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Analysis of the Interaction of Viral RNA Replication Proteins by Using the Yeast Two-Hybrid Assay

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The yeast two-hybrid system has been a useful tool in the genetic evaluation of protein-protein interactions. However, the biological relevance of these two-hybrid interactions to viral positive-strand RNA replication has not been demonstrated. The brome mosaic virus (BMV) system has been characterized extensively both genetically and biochemically, providing numerous mutations in the BMV 1a helicase-like and 2a polymerase-like proteins. We have tested wild-type 1a and 18 insertion mutations of 1a and found a perfect correlation between the in planta phenotypes and their ability to interact with 2a in the two-hybrid system. This finding allowed further characterization of the interaction between and among the BMV viral proteins. Using the two-hybrid assay, we have found that the interaction between the helicase-like region of 1a and the N terminus of 2a is stabilized by the presence of the centrally conserved polymerase-like domain of 2a. We have also identified a novel interaction between the 1a helicase-like protein and itself. Additionally, we have found this interaction in two related tripartite RNA viruses, cowpea chlorotic mottle virus and cucumber mosaic virus. We have demonstrated that this protein-protein interaction is specific to homologous pairings of the protein.

Replicases of positive-strand RNA viruses are thought to function as complexes of both viral and host proteins (5, 31). While the sequences of many viral replication proteins have been identified, we have only a minimal understanding of the higher-order interactions between them. The best-studied viral replicase, from the coliphage Qβ, is known to be composed of one viral protein (subunit β) and four host-derived proteins (S1, EF-Tu, EF-Ts, and HF-1) (7). However, the interaction between the subunits is not defined. We have focused on dissecting the interactions between the replication proteins of the monocot-infecting brome mosaic virus (BMV) in order to further understand RNA replicase structure and function.

The BMV genome is composed of three genomic positive-strand RNAs designated RNA1, RNA2, and RNA3. The genomic RNAs serve dual functions as mRNAs for translation and as templates for the synthesis of the complementary negative-strand RNAs. RNA1 and RNA2 encode the replication proteins, 1a (109 kDa) and 2a (96 kDa). Expression of 1a and 2a from transfected RNAs is sufficient for RNA replication in protoplasts (21).

Several domains of 1a and 2a have been identified based on sequence similarity. The N terminus of 1a resembles the nsP1 protein of Sindbis virus, suggesting a role in RNA capping (14, 27, 35). The 1a C terminus has sequence homology to many viral and cellular helicases (13). 2a contains two nonconserved regions flanking a centrally conserved domain which shares sequence motifs with many polymerases, including the presence of the Mg^{2+}-binding GDD motif (4). Mutations in 1a and 2a have been shown to abolish or greatly reduce RNA replication levels (22, 40).

The BMV 1a and 2a proteins have been demonstrated to interact both in vitro and in the yeast two-hybrid system (20, 29). The two-hybrid system detects protein-protein interactions in yeast by taking advantage of the modular nature of transcription factors (25). A protein of interest is fused to a DNA binding domain, while a second protein is fused to a transcription activation domain. If two proteins interact, the chimeric protein complex can bind to a promoter region containing the DNA recognition site, and the activation domain stimulates transcription initiation of a reporter gene(s). In the system that we use, the two genes activated are lacZ and *HIS3* (41). We seek to develop the two-hybrid assay as a genetic system to further characterize 1a-2a interactions as well as look for new ones. However, of all of the two-hybrid results presented thus far, their relevance to viral replication has not been rigorously demonstrated (15, 23, 29). In this study, we have used an extensive set of previously characterized mutants of 1a and shown an absolute correlation between the in planta phenotypes of these mutants and their ability to interact in the two-hybrid system. These findings, along with the fact that BMV can replicate in yeast (17), indicate that the two-hybrid system is suitable for further dissection of the interactions between BMV RNA replication proteins.

Using the two-hybrid assay, we found that a longer version of 2a, including the centrally conserved polymerase-like domain, forms a more stable complex with 1a than does the N terminus of 2a alone. We also identified a previously unreported interaction between the BMV 1a helicase-like protein and itself. We analyzed the specificity of the 1a-1a interaction in two additional tripartite viruses, cowpea chlorotic mottle virus (CCMV) and cucumber mosaic virus (CMV). Homologous pairings of all three tripartite viruses resulted in strong 1a-1a interactions, while heterologous combinations resulted in much reduced interactions. This observation suggests that a specific interaction between 1a and itself may be a common theme for the positive-strand tripartite RNA viruses.

