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Formation of brome mosaic virus RNA-dependent RNA polymerase in yeast requires coexpression of viral proteins and viral RNA

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ABSTRACT **In this report we show that yeast expressing brome mosaic virus (BMV) replication proteins 1a and 2a and replicating a BMV RNA3 derivative can be extracted to yield a template-dependent BMV RNA-dependent RNA polymerase (RdRp) able to synthesize (−)-strand RNA from BMV (+)-strand RNA templates added *in vitro*. This virus-specific yeast-derived RdRp mirrored the template selectivity and other characteristics of RdRp from BMV-infected plants. Equivalent extracts from yeast expressing 1a and 2a but lacking RNA3 contained normal amounts of 1a and 2a but had no RdRp activity on BMV RNAs added *in vitro*. To determine which RNA3 sequences were required *in vivo* to yield RdRp activity, we tested deletions throughout RNA3, including the 5′, 3′, and intercistronic noncoding regions, which contain the cis-acting elements required for RNA3 replication *in vivo*. RdRp activity was obtained only from cells expressing 1a, 2a, and RNA3 derivatives retaining both 3′ and intercistronic noncoding sequences. Strong correlation between extracted RdRp activity and BMV (−)-strand RNA accumulation *in vivo* was found for all RNA3 derivatives tested. Thus, extractable *in vitro* RdRp activity paralleled formation of a complex capable of viral RNA synthesis *in vivo*. The results suggest that assembly of active RdRp requires not only viral proteins but also viral RNA, either to directly contribute some nontemplate function or to recruit essential host factors into the RdRp complex and that sequences at both the 3′-terminal initiation site and distant internal sites of RNA3 templates may participate in RdRp assembly and initiation of (−)-strand synthesis.**

Brome mosaic virus (BMV), a plant-infecting virus, is a representative member of the alphavirus-like superfamily of (+)-strand RNA viruses of animals and plants. The BMV genome consists of three separately encapsidated RNAs, designated RNA1–3. Monocistronic RNA1 and RNA2, respectively, encode proteins 1a (109 kDa) and 2a (94 kDa). 1a and 2a are essential mutually interacting components of the RNA-dependent RNA polymerase (RdRp) involved in BMV RNA replication (1, 2). This RdRp, whose functions have been studied *in vivo* and *in vitro*, is a complex of 1a, 2a, and host proteins (2–6). RNA3 contains two genes separated by an ≈250-nt intercistronic region. The 5′-proximal gene, encoding the 3a infection movement protein (7, 8), is translated directly from RNA3. The 3′-proximal coat protein gene is expressed via a subgenomic mRNA, RNA4. The 3a and coat proteins are dispensable for RNA replication but are required for infection spread in plants (8).

BMV-directed replication of RNA3 *in vivo* depends on cis-acting sequences in three regions of RNA3 (see Fig. 3*A*): the 3′ and 5′ noncoding regions (NCRs) and the intercistronic NCR (9). The last 160–200 nt of the 3′ NCR contain signals for initiation of (−)-strand RNA synthesis *in vitro* and are essential for RNA3 replication *in vivo*. The 91-nt 5′ NCR is thought to contain signals for initiation of (+)-strand synthesis and is similarly essential for RNA3 replication *in vivo* (9, 10). In addition, deletions in a 150-nt intercistronic element inhibit RNA3 replication *in vivo* by 50- to 100-fold (9–11). The remaining 100 nt of the intercistronic NCR, 3′ to the replication element (see Fig. 3*A*), contains a promoter for transcription of a subgenomic mRNA, RNA4, from the complementary (−)-strand copy of RNA3 (11–13). This region includes an ≈20-nt core subgenomic promoter directly upstream of the RNA4 initiation site and further upstream activating elements necessary for wild-type (wt) levels of RNA4 transcription.

