RNA-binding activities of barley stripe mosaic virus γb fusion proteins

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The barley stripe mosaic virus (BSMV) γb gene encodes a 17 kDa cysteine-rich protein known to affect virulence and to have a role in regulating viral gene expression. We have constructed recombinant γb-glutathione S-transferase fusion proteins in Escherichia coli and have determined the ability of the purified fusion proteins and various mutant derivatives to bind nucleic acids in vitro. Gel-shift analyses revealed that the wild-type γb-fusion protein is able to bind RNA cooperatively. The binding affinity is highly selective for single-stranded RNA because double-stranded RNA, single-stranded and double-stranded DNA, and transfer RNA were unable to compete for binding with the labelled RNA probes. However, BSMV-specific sequence binding was not observed since a chloroplast RNA competed for binding with 32P-labelled transcripts derived from the BSMV genome. The first 44 amino acids of the 152 amino acid γb fusion protein encompassing one of two cysteine-rich 'zinc finger-like' motifs, and a basic region separating the finger-like motifs are required for RNA binding. Site-specific amino acid substitutions within two groups of lysine and arginine residues located in the basic motif reduced the binding affinity of the fusion protein greatly, but cysteine and histidine substitutions designed to disrupt the finger-like motifs failed to have appreciable effects on binding. These findings indicate that the regulatory properties of γb may be mediated in part by RNA binding activities.

Introduction

Barley stripe mosaic virus (BSMV) is a rod-shaped plus-strand RNA virus with an unusual tripartite genome consisting of RNAs α, β and γ that collectively encode seven major proteins (Jackson et al., 1989, 1991). Two proteins, αa and γa, constitute the BSMV-encoded replicase components (Petty et al., 1990a). Three genes organized in a 'triplex gene block' at the 3' end of RNAβ encode proteins that are required for cell-to-cell movement (Petty & Jackson, 1990; see Donald et al., 1995 for review). These consist of the 58 kDa (βb), the 17 kDa (βc) and the 14 kDa (βd) proteins, plus an additional minor protein designated βd', which is expressed in infected plants and has recently been characterized in vitro (Zhou & Jackson, 1996). The role, if any, of βd' in local or long distance movement has not yet been determined. The capsid protein, βa, is dispensable for systemic spread of BSMV (Petty & Jackson, 1990). The γb gene, which encodes a 17 kDa cysteine-rich protein, is the 3' proximal cistron of the bicistronic RNAγ (Gustafson et al., 1987). Mutations within γb have been shown to affect pathogenesis and to have regulatory effects on gene expression (Petty et al., 1994; Donald & Jackson, 1994). The γb protein is also dispensable for systemic invasion of barley in some BSMV strains, but not in others (Petty et al., 1990a).

Several small cysteine-rich proteins that appear to be involved in regulation of viral gene expression and pathogenesis are encoded by the hordei-, furo-, tobra- and carlaviruses (Morozov et al., 1989; Gramstat et al., 1990). These include the BSMV γb protein, the 16 kDa protein of tobacco rattle virus (TRV), the 14 kDa protein of beet necrotic yellow vein virus (BNYVV) and the 12 kDa protein of potato virus M. A cysteine-rich, 'zinc finger-like' motif is also found in the capsid proteins of tobacco streak virus and alfalfa mosaic virus (Sehnke et al., 1989). Although these proteins share little obvious amino acid sequence identity, several of them have some similarity to nucleic acid binding proteins (Koonin et al., 1991).

In the case of BSMV γb, the cysteine-rich domain is organized into two clusters that resemble the metal-binding 'zinc finger' motifs characteristic of many eukaryotic transcription factors (Morozov et al., 1989; Struhl, 1989). The two γb clusters are separated by a basic region related in sequence to putative chloroplast tRNA intron-encoded proteins (ClPs) and positively charged residues similar to those of the 'tether' domains

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of reverse transcriptases (Koonin et al., 1991). BSMV \( \gamma b \) also has considerable homology to an analogous protein encoded by poa semilatent virus (PSLV) (Agranovsky et al., 1992), a closely related hordeivirus (Hunter et al., 1986).

Understanding the function of \( \gamma b \) during BSMV infection is of special interest because of its role in pathogenesis and its regulatory effects on gene expression. Deletions and substitutions within the \( \gamma b \) gene that are designed to affect amino acids in the \( \gamma b \) protein can have profound effects on the symptom phenotype (Donald & Jackson, 1994; Petty et al., 1994). Several mutations within \( \gamma b \) also affect accumulation of the \( \gamma b \) protein in infected barley (Donald & Jackson, 1994) and modulate expression of RNA\( \beta \) encoded proteins during infection (Petty et al., 1990a; Donald & Jackson, 1994).