MATERIALS AND METHODS

Manipulation of *Saccharomyces cerevisae*. Y835 (*lys2::lexAop-HIS3 ura3::lexAop-lacZ trp1-901 his3 leu2-3,-112 ade2 Δgal14 Δgal80* [41]) was used in our experiments. Yeast cells were transformed by the method of Becker and Guarente (6). Production of β-galactosidase was quantitated by the filter colony lift assay of Chevray and Nathans (9). Quantitative assays of β-galactosidase specific activity were performed by the method of Miller (28) and as previously described (29) except that we incubated the cultures with shaking for 3 h at 30°C before pelleting the cells.

Two-hybrid interactions were also detected in two ways by growth on selective media lacking histidine (36). First, a qualitative assay was performed by simply

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TABLE 1. Summary of plasmids and encoded polypeptides

Plasmid[a]	Change in polypeptide[b]	Encoded fusion polypeptide	Source or reference
DNA binding domain fusions			
pBTM116	NA	LexA (amino acids 1–211)	Stan Fields
pB-B1a	None	LexA-BMV 1a	This work
pB-CC1a	None	LexA-CCMV 1a	This work
pB-C1a	None	LexA-CMV 1a	This work
1a insertional mutations—amino acids inserted			
pB-PK1	G-S (267)	LexA-PK1	This work
pB-PK2	D-P (507)	LexA-PK2	This work
pB-PK3	G-S (5)	LexA-PK3	This work
pB-PK4	G-S (492)	LexA-PK4	This work
pB-PK6	G-S (95)	LexA-PK6	This work
pB-PK7	G-S (239)	LexA-PK7	This work
pB-PK9	G-S (154)	LexA-PK9	This work
pB-PK10	G-P (198)	LexA-PK10	This work
pB-PK11	G-P (203)	LexA-PK11	This work
pB-PK13	W-A-H (403)	LexA-PK13	This work
pB-PK14	G-P (556)	LexA-PK14	This work
pB-PK15	W-A-H (651)	LexA-PK15	This work
pB-PK16	G-P-T (464)	LexA-PK16	This work
pB-PK17	D-P (869)	LexA-PK17	This work
pB-PK18	G-S (912)	LexA-PK18	This work
pB-PK19	G-S (670)	LexA-PK19	This work
pB-PK20	D-P (905)	LexA-PK20	This work
Transcription activation domain fusions—amino acids deleted			
pGAD424	NA	GAL4 activation domain	Stan Fields
pG-B1a	None	GAL4-BMV 1a	This work
pG-2a-N	141–961	GAL4-2aN	29
pG-2aΔC	699–961	GAL4-2aΔC	This work
pG-2aΔN	1–161	GAL4-2aΔN	This work
pG-2aΔN/C	1–161, 698–961	GAL4-2aΔN/C	This work
pG-3a	None	GAL4-BMV 3a	This work
pG-CP	None	GAL4-BMV CP	This work
pG-CC1a	None	GAL4-CCMV 1a	This work
pG-C1a	None	GAL4-CMV 1a	This work

[a] Insertional mutations were first described by Kroner et al. (22). "pB-" indicates fusions created in pBTM116; "pG-" indicates fusions created in pGAD424.
[b] NA, not applicable. For 1a insertional mutations, numbers in parentheses indicate the amino acid which occurs immediately prior to the insertion.

patching strains out onto selective plates which either lacked or contained histidine. Second, a quantitative assay was done by calculating the percent survival of each strain on media lacking histidine. For the latter assay, 3-day-old yeast colonies were scraped off agar plates, washed twice in ice-cold water, and suspended in a final volume of 1.0 ml to an optical density at 600 nm of 0.1, corresponding to approximately 2×10^5 viable cells per ml. At appropriate dilutions, the cell suspensions were plated onto media either with or without histidine. Colonies were counted after 3 days of incubation, and the percent survival of strains was determined from the number of colonies which grew on plates lacking histidine versus the number of colonies which grew on plates amended with histidine.

