approx. 100K was detected in addition to some non-specific bands. These specific bands showed almost the same electrophoretic mobility as the 1a and 2a proteins translated in vitro. Therefore, the anti-1a and anti-2a sera raised against the fusion proteins were shown to react specifically with the 1a and 2a proteins, respectively, translated in vitro. However, these bands were faint. Their intensities were not enhanced by changing the concentration of each antiserum, but were increased by increasing the amount of protein subjected to electrophoresis (data not shown), suggesting that the cause was not due to the low titre of each antiserum, but to the low amounts of translation products of BMV RNAs 1 and 2 in barley cells.

Detection of the 1a and 2a proteins in the BMV replicase fraction

A DEAE fraction was prepared from BMV-infected barley leaves and analysed by the immunoblotting procedure using the anti-1a and anti-2a sera. Both the 1a and 2a proteins were detected in the fraction (Fig. 4, lanes 1 and 4). Some other bands were probably due to non-specific reactions, because they were also detected in the same fraction from mock-infected barley leaves (Fig. 4, lanes 2 and 5). When portions with equal replicase activity were analysed, the more highly purified PC fraction contained lower levels of the 1a and 2a proteins than the DEAE fraction (Fig. 4, lanes 3 and 6). The reason might be that inactive forms of the 1a and 2a proteins, or some materials that inhibited replicase activities, were removed by phosphocellulose column chromatography.
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Fig. 4. Immunoblot analysis of the BMV replicase fraction with the anti-la serum (lanes 1, 2 and 3) or anti-2a serum (lanes 4, 5 and 6). Lanes 1 and 4, DEAE fraction from BMV-infected leaves; lanes 2 and 5, DEAE fraction from mock-infected leaves; lanes 3 and 6, PC fraction from BMV-infected leaves with the same replicase activity as the DEAE fraction. The positions of the 1a and 2a proteins are shown on the left.

Fig. 5. Effects of the anti-la (○) and anti-2a (○) sera on BMV replicase activity. Individual antisera and normal serum (to a final combined volume of 2 μl) were added to the PC fraction (20 μl). After pre-incubation at 4 °C for 1 h, the ingredients for the replicase assay were added to the mixtures and BMV replicase activity was measured.

Fig. 6. BMV replicase activity after removal of immunocomplexes. Sera (3 μl) were added to the PC fraction (30 μl) and incubated as in Fig. 5. Then, 50% (v/v) Protein A-Sepharose was added to a final concentration of 10%. After further incubation at 4 °C for 1 h with gentle rocking, immunocomplexes were removed by centrifugation. Ingredients for the replicase assay were added to the supernatants which corresponded to 20 μl replicase and 2 μl sera. The amount of the anti-la (○) and anti-2a (○) sera in the supernatants are indicated. Normal serum was also used as in Fig. 5.

Effect of the anti-la and anti-2a sera on BMV replicase activity

To investigate the biochemical roles of the 1a and 2a proteins in the BMV replicase fraction, anti-la or anti-2a serum was added to the PC fraction. After incubation at 4 °C for 1 h, relative replicase activities were measured. The anti-la serum inhibited replicase activity in proportion...
to the amount added but the anti-2a serum did not show such an inhibitory effect (Fig. 5). These results suggested that the 1a protein was involved in the BMV replicase as a subunit.

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As described above, the anti-2a serum did not inhibit replicase activity. One reason for this is that the 2a protein might be involved in the BMV replicase as a non-catalytic subunit, and that the binding of antibodies to the 2a protein had no inhibitory effect on activity. To examine this possibility, Protein A–Sepharose was added after incubation of the enzyme fraction and antisera, and centrifuged to remove immunocomplexes. Removal of the 1a protein strongly inhibited replicase activity (Fig. 6). On the other hand, removal of the 2a protein did not have such a marked inhibitory effect on replicase activity (Fig. 6), template dependence, specificity and production of RNAs in vitro (data not shown). These results also did not support the involvement of the 2a protein in the BMV replicase preparation.

**DISCUSSION**

The non-structural proteins 1a and 2a of BMV have been shown to be associated with BMV RNA replication (Kiberstis *et al.*, 1981; French *et al.*, 1986). However, direct involvement of these proteins in the BMV replicase complex was not certain. Bujarski *et al.* (1982) demonstrated the specific presence of the 1a protein in the BMV replicase fraction and Gorbalenya *et al.* (1985) reported that this protein contained a nucleotide-binding domain sequence. Kamer & Argos (1984) observed homology of the amino acid sequences of the 2a protein and RNA-dependent RNA polymerase of animal viruses and phages. In this study we prepared antisera specific to the 1a and 2a proteins in order to test for the involvement of either or both of these proteins in the BMV replicase complex. These antisera enabled us to identify the involvement of at least the 1a protein as a subunit in the BMV replicase which initiated minus strand synthesis de novo on BMV RNA. No data have been obtained so far to support the involvement of the 2a protein. These results are consistent with the recent report by Quadt *et al.* (1988), who also suggested that at least the 1a protein was a component of the enzyme complex.

However, there is a possibility that the anti-2a serum prepared here did not react with the 2a protein in the BMV replicase for some reason. To ascertain whether the 2a protein is involved, it would be necessary to purify the BMV replicase extensively and identify the subunits composing this complex. The BMV replicase fraction might be further purified by immunoaffinity chromatography using the anti-1a serum prepared here.

If the 2a protein is not involved in the BMV replicase we prepared, what is the function of this protein? The BMV replicase fraction prepared by Miller *et al.* (1985) showed activity for subgenomic RNA synthesis. The BMV replicase fraction prepared here also showed this activity (M. Horikoshi *et al.*, unpublished data). Preliminary experiments suggested that the 1a protein was involved in such activity but that 2a was not. The 2a protein might be involved in plus strand RNA synthesis, although no experimental data in support of this hypothesis have yet been produced.

We previously reported the involvement of the chloroplast as a site for BMV RNA replication (Nakayama *et al.*, 1987). The replication site of BMV RNA might be clearly identified by immunocytochemical methods using the anti-1a serum, as in the cases of turnip mosaic virus (Garnier *et al.*, 1986) and tobacco mosaic virus (Saito *et al.*, 1987). Similar experiments using the anti-2a serum to identify the localization of the 2a protein might give some suggestions as to its function.

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