As with BSMV, the cysteine-rich proteins of TRV (Guilford et al., 1991) and BNYVV (Gilmer et al., 1992) are also dispensable for systemic infection. Moreover, local lesions elicited by BNYVV mutants containing deletions of the gene encoding the cysteine-rich 14 kDa protein are reduced in size and the accumulation of mutant viral RNA in infected leaves is an order of magnitude lower than in comparable wild-type infections (Gilmer et al., 1992; Hehn et al., 1995).

Proteins encoded by other plant viruses also contain cysteine-rich motifs that may contribute to their biological properties. For example, the proteinase (HC-Pro) that is involved in aphid transmission of potato virus Y (Robaglia et al., 1989) and other potyviruses contains a zinc finger-like motif. In tobacco vein mottling virus (TVMV), mutations involving a histidine, a lysine and a cysteine residues of the HC-Pro finger motif affect aphid transmission and symptom development in plant hosts (Atreya & Prone, 1993). In addition, highly conserved zinc finger motifs are present in the nucleocapsid proteins encoded by plant pararetroviruses (Covey, 1986) and, in figwort mosaic virus, this motif is required for infectivity (Scholthof et al., 1993). Nevertheless, despite the evidence that proteins encoding the zinc finger-like motifs contribute substantially to the pathogenicity of several RNA and DNA plant viruses, essentially no information currently exists about the biochemical functions of these proteins, or their possible interactions with nucleic acids or other viral and host proteins during infection.

To provide a more detailed insight into the role of \( \gamma b \) during BSMV infection, we have studied the ability of a \( \gamma b \) fusion protein and deletion derivatives to bind RNA in vitro. In addition, the binding abilities of fusion proteins containing site-specific mutations in the two cysteine-rich motifs of the BSMV \( \gamma b \) protein, and in the basic residue motif separating the two clusters, were evaluated. We suggest that the \( \gamma b \) protein may function in part through its RNA binding activities to regulate processes involved in BSMV replication and pathogenesis.

**Methods**

Plasmids for the expression of recombinant \( \gamma b \) protein. DNA manipulations were carried out according to protocols described by Sambrook et al. (1989) and Ausubel et al. (1987). The expression vector pGEX-2T (Pharmacia) was used for construction of recombinant BSMV \( \gamma b \)-glutathione S-transferase (GST) fusion proteins (Smith & Johnson, 1988). Sense and anti-sense PCR primers, PCR1 and PCR2, which are complementary to the 5' and 3' ends of the \( \gamma b \) ORF and contain Smal and EcoRI restriction enzyme cleavage sites (Table 1), were used to facilitate construction of a translational fusion of \( \gamma b \) with GST in pGEX-2T. This plasmid (pGEX\( \gamma b \)) retained the full amino acid sequence of the \( \gamma b \) protein fused to the C terminus of GST by an additional glycine residue. The plasmid pGEX\( \gamma b \Delta 1 \), which expressed a GST--\( \gamma b \) fusion protein lacking the first 12 amino acids of the \( \gamma b \) protein, was constructed by cloning a \( \gamma b \) gene fragment from a BSMV cDNA clone into pGEX-2T. The \( \gamma b \Delta 1 \) fragment was prepared by digestion with KpnI, treated with Klenow enzyme to trim back the 3' overhang, and digested with EcoRI. The fragment was then purified by electrophoresis in low-melting point agarose and ligated into gel purified pGEX-2T that had been digested with Smal and EcoRI.

Two other GST fusions lacking additional N-terminal \( \gamma b \) sequences were amplified with the plus-sense primers, PCR1 and PCR2 (Table 1), which are complementary to the 5' ends of targeted deletions of the \( \gamma b \) gene, together with an anti-sense primer, PCR2. The resulting fragments were gel purified and cloned into pGEX-2T to produce the plasmids pGEX\( \gamma b \Delta 2 \) and pGEX\( \gamma b \Delta 3 \), which have N-terminal deletions of 44 and 87 amino acids, respectively (Fig. 1).