Construction of plasmids. The plasmids and their encoded proteins are detailed in Table 1. Viral sequences were usually cloned into two vectors: pGAD424, which contains a GAL4 transcription activation domain; and pBTM116, which contains a LexA DNA binding domain (kind gifts of Stan Fields).

PCR was used to generate DNA fragments flanked by appropriate restriction sites for cloning into two-hybrid plasmids. Table 2 lists the oligonucleotides and templates used in the PCRs along with the restriction sites employed. All amplified DNA fragments were directly ligated into plasmid pCRII (Invitrogen Inc., San Diego, Calif.). The resulting recombinant plasmids were then digested with the appropriate restriction enzymes and cloned as in-frame fusions to LexA and GAL4 in pBTM116 and pGAD424, respectively. Production of the plasmid containing the N terminus of 2a, pG-2a-N, has been previously reported (21). All clones were subject to restriction analysis for correct orientation and internal site composition. In most cases, at least two independently derived clones were tested.

RESULTS

Correlation between yeast two-hybrid protein-protein interactions and in planta replication. The helicase-like portion of 1a and the N-terminal 140 residues of 2a can interact in vitro and in the yeast two-hybrid system (20, 29). Additionally, potyviral proteins including the NIa and NIb proteins of tobacco etch virus and tobacco vein mottling virus have been shown to interact in the two-hybrid system (15, 23). While the relevance of these interactions to viral replication has been assumed, it has not been rigorously demonstrated for any of them. We wanted to demonstrate the relevance of the two-hybrid system for BMV RNA replication proteins. BMV is a good system for a thorough analysis of the efficacy of the two-hybrid system since there exist numerous well-characterized mutations in the RNA replication proteins. One series, the PK mutants, each contain an insertion of two or three amino acids in the BMV 1a coding sequence (22). The abilities of these mutants to replicate in protoplasts and to coimmunoprecipitate with 2a have been previously characterized (19, 22) and are summarized in Table 3.

We tested the abilities of wild-type 1a (wt1a) and the PK mutants to interact with two truncations of 2a. The first, 2a-N, contains the first 140 residues of 2a, which have been shown to be sufficient for interaction with the helicase-like region of 1a (20). The second, 2aΔC, lacks the C-terminal 124 residues, which have been shown to be dispensable for RNA replication in protoplasts (40).

We observed an absolute correlation between the replication phenotypes of the PK mutants in barley protoplasts and

TABLE 2. Names and descriptions of oligonucleotides used

Name	Sequence[a]	Complementary viral sequences[b]	Restriction site	PCR template	Reference for template
B103-2	5′ATA*GAATTC*ATGTCAAGTTCTATCCG3′	75–89	*Eco*RI	pB1TP3	17
B102	5′ATA*GGATCC*TCACTCAGAGACAAGCG3′	2979–2963	*Bam*HI	pB1TP3	
B1PK3′	5′A*CTGCAG*TCACTCAGAGACAAGGC3′	2979–2965	*Pst*I	pB1PKN	22
B3a 5′	5′A*GAATTC*CATATGTCTAACATAGTTTCTCC3′	92–111	*Eco*RI	pB3TP8	17
B3a 3′	5′A*GGATCC*CTATTTAATTCTAAGCGTAG3′	1003–984	*Bam*HI	pB3TP8	17
BCP 5′	5′A*GAATTC*ATGTCGACTTCAGGAAC3′	1251–1267	*Eco*RI	pB3TP8	17
BCP 3′	5′A*GGATCCC*TACCTATAAACCGGGGTGA3′	1820–1802	*Bam*HI	pB3TP8	17
CC101	5′ATA*GGATCC*TGGCAAGTTCTTTAG3′	73–86	*Bam*HI	pCC1TP1	3
CC102	5′ATA*GTCGAC*TTCGACTAGGCACTG3′	3145–3131	*Sal*I	pCC1TP1	3
C101	5′A*GAATTC*ATGGCGACGTCCTCG3′	95–109	*Eco*RI	pFny106	34
C102	5′A*GGATCC*CTAAGCACGAGCAAC3′	3076–3062	*Bam*HI	pFny106	34
B2a 5′ ΔN	5′AA*GAATCC*ATGGATACCCCCAAGGAG3′	587–601	*Eco*RI	pB2TP5	17
B2a 3′ ΔCa	5′AA*GGATCC*ACAGTAGCAATCAGAGAA3′	2194–2177	*Bam*HI	pB2TP5	17
B2a 3′ ΔCb	5′ATA*GGATCC*TCAGGTACAGTAGC3′	2197–2187	*Bam*HI	pB2TP5	17
B2a 3′	5′AA*GGATCC*TCATCTCAGATCAGAGGG3′	2555–2572	*Bam*HI	pB2TP5	17

[a] Restriction sites are in italics; while viral sequences are underlined.
[b] Nucleotide numbers are taken from references 2 (BMV RNA1 and -2), 1 (BMV RNA3), 11 (CCMV RNA1), and 33 (CMV1).

their abilities to interact with both versions of 2a (Fig. 1 and Table 3). All replicating 1a mutants (PK1, -2, -4, -9, -14, -19, and -21) can still interact with both versions of 2a, while the nonreplicating mutants (PK3, -6, -7, -10, -11, -13, -15, to -18, and -20 [Table 3]) cannot. These experiments were performed qualitatively in two different ways. Colonies were scored as + for the induction of β-galactosidase activity, which turns cells blue in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), while colonies which did not change color were scored as − (Table 3). The interaction was also scored for *HIS3* expression by growing transformed strains on plates which either contained or lacked histidine (Fig. 1A). PK1 was scored as a +/− in Table 3 because it turned blue in the filter assay but could not grow on plates lacking His (Fig. 1B). This difference will be addressed in greater detail later. The filter assay may be a more sensitive method of detection since it can pick up signal from the accumulation of β-galactosidase, a very stable enzyme. As a negative control, all of the plasmids were assayed in the absence of potential fusion partners and, as expected, did not result in β-galactosidase production (data not shown). PK5 and PK12 were not tested in our experiments because PCR-generated clones and the original plasmid stocks did not yield the expected digestion patterns.

Biochemical assays have been used to identify a protease-resistant structure in 1a and for 1a-2a coimmunoprecipitations (19, 29). This protease-resistant structure exists in the absence of the N-terminal methyltransferase-like half of 1a and appears to be necessary in binding 2a. PK19, a temperature-sensitive replicating mutant, was shown to be temperature sensitive with respect to protease-resistance and coimmunoprecipitation of 2a. We did not observe this temperature sensitivity of PK19 in our two-hybrid assays because at the nonpermissive temperature (35°C) tested by Kroner et al. (22), our positive control interactions were greatly reduced. All other replicating mutants (PK1, -2, -4, -9, -14, and -21) retained this protease-resistant structure and could bind to 2a in coimmunoprecipitation assays. Some of the nonreplicating mutants (PK3, -6, -7, -10, -11, -13, and -15) were resistant to protease digestion and could coimmunoprecipitate 2a. However, PK16, -17, -18, and -20, which are insertions into the C-terminal helicase-like half of the protein, lost the protease-resistant structure and the ability to bind 2a in vitro (19, 29).

In interpreting the results of these biochemical assays, it is important to stress that the presence of a structure or coimmunoprecipitation in vitro does not mean that a mutant protein has retained all of its normal activities. Additional in vivo requirements of the 1a-2a interaction may not be detected in the in vitro experiments. The two-hybrid results we observe are most consistent with the results from BMV replication in protoplasts.

Analysis of other protein-protein interactions in the two-hybrid assay. We used the two-hybrid system to look for additional interactions between and among BMV proteins. Constructs suitable for two-hybrid analysis of BMV 1a, 2a-N, 2aΔC, 3a, and CP were made and analyzed in pairwise combinations. At least two independently transformed strains were