To amplify the BSMV \( \gamma b \) derivatives for cloning, 100 pmol of each PCR primer was mixed with 10 ng of the phagemid cDNA clone in a 100 \( \mu l \) PCR reaction containing 250 \( \mu M \) dNTPs, 25 mM-KCl, 20 mM-Tris--HCl (pH 8.5), 3 mM-MgCl\(_2\), 0.01% gelatin and 2-5 units of Taq polymerase (Perkin-Elmer). Amplification was carried out in a Perkin-Elmer temperature cycler for 25 cycles using the following regimen: 25 s at 95 °C, 55 s at 50 °C, 2 min at 72 °C. After subcloning PCR fragments into pGEX-2T, the DNA sequence encompassing the \( \gamma b \) N-terminal 90 amino acids was verified by sequence analyses.

Specific oligonucleotides used to introduce missense mutations (Kunkel, 1985; McClary et al., 1989) into the expression vector construct pGEX\( \gamma b \) are shown in Table 1. Five oligonucleotides, \( \gamma \)MO21--\( \gamma \)MO25, were used to introduce degeneracies at the underlined nucleotide positions in order to generate multiple mutations. The oligonucleotides \( \gamma \)MO26--\( \gamma \)MO29 were used to introduce multiple missense mutations into the basic motif of \( \gamma b \). Mutations were verified by double-strand DNA sequencing with Sequenase (USB) using oligonucleotide primers (Table 1) complementary to regions near the target site (Kraft et al., 1988).

Purification of recombinant \( \gamma b \) proteins. The GST--\( \gamma b \) proteins were purified from E. coli essentially as described by Smith & Johnson (1988), suspended in PBS (150 mM-NaCl, 15 mM-NaH\(_2\)PO\(_4\), pH 7.4), supplemented with 10% glycerol and 1 mM-DTT, and frozen at -70 °C. For each pGEX\( \gamma b \) plasmid construct, protein was purified from at least two independently isolated transformants. Although the yield of purified protein varied with the expression plasmid used, 10-20 \( \mu g \) of purified GST--\( \gamma b \) or its site-specific mutated derivatives were usually recovered from a 200 ml cell culture. Successive N-terminal deletions of the \( \gamma b \) protein resulted in improved yields of the fusion proteins. For the constructs GST--\( \gamma b \Delta 1 \), GST--\( \gamma b \Delta 2 \) and GST--\( \gamma b \Delta 3 \), the respective yields of fusion protein were 40-50 \( \mu g \).
Table 1. Oligonucleotide primers used for cloning and mutagenesis of yb

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'-3')</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>ySEQ1, 21-mer</td>
<td>GTCTAGATTTAAATCTACT</td>
<td>Sequencing primer: 5' to yb ORF</td>
</tr>
<tr>
<td>PCRy1, 30-mer</td>
<td>CGCCGGGAAATGATGGCTAATTCTTGTTG</td>
<td>PCR primer/adapter for subcloning yb into pGEX-2T fusion vector.</td>
</tr>
<tr>
<td>PCRy2, 30-mer</td>
<td>CGAAATCCATATGGTGATTGGCACCATC</td>
<td>PCR primer (anti-sense)/adapter for subcloning yb into pGEX-2T fusion vector.</td>
</tr>
<tr>
<td>PCRy3, 29-mer</td>
<td>CGCCGGGATTTGGAACCCAAATAGGCC</td>
<td>Contains SmaI site.</td>
</tr>
<tr>
<td>PCRy4, 31-mer</td>
<td>CGCCGGGTCTGATTGCATTCTTCAAC</td>
<td>PCR primer/adapter for subcloning ybA2 into pGEX-2T fusion vector.</td>
</tr>
<tr>
<td>yMOb21, 32-mer</td>
<td>GGTACAGTT (G/C) TGGATGCCAT (G/C) CTCCATTGC</td>
<td>Contains Sinai site.</td>
</tr>
<tr>
<td>yMOb22, 34-mer</td>
<td>ATGTAGCCTCT (G/C) TGCCCAAAGGCC (A/T) TGGCGATCTTG</td>
<td>PCR primer (anti-sense)/adapter for subcloning yb into pGEX-2T fusion vector.</td>
</tr>
<tr>
<td>yMOb23, 33-mer</td>
<td>GAGAGGCTCT (G/C) TAGATCACTGCG (G/T) AATCGTGAAT</td>
<td>Contains EcoRI site.</td>
</tr>
<tr>
<td>yMOb24, 36-mer</td>
<td>GCTACTTCTCTT (G/C) TGTGT (G/C) TT (G/C) TGGTACCTTAACT</td>
<td>PCR primer/adapter for subcloning ybA2 into pGEX-2T fusion vector.</td>
</tr>
<tr>
<td>yMOb25, 34-mer</td>
<td>AGTACCTACT (G/C) TGTGAAGAGAT (G/C) TGAGCGAAAGC</td>
<td>Contains SmaI site.</td>
</tr>
<tr>
<td>yMOb26, 30-mer</td>
<td>AAGAGATGGGCAAAACATGTATATCTT</td>
<td>yb mutagenesis: C2 cluster (C60,C64) - (S,S)</td>
</tr>
<tr>
<td>yMOb27, 31-mer</td>
<td>TGCCAACTCTACATATGGGAAAC</td>
<td>yb mutagenesis: C2 cluster (C81,H85) - (S,L)</td>
</tr>
<tr>
<td>yMOb28, 31-mer</td>
<td>TGGGAACACTCCAGAGAGTCTATTGGGAAAC</td>
<td>yb mutagenesis: C2 cluster (C71,C75) - (S,L)</td>
</tr>
<tr>
<td>yMOb29, 36-mer</td>
<td>TATTTGAAACACAAAATATTTTT</td>
<td>yb mutagenesis: C1 cluster (C79,C83) - (S,S)</td>
</tr>
</tbody>
</table>