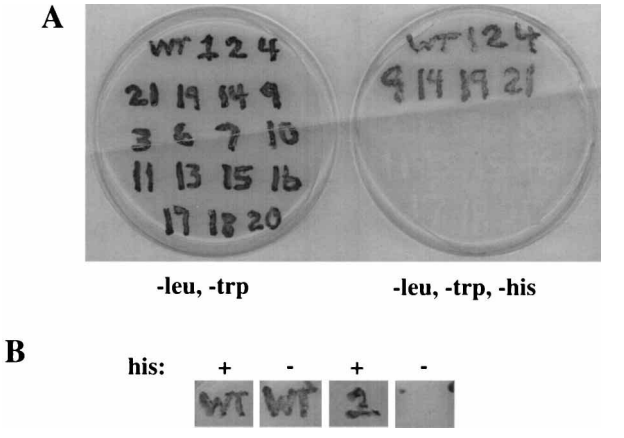

FIG. 1. Growth of yeast strains on media with or without histidine. (A) Strains transformed with pG-2aΔC and either pB-B1a or a comparable PK mutant. (B) Strains transformed with pG-2a-N and either pB-B1a or pB-PK1. The yeast transformants were written onto plates in a manner which indicates the identity of the mutant 1a protein. As explained in the text, PK5 and PK12 are not included in our experiments.

TABLE 3. Summary of in vivo, in vitro, and two-hybrid interaction properties of PK mutants

1a construct[a]	Protoplast replication	Two-hybrid binding to[b]:		Protease resistance[c]	Co-IP with BMV 2a[d]
		2a-N	2aΔC		
Replicating					
wt1a	+	+	+	+	+
PK1	TS[f]	+/−	+	NA[e]	NA
PK2	+	+	+	+	+
PK4	TS	+	+	+	+
PK5	+	NA	NA	+	+
PK9	+	+	+	+	+
PK14	+	+	+	+	+
PK19	TS	+	+	TS	TS
PK21	+	+	+	+	+
Nonreplicating					
PK3	−	−	−	+	+
PK6	−	−	−	+	+
PK7	−	−	−	+	+
PK10	−	−	−	+	+
PK11	−	−	−	+	+
PK12	−	NA	NA	+	+
PK13	−	−	−	+	+
PK15	−	−	−	+	+
PK16	−	−	−	−	−
PK17	−	−	−	−	−
PK18	−	−	−	−	−
PK20	−	−	−	−	−

[a] The PK mutants and their phenotypes were originally described by Kroner et al. (22).
[b] +, blue colony color; −, no color change.
[c] Data from reference 29.
[d] Coimmunoprecipitation (Co-IP) data from reference 20.
[e] NA, not applicable.
[f] TS, temperature sensitive.

TABLE 4. Summary of interactions between 1a and other BMV proteins, including various truncations of 2a

Plasmid(s) carried by yeast strain Y835[a]	Appearance of colonies in filter assay[b]	Relative β-galactosidase specific activity[c]	
		Exp 1	Exp 2
pB-B1a only	W	1.00	1.00
pB-B1a and pGAD424	W	1.69	1.53
pBTM116 and pG-B1a	W	1.08	1.20
pB-B1a and pG-3a	W	1.30	1.17
pB-B1a and pG-CP	W	1.10	0.96
pB-B1a and pG-2a-N	B	4.69	3.82
pB-B1a and pG-2aΔC	B	15.2	8.21
pB-B1a and pG-B1a	B	8.45	6.26
pB-B1a and pG-2aΔN/C[d]	W	1.00	0.56
pB-B1a and pG-2aΔN	W	0.90	1.04

[a] Plasmids are described in Tables 1 and 2.
[b] W, white; B, blue.
[c] Specific activity is calculated as micromoles of ONPG hydrolyzed per min per milligram of protein. Data are shown as fold activity relative to the activity in cells containing only pB-FL1a. Results are averages of two independent assays.
[d] Data for the interactions of B1a with 2aΔN and 2aΔN/C are shown as fold activity relative to "pB-B1a and pG-2aΔN/C" in experiment 1.

tested for β-galactosidase activity by using the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis assay in at least two independent sets of ONPG tests and/or filter lift assays (Table 4).

No interactions were observed between the following heterologous pairs of proteins: 1a with 3a or CP, CP with 2a-N or 2aΔC, and 3a with 2a-N or 2aΔC or CP (Table 4 and data not shown). Also, no interactions were observed in the 3a-3a and CP-CP homologous pairings. The fact that we did not see 3a-3a or CP-CP interactions does not mean that they do not occur. It is possible that these interactions are mediated in some way by the viral RNA or other requirements which are not duplicated in the yeast two-hybrid system. The homologous pairing of 2aΔC with itself could not be tested because the 2a protein, when fused to the DNA binding domain, resulted in some transcription activation in the absence of a fusion partner. When 2aΔC was fused to the transcription activation domain, no β-galactosidase activation was observed in the absence of fusion partners. This phenomenon has been observed with proteins which are activators of transcription or contain sequences resembling transcription activation motifs (12).

Additional site of contact between 1a and 2a. We have previously reported that the helicase-like portion of 1a interacts with 2a-N in the two-hybrid system, yielding β-galactosidase activities of 100-fold over background (29). In the experiments in this report, wt1a interacted with 2a-N, generating a relative activity of fourfold over background (Table 4). Smaller fusion proteins generally work better in the two-hybrid system, possibly because they can enter the nucleus more efficiently (12). It is therefore unexpected that wt1a interacts with 2aΔC with a relative activity 8- to 15-fold over background. This two- to threefold enhancement was reproduced in more than four independent experiments and was observed qualitatively with the majority of the replicating PK mutants (Table 5 and data not shown). One replicating mutant showed a dramatic difference in its ability to interact with 2a-N versus 2aΔC. PK1 interacted with both versions of 2a in the filter assays (Table 3); however, the interaction with 2a-N appeared to be weaker, as indicated by a much weaker blue signal (data not shown). In further support of this difference, PK1 when expressed with 2a-N could not grow on plates lacking histidine whereas transformants containing PK1 and 2aΔC could grow on plates lacking histidine (Fig. 1B, Table 3, and data not shown). We quantitated the interaction of 2a-N and 2aΔC with different versions of 1a by the number of colonies formed on plates lacking

TABLE 5. Differentiation in the ability of wt1a and 1a mutants to interact with 2a-N versus 2aΔC

1a construct[a]	β-Galactosidase activity[b]		% survival[c]	
	GAL4–2a-N[a]	GAL4–2aΔC	GAL4–2a-N	GAL4–2aΔC
LexA-BMV1a				
A	++	+++	43	112
B	++	+++	44	100
LexA-PK9				
A	++	+++	48	78
B	++	+++	68	100
LexA-PK1				
A	+	+++	<0.02	60
B	+	+++	<0.02	48
LexA-PK18				
A	−	−	<0.02	<0.02
B	−	−	<0.02	<0.02

[a] Polypeptide fusions are described in Table 1. A and B indicate independently derived transformants.
[b] Determined by filter assays. +++, rapid (<4 h), strong reaction; ++, slower (<8 h) but still strong reaction; +, very slow (>20 h) and weak reaction; −, no detected β-galactosidase activity after >24 h of incubation.
[c] Determined after 3 days of incubation as total number of colonies growing on plates with histidine/total number of colonies growing on plates lacking histidine × 100.

histidine. As can be seen in Table 5, wt1a had at least 100% survival when expressed with 2aΔC and only about 43% survival when expressed with 2a-N. This trend was also observed for another replication-competent PK mutant, PK9. PK18, a nonreplicating mutant, had less than 0.02% survival with both versions of 2a. PK1 exhibited less than 0.02% survival when expressed with 2a-N and approximately 50% survival when expressed with 2aΔC. Thus, in several independent assays, wt1a and the majority of the replicating PK mutants interacted better with 2aΔC than 2a-N (Table 5 and data not shown).

The different binding ability of PK1, along with the fact that 2aΔC reproducibly shows higher activities than 2a-N, further suggests that there may be an additional site(s) of 1a-2a interaction besides the 2a N terminus. Alternatively, an inhibitory sequence of the 1a-2a interaction may exist in the methyltransferase-like portion of 1a which is overcome in the presence of the longer version of 2a. In support of a possible additional binding site, Smirnyagina et al. (37) have shown that a deletion mutant of 2a which is missing both the N and C termini (2aΔN/C) can replicate RNA3 in protoplasts.