50–60 µg, and 200 µg per 200 ml culture. Under the same conditions, between 1 and 2 mg of purified GST were obtained from the parental expression vector, pGEX-2T.

Nucleic acid binding assays. To initiate native gel-mobility shift binding assays, 32P-labelled RNA probes were diluted to 10 ng/µl in incubation buffer (10 mM-Tris-HCl, pH 8.0; 200 mM-NaCl, 1 mM-EDTA, 10% glycerol, 1 mM-DTT), heated at 70 °C for 3 min and cooled on ice. Various amounts of purified protein were mixed with 10 ng of probe RNA in incubation buffer (up to a 300:1 molar ratio of protein:RNA under saturating conditions) to a final volume of 20 µl and incubated at 4 °C for 30 min before electrophoresis. In competition experiments, protein was pre-incubated with unlabelled RNA or DNA competitor for 20 min before adding the radioactive probe. After an additional 20 min incubation at 4 °C, samples were separated on 4% native polyacrylamide gels, dried and exposed to X-ray film.

For filter binding assays, purified fusion proteins were loaded onto duplicate SDS–polyacrylamide gels. One gel was stained with Coomassie blue and dried to visualize the purity and electrophoretic mobility of the proteins, and the other was electro-blotted onto nitrocellulose (Towbin et al., 1979). The nitrocellulose-bound protein was washed, renatured and incubated with 32P-labelled RNA probes as described by Grammat et al. (1990) before exposure to X-ray film.

Preparation of nucleic acid probes used for binding assays. In order to provide a nonspecific RNA probe, petD RNA was generated with T7 polymerase using a linearized pBluescript (Stratagene) plasmid containing a 182 bp fragment corresponding to the 3' end of the spinach...
chloroplast petD gene (Stern et al., 1989). BSMV-specific RNA probes were prepared from linearized transcription vector plasmids containing subcloned BSMV fragments. Plus- and minus-sense probes corresponding to the common 3' end (215 nt) of BSMV RNAs were prepared from the vector pTZ18U and pTZ19U (Pharmacia) subclones constructed for hybridization analyses (Petty et al., 1990a). An RNA-specific probe was obtained by subcloning a 210 bp EcoRV-KpnI 'blunt-ended' fragment containing the γb-γb intergenic region of the genomic ND18 strain y42 cDNA clone (Petty et al., 1990b) into the HindIII site of pGEM-4Z (Promega), that had been treated with Klenow polymerase. An additional 167 bp 'blunt ended' BstBI-StyI DNA fragment corresponding to the βa-βb intergenic region was subcloned in both orientations from the ND18 strain β42 cDNA genomic clone. Run-off transcription yielded a 190 nt probe containing the 167 nt intergenic sequence plus 23 residues of plasmid sequence. For filter binding assays, a full-length BSMV genome transcript was prepared by run-off transcription from the ND18 strain cDNA clone β42Sp1 after linearization with SpeI (Petty et al., 1989).

Radiolabelled probes were transcribed in the presence of [α-32P]UTP (3000 Ci/mmol; Amersham) in reactions containing 200 μM unlabelled UTP. After 1 h at 37 °C, the transcription reactions were diluted to 500 μl with 5 × TE buffer (50 mM-Tris-HCl, pH 8.0, 5 mM-EDTA), loaded onto a Sephadex G-50 spin-column (Pharmacia) to remove unincorporated nucleotides, and extracted with phenol-chloroform. The nucleic acids were then precipitated with ammonium acetate buffer (Sambrook et al., 1989). Single-stranded [poly(U), poly(C), poly(A), poly(A:U) and poly(I:C)] for use as RNA competitors in native-gel mobility-shift assays were purchased from Pharmacia and yeast tRNA was obtained from Boehringer.

DNA competitors were prepared by digesting DNA from Riboprobe vectors (Promega) with restriction enzymes, purifying the fragments on 5% preparative polyacrylamide gels, and eluting them from gel slices into ammonium acetate buffer (Sambrook et al., 1989). Single-stranded DNA was generated from the fragments by boiling for 2 min, followed by rapid cooling to 4 °C.

Results

Recombinant BSMV γb protein exhibits cooperative RNA binding activity in vitro

Initial attempts to detect RNA binding were conducted with a BSMV 17 kDa γb protein derived from pET3a, a T7 RNA polymerase expression vector (Donald et al., 1993). However, we were unable to obtain biochemically active protein from the purified un Used γb preparations, which tended to form insoluble precipitates. To circumvent this problem, chimeric proteins were constructed in which γb was fused to the carboxy-terminus of GST. We first fused GST to wild-type γb, and subsequently engineered three successive N-terminally deleted derivatives of γb (Fig. 1). The resulting fusion proteins were purified from clarified E. coli extracts by glutathione-affinity chromatography and their purity was measured by SDS-polyacrylamide gel electrophoresis (Fig. 2). We could not release native γb efficiently by thrombin cleavage, so the purified γb fusion protein are minor degradation products. Molecular mass markers (lane M) are indicated.

For filter binding assays, a full-length BSMV fusion protein before adding the radioactive fla-flb probe and completely retarded probe near the top of the gel. The results of this experiment indicated cooperative binding of the 'wild-type' fusion protein to the RNA probe because the products consisted primarily of free probe and completely retarded probe near the top of the gel. The less abundant heterodisperse products migrating at intermediate positions probably represent partially complexed probes. Additional experiments (Fig. 3b) revealed that the fusion protein encoded by GST-γbA1, in which the N-terminal 12 amino acids of γb were removed, was also able to bind the labelled RNA probe. However, the binding affinity and cooperativity appeared to be lower than that of the 'wild-type' GST-γb fusion protein as evidenced by the heterogeneous and more rapid electrophoretic mobility of the GST-γbA1 protein-RNA complexes. In contrast, the GST-γbA2 and GST-γbA3 deletions, which lacked the N-terminal 44 and 87 amino acids, respectively, failed to bind appreciable amounts of the radioactive probe (Fig. 3b). These data suggest that the N-terminal 44 amino acids of the 152 amino acid γb protein are required for RNA binding.

To assess the specificity of RNA binding, competition experiments were performed in which a 10-fold excess (by mass, relative to radioactive probe) of unlabelled competitor RNA or DNA was preincubated with the GST-γb protein before adding the radioactive βa-βb...
RNA binding by the BSMV yb protein

Fig. 3. Nucleic acid binding of the recombinant GST-yb protein and N-terminal deletions of the yb portion of the fusion protein. (a) Autoradiogram of a 4% native polyacrylamide gel illustrating the gel retardation patterns formed after a 30 min incubation at 4°C in reactions containing 10 ng of the 32P-labelled 190 nt βa-βb intergenic BSMV RNA probe with increasing amounts (0, 50, 100, 250, 500 and 750 ng/μl) of ‘wild-type’ GST-yb protein. The designation ‘P’ along the side of the gel indicates the electrophoretic position of the free probe, and ‘C’ shows the fully complexed probe migrating near the top of the gel. (b) Gel-shift assay comparing nucleic acid binding by 500 ng of the purified GST-yb fusion proteins after a 30 min incubation at 4°C with the labelled 190 nt BSMV RNAβ probe. The lane designated ‘F’ indicates a sample of the control probe that was incubated without added protein. (c) Competition experiment conducted by preincubating 750 ng of ‘wild-type’ yb fusion protein with various unlabelled competitor nucleic acids followed by a 20 min incubation with the 32P-labelled 190 nt BSMV RNAβ probe. The preincubated competitors, present at 10:1 mass ratios relative to the 190 nt BSMV probe, were: (1) poly(U); (2) double-stranded poly(U):poly(A); (3) yeast tRNA; (4) the unlabelled 190 nt BSMV RNAβ probe; (5) a 182 nt nonspecific petD RNA probe; (6) single-stranded DNA; (7) double-stranded DNA fragments, homologous to the BSMV probe. (a) (lane 750 ng) and (c) can be compared directly as the samples were separated on the same gel.