To determine if an additional 1a binding site(s) in 2a can function independently of the 2a N terminus, we constructed two additional 2a truncations. The first, 2aΔN, lacks the first 162 residues of the N-terminal domain of 2a, including those that have been found to be sufficient for interaction with the helicase-like region of 1a (19). The second, 2aΔN/C, was the same as the truncation used by Smirnyagina et al. (37), lacking the first 162 residues as well as the last 125 residues of the nonessential 2a C terminus (40). As shown in Table 4, neither of these mutant 2a proteins could interact with wt1a. Therefore, the N terminus of 2a is necessary to mediate the 1a-2a interaction. It is possible that the longer version of 2a acts to stabilize the interaction between the two proteins.

Interaction between the 1a protein and itself. An additional interaction was observed between the 1a protein and itself. The relative β-galactosidase activity of pB-B1a with pG-B1a was reproducibly six- to eightfold higher than that of a related strain containing pB-B1a only (Table 4). Additional controls for these experiments include cells transformed with 1a fused to the LexA DNA binding domain (pB-B1a) by itself or with two-hybrid plasmids lacking BMV sequences. All of these controls yielded activities similar to the amounts observed with the yeast strain lacking any plasmids (Table 4). The transformants also grew on plates lacking histidine (data not shown). Mapping of the binding domain(s) will be presented elsewhere (29a).

1a-1a interaction in other tripartite RNA viruses. To determine whether the 1a-1a interaction is a general property for all tripartite RNA viruses, we examined whether the 1a-1a interaction was observed in closely related tripartite RNA viruses. DNAs encoding 1a homologs from CCMV and CMV were cloned into two-hybrid plasmids and tested for 1a-1a interaction. CCMV is a bromovirus which is closely related to BMV, and their 1a proteins are 68% identical and 80% similar at the amino acid level, based on the computer alignment program Clustal W (39); CMV, a cucomovirus more distantly related to BMV, has 45% identity and 63% similarity to the BMV 1a protein at the amino acid level. The 1a proteins of both of these viruses were found to interact with themselves. The interaction of CCMV 1a fusion proteins has 5-fold activity above background, while CMV's 1a-1a interaction is 15- to 30-fold above background (Fig. 2). As a further indication of these interactions, these transformants readily turned blue in the presence of X-Gal and could grow on defined medium lacking histidine, while cells containing either plasmid alone did not (data not shown).

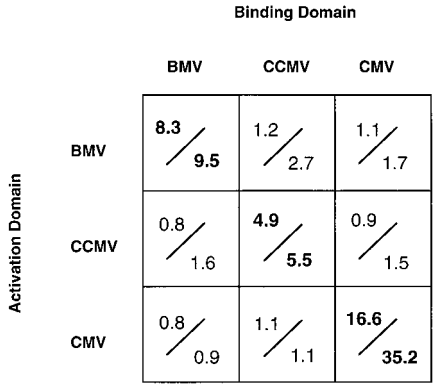

FIG. 2. Summary of the 1a-1a interaction in three different tripartite RNA viruses. β-Galactosidase specific activities are calculated as micromoles of ONPG hydrolyzed per minute per milligram of protein. Numbers are shown as fold activity relative to the background activity of cells harboring only pB-C1a. Numbers above and below the slash marks were derived from two independent experiments. "Binding Domain" indicates 1a fusions to the LexA DNA-binding domain; "Activation Domain" indicates 1a fusions to the GAL4 transcription activation domain.

In contrast to homologous combinations, heterologous combinations of 1a proteins of different viruses gave reduced relative β-galactosidase and the transformants did not turn blue (Fig. 2 and data not shown). After extensive incubation (>24 h), the CCMV 1a-BMV 1a transformants turned slightly blue, which indicates weak protein-protein interaction. This weak heterologous interaction between the CCMV and BMV 1a proteins is not unexpected, considering their similarity. However, quantitation of the β-galactosidase activity revealed that the interaction resulted in only approximately twofold activity over background. Transformants with CMV 1a-BMV 1a and CCMV 1a-CMV 1a did not have detectable β-galactosidase even after prolonged incubation.