intergenic probe (Fig. 3c). Unlabelled substrates that were able to compete most effectively for BSMV β-RNA binding activity were poly(U) (Fig. 3c, lane 1) and the homologous BSMV-specific RNAβ probe (Fig. 3c, lane 4). Both substrates exhibited similar high levels of competition, seen as reductions in the amount of label appearing near the top of the gel. We also obtained similar results in competition experiments with plus- and minus-sense derivatives of the BSMV RNAγ probe, and with the plus- and minus-sense BSMV probes homologous to the common 3’ ends of BSMV RNAs (data not shown). The non-viral 182 nt RNA probe petD (Fig. 3c, lane 5), which corresponds to the 3’ end of the chloroplast petD gene (Stern et al., 1989), appeared to be a weaker competitor in this particular experiment. However, in several other titration and competition experiments, the BSMV and petD probes had similar band-retardation profiles, and unlabelled BSMV probes were also able to compete effectively with the radioactive petD probe (not presented). Therefore, these results in toto suggest that the differences, if any, between the binding affinities of GST-yb for the petD and BSMV RNA probes are negligible. In contrast, synthetic double-stranded RNA [poly(U):poly(A)], yeast tRNA, and single- or double-stranded DNA of similar size to and sharing common sequence with the BSMV RNAβ probe were unable to compete for binding (Fig. 3c, lanes 2, 3, 6 and 7). Competition experiments with the GST-γbΔ1 encoded fusion protein (not shown) yielded results similar to those obtained with GST-γb. From these results, we conclude that the GST-γb fusion protein has a single-stranded RNA binding activity which appears to have little sequence specificity under the in vitro conditions used in our experiments.

Mutations within a basic motif of the GST-γb protein affect its RNA binding activity

We have previously shown that amino acid substitutions within the cysteine-rich C1 and C2 clusters of yb, and the basic motif separating the clusters, have profound effects on the disease phenotype in barley (Donald & Jackson,
1994). To investigate the contribution of these amino acids to RNA binding, a series of substitutions identical to those previously used in the pathogenesis study were introduced into the pGEX-γb plasmid (Fig. 4). These substitutions targeted each cysteine and histidine residue in clusters 1 and 2, both individually and in combination. Cysteine residues were changed to serines, and the single histidine in cluster 2 was changed to a leucine. Additional mutations were introduced into the basic motif separating the two cysteine-rich clusters to modify amino acids that are conserved when compared to sequences of the related PSLV γb protein (Agranovsky et al., 1992), or the ‘tether’ domain of the chloroplast intron proteins (CIP) (Koonin et al., 1991). Mutation 26 substitutes glutamine and asparagine for the adjacent arginine and lysine residues that are located at the carboxy-terminal side of the last cysteine residue of cluster 1. Mutation 27 targets two lysine residues that are sandwiched between two tyrosine residues, and in mutation 28, the two tyrosines are replaced with serines. These four residues are conserved in the CIP proteins, but are less stringently maintained in the PSLV motif (Fig. 4), which retains only one lysine and one tyrosine residue. Mutation 29 substitutes three basic residues similarly placed in the CIP proteins and in PSLV (Fig. 4).

The effects of the γb mutations on RNA binding in vitro by the mutant GST-γb fusion proteins were assessed in several mobility-shift experiments, one of which is shown in Fig. 5. The results show that two of the substitution mutations in the basic motif between the two cysteine-rich clusters (Fig. 4) reduced the RNA binding affinity of the fusion protein greatly, while a third mutation had moderate effects on binding (Fig. 5). The basic motif mutants BM26 and BM29, which contain substitutions neutralizing two of the three groups of basic residues, had the most pronounced effects on the ability of the GST-γb fusion protein to form retarded complexes with the labelled probe. However, these substitutions did not completely eliminate RNA binding because small amounts of the fully shifted band, plus a minor band with a mobility slower than that of the probe, were present. These minor bands migrated similarly to those of the more easily visualized bands in adjacent lanes containing the more active mutant