DISCUSSION

In this report, we validated the use of the two-hybrid system to study the protein-protein interactions between viral RNA replication proteins. We found a perfect correlation between our observed two-hybrid interactions and the in planta replication phenotypes of 18 BMV 1a insertional mutants previously described by Kroner et al. (22). Our two-hybrid results are consistent with previously reported in vitro coimmunoprecipitation and protease digestion studies (19, 29). The 1a-2a interaction is thought to be required for at least some steps of RNA replication, and the protease-resistant structure has been found to be necessary for the binding of the 2a protein (29). All of the replicating mutants scored positive in three different tests: binding to 2a in coimmunoprecipitation assays, binding to two different deletions of 2a in the two-hybrid system, and retention of the protease-resistant structure (Table 3) (19, 29). Since results from all three assays are consistent, the ease of manipulating the two-hybrid system makes it the method of choice for further studies of the interaction of BMV RNA replication proteins.

wt1a interacted better with 2aΔC than 2a-N. This result was initially surprising since we would expect smaller fusion proteins to work better in the two-hybrid system (12). Instead, the fusion with 2aΔC (135 kDa) reproducibly interacted with wt1a with an activity two- to threefold higher than that of the smaller LexA–2a-N fusion (42 kDa). In addition, PK1 interacted well with 2aΔC but poorly with 2a-N. Finally, Smirnya-

gina et al. (37) have shown that 2aΔN/C and wt1a were able to replicate RNA3 when expressed from cDNAs using the CMV 35S promoter. All of these observations are consistent with a possible additional site of interaction in the central domain of 2a. However, we found that 2aΔN and 2aΔN/C were not able to interact with either wt1a or the helicase-like region of 1a. Therefore, the central domain of 2a is not sufficient for two-hybrid interaction with 1a and may instead stabilize the interaction. The observed replication of RNA3 seen by Smirnyagina et al. (37) could be due to the high levels of 2aΔN/C expressed from the 35S promoter or to the less likely possibility that the 1a-2a interaction is not necessary for replication of RNA3.

Results from two-hybrid analyses must be interpreted cautiously. A positive interaction needs to be confirmed with an extensive series of negative controls. In the case of the 1a-1a interaction, we obtained positive results only when the 1a fusion proteins were present in yeast with their respective fusion partners. No interactions were observed when either fusion partner was present alone or in combination with plasmid vectors. Additionally, no other interactions were observed between or among any of the BMV proteins, including 3a and CP. However, negative results are not as meaningful as positive results, and we do not mean to imply that other interactions do not occur.

The observation that more than one 1a is needed in RNA replication is consistent with previous genetic observations. First, Rao and Hall (32) observed that RNA1 was needed in excess of RNA2 during protoplast replication experiments. Second, the 1a protein contains a helicase-like domain, and helicases tend to function as multimers (24, 38). Third, even though the tobacco mosaic virus 183 kDa read-through protein already contains the conserved helicase-like and polymerase-like domains, the virus still needs to separately express the 126-kDa (helicase-like) protein in stoichiometric excess of the 183-kDa protein (16). Fourth, Smirnyagina et al. (37) showed that the BMV 1a protein was needed in *trans* when a fusion of the BMV replication proteins was constructed in a manner reminiscent of tobacco mosaic virus (37). Pogue and Hall (30) have proposed a model which includes a need for more than one BMV 1a protein. Finding 1a-1a interactions in other tripartite viruses (CCMV and CMV) indicates that the 1a-1a interaction may be a part of the general replication strategy of this group of viruses.

Although the 1a-like proteins of tripartite RNA viruses are very similar to one another at the amino acid level, the interactions appear to be quite species specific. Despite the fact that BMV and CCMV are approximately 80% similar to one another at the amino acid level, heterologous combinations of RNA1 and -2 cannot replicate in protoplasts (3). Even when the *cis*-acting replication sequences are removed and the proteins are expressed from DNA promoters, heterologous combinations of 1a and 2a are severely inhibited in their ability to direct replication of RNA3 derivatives (10). In this study, we have shown that the 1a proteins from these different viruses cannot interact with one another in the two-hybrid assay. We are currently examining other heterologous protein-protein interactions between the replication proteins of tripartite RNA viruses.

Enzymatically active fractions of the BMV RNA-dependent RNA polymerase (RdRp) have been reported to elute at a molecular mass of over 2 million Da (20). One attractive feature of the observed 1a-1a interaction is that RdRp purified in vitro may contain multimers of the various viral and possibly cellular subunits. The exact stoichiometry of the subunits within RdRp awaits improvements in its purification and further biochemical analysis.

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