![Diagram of the BSMV γb cysteine-rich and basic motifs with comparison to PSLV and CIP sequences.](image-url)
proteins BM27 and BM28 (Fig. 5). Therefore, mutations BM26 and BM29 appear to affect cooperative interactions that normally result in formation of the highly retarded protein–RNA complexes with the unmutated GST–yb fusion proteins. Mutation 27, which targets two lysines in the most distal of the three groups of basic residues, caused a moderate reduction in binding activity, while mutation 28 was able to form retarded complexes similar to those of the wild-type GST–yb fusion protein (Fig. 5). These results thus suggest that the basic residues modified by mutations 26 and 29 may interact directly with the negatively charged nucleic acid, while residues substituted in mutations 27 and 28 have more indirect interactions with the nucleic acid during binding.

In contrast, proteins containing single amino acid substitutions within the C1 and C2 motifs (Fig. 4) had RNA binding activities similar to those of the ‘wild-type’ fusion protein, GST–yb (data not shown). The double mutant C2 (64, 71), which alters nonconserved cysteines at position 64 and 71, also failed to affect binding. In addition, two cluster 2 double substitution mutants, C2 (60, 85), and C2 (81, 85), that were designed to disrupt the finger motif by targeting conserved cysteines 60, and 81 and histidine 85 (Fig. 4), also retained binding activity similar to that of GST–yb (Fig. 5). The cluster 1 triple mutant C1 (9, 10, 19) reduced the proportion of retarded probe near the top of the gel, but this mutant was still able to form protein–probe complexes with intermediate migration patterns (Fig. 5). Thus, multiple cysteine substitutions that were designed to destroy the finger-like motifs in the two clusters had only moderate effects on RNA binding activity.
Although there was some variability in the profiles of retarded protein–RNA complexes from different protein preparations, the relative abilities of the individual mutant γb fusion proteins to form complexes with labelled RNA probes were quite reproducible. In addition, separate filter-binding experiments were also conducted in which recombinant proteins were transferred from SDS–polyacrylamide gels, immobilized on nitrocellulose, renatured and incubated with radioactive probes (Gramstat et al., 1990). The binding activity in these experiments (Fig. 6) confirmed the results of the mobility gel-shift assays in two important respects. First, the basic motif mutant proteins BM26 and BM29 lacked substantial RNA binding activity. Second, the unmutated GST–γb fusion protein, all fusions containing cysteine or histidine residue substitutions in either cluster 1 or cluster 2, and mutant protein BM28 exhibited RNA binding activity. Interestingly, mutant protein BM27, which had low RNA binding activity in the gel-shift assay, failed to show activity in the filter binding assay. However, the inability of mutant BM27 to bind RNA in this assay may reflect compromised protein stability as much as lowered RNA binding activity, since, unlike the more sensitive gel-shift assay, the filter binding assay requires renaturation of detergent-treated protein as a prerequisite for RNA binding.

Discussion

We have demonstrated that a recombinant GST–γb fusion protein has the ability to bind single-stranded RNA in vitro and to discriminate between single-stranded and double-stranded RNA, tRNA and DNA in competition experiments. Deletion analyses suggest that the RNA binding domain is located within the amino-terminal third (44 amino acids) of the γb protein that encompasses the first of two cysteine-rich clusters and a basic motif separating the two clusters. The basic motif appears to contribute substantially to RNA binding because substitution of two groups of basic amino acids within the motif that are immediately adjacent to the C1 cluster reduced the binding activity greatly. Mutations within a third group of amino acids proximal to the C2 cluster resulted in a more moderate reduction in RNA binding activity. Additional site-specific substitutions revealed that conserved amino acids within the two cysteine-rich clusters are dispensable for the in vitro RNA binding activity. We were unable to detect BSMV-specific RNA binding, but the results do show that the amino acid sequences affecting binding reside within one of the regions that we have previously shown to contribute to virulence (Donald & Jackson, 1994; Petty et al., 1990a, 1994).

The biochemical effects of mutations affecting the basic motif are reminiscent of similar mutations within a basic domain of retroviral nucleocapsid proteins that have RNA binding activities. These effects were first reported by Prats et al. (1991), who found that the zinc finger motif in the nucleocapsid protein of Moloney murine leukaemia virus (MoMLV) was dispensable for RNA binding in vitro despite modifications that destroy the affinity of the motif for Zn\(^{2+}\). Instead, a pair of lysines adjacent to the zinc finger motif, in positions analogous to those of the lysine and arginine residues substituted in γb mutant BM26 (Fig. 4), were required for non-specific RNA binding. Another similarity to the BSMV γb results was that mutations in the MoMLV nucleocapsid zinc finger motif, like those in the C1 and C2 clusters, had no obvious effects on RNA associations in vitro. From these results, Prats et al. (1991) postulated that interactions of the finger motif with zinc influence the conformation requirements of the nucleocapsid protein for specific packaging of the retroviral RNA genome. Additional studies that are relevant to our findings with BSMV suggest that the human immunodeficiency virus (HIV) nucleocapsid protein, which contains two zinc finger-like motifs flanked by basic amino acid residues, has RNA annealing activities that are independent of the activity of the two zinc fingers (Berkowicz et al., 1993; Berkowicz & Goff, 1994; De Rocquigny et al., 1992; Summers et al., 1992). Nonspecific RNA binding is thought to occur via electrostatic and hydrophobic interactions involving basic amino acids with some similarity to those composing the BSMV basic motif, whereas the finger-like regions appear to govern base-specific RNA sequence interactions.

Mutations within the BSMV γb gene have a number of pleiotropic effects on the symptom phenotype elicited in both systemic and local lesion hosts (Petty et al., 1990a, 1994; Donald & Jackson, 1994). Corresponding analyses of protein accumulation suggest that γb also has a role in expression of BSMV genes during infection, particularly a trans-acting effect on proteins encoded by RNAβ (Petty et al., 1990a; Donald & Jackson, 1994). Nevertheless, the RNA binding properties of the γb mutations were only partly correlated with the abnormal pathological effects that we have observed previously. This was most clearly evident with the single mutations within the C1 and C2 clusters which failed to affect RNA binding in vitro, yet elicited abnormal infection phenotypes (Donald & Jackson, 1994). A more complicated observation, however, was that the mutations within the basic motif that caused the most pronounced reductions in RNA binding affinity resulted in profound alterations in the symptom phenotype, but in different ways. Mutation 26, which altered the arginine and lysine residues proximal to the C1 cluster, induced a bleached phenotype that was somewhat more extreme than that produced by the C1
mutations, whereas mutation 29, which targeted the three central lysine and arginine residues, elicited the null phenotype characteristic of the C2 mutants and the yb deletion mutants (Donald & Jackson, 1994). However, mutation 27, which changed two lysine residues between two tyrosines, had reduced RNA binding affinity, yet this mutation failed to have a substantial effect on the disease phenotype. In contrast, substitution of the tyrosines in BM28 had no discernible effect on RNA binding, yet they led to a pronounced bleached phenotype (Donald & Jackson, 1994). Taken together, these results suggest that yb is a multifunctional protein, and that the biological effects of the yb mutations result from disruption of different functions, one of which involves RNA association activities.

Our inability to make more direct correlations between our in vitro and in vivo observations may be caused to some extent by the conditions used to assess RNA binding. For example, various virus or host proteins not present in the reactions may contribute to the specificity of binding in ways that we are presently unable to predict. It would also have been preferable to use native yb in the binding assays, but we were unable to release yb from GST in sufficient amounts for biochemical assays by thrombin treatment or to purify a soluble biochemically active yb protein by expression from the pET3a vector. Therefore, the results derived from the fusion protein experiments, particularly the lack of sequence specific RNA binding, must be interpreted with some caution because protein folding, protein–protein interactions and protein–RNA interactions that could have influenced binding activity may have been affected by the fusions. Nevertheless, a strategy similar to the one used in the present study has been described that permits sequence specific binding of GST fusions with the HIV Gag and nucleocapsid proteins (GST–Gag, GST–NC) to probes containing cis-elements known to be involved in packaging of HIV genomic RNA (Berkowicz et al., 1993; Berkowicz & Goff, 1994). A less plausible alternative possibility is that yb is sequestered in the cell in such a way as to eliminate the necessity for specific RNA binding, as has been suggested for several viral movement proteins (Citovsky & Zambryski, 1993). We are presently attempting to isolate native recombinant yb for in vitro binding assays and are also attempting to use protoplasts to develop a biological assay to assess RNA binding. These experiments may provide more pertinent information to explain how the RNA binding activities of yb contribute to its diverse regulatory and phenotypic properties.

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