Recently, it was demonstrated that BMV RNA3 derivatives can be replicated and direct subgenomic mRNA transcription in yeast expressing BMV proteins 1a and 2a from DNA plasmids (14). Here we show that yeast expressing 1a and 2a and replicating such RNA3 derivatives can be extracted to yield BMV-specific template-dependent RdRp activity. Moreover, even though RdRp activity was assayed on *in vitro*-supplied BMV RNA templates, we find that RdRp can only be isolated from cells expressing certain BMV RNA template sequences as well as 1a and 2a. These and other results reported below suggest that active BMV RdRp may only assemble on or in the presence of viral RNA templates, because the RNA directly contributes some function(s) to the RdRp or is required to recruit some essential host factor(s) into a functional complex with 1a and 2a. Deletion results further show that *in vivo* synthesis and accumulation of (−)-strand RNA3 depends not only on the cis-acting signals adjacent to the 3′-terminal (−)-strand initiation site but also on the distant intercistronic region.

MATERIALS AND METHODS

BMV RdRp Preparation from Transgenic Yeast. Yeast coexpressing BMV proteins 1a and 2a (B12 cells) and B12 cells replicating B3URA3 RNA (B123 cells) were as described (14). Yeast cultures (100 ml) were grown at 30°C in selective galactose medium as described (14) to 2×10^7 cells per ml. Cells were harvested by centrifuging 10 min at $500 \times g$, washed with water, and resuspended in 2.5 ml of ST (1 M sorbitol/50 mM Tris·HCl, pH 7.6). Cell walls were partially digested by adding 1000 units of lyticase (Sigma) and incubating 1 hr at 30°C. Cells were washed twice with ST and resuspended in buffer A (5). Further steps were performed at 4°C. An equal volume of glass beads (400–600 μm; Sigma) was added and cells were disrupted by vigorous shaking for 10 min. Membranes were isolated and RdRp activity was released with Triton X-100 as described (6). The resulting detergent extracts were used for RdRp assays. Preparation of highly purified RdRp from BMV-infected barley and RdRp activity assays were performed as described (5).

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Abbreviations: BMV, brome mosaic virus; dsRNA, double-stranded RNA; NCR, noncoding region; RdRp, RNA-dependent RNA polymerase; wt, wild type.

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Protein and RNA Analysis. Proteins were electrophoresed, blotted, and probed with polyclonal antisera against 1a and 2a as described (2). Total RNA was extracted, glyoxalated, electrophoresed, blotted, and hybridized as described (15). Northern blots were probed with strand-specific ^{32}P-labeled transcripts complementary to the conserved 200 nt at the 3′ end of BMV RNA or the coat protein gene (15, 16). Quantitative results were obtained by analyzing multiple experiments with a Molecular Dynamics digital radioactivity imaging system.

Expression of RNA3 Deletion Mutants in Yeast. wt RNA3 cDNA was inserted between the yeast *GAL1* promoter and a hepatitis δ virus ribozyme in a yeast DNA plasmid to allow transcription of full-length 3′-processed (+)-strand RNA3. Construction of this plasmid will be described elsewhere (M.I., M.J., M. Krol, and P.A., unpublished data). Plasmids ΔCSP and Δ3′ (laboratory designations pB3RQ50 and pB3RQ64) were constructed by deleting the *Bgl* II–*Sal* I (nt 1217–1249) and *Nsi* I–*Kpn* I (nt 1720–2071) fragments from the wt RNA3 plasmid (pB3RQ39). Plasmids Δ301 (pB3RQ57), Δ600 (pB3RQ77), Δ817 (pB3RQ78), and Δ1249 (pB3RQ81) were constructed from pB3RQ39 by deleting RNA3 cDNA sequences between a *Sna*BI site adjacent to the RNA3 5′ end and downstream *Afl* III, *Cla* I, *Pfl*MI, and *Sal* I sites, respectively. To construct plasmids Δ1003 (pB3RQ79) and Δ1217 (pB3RQ80), partial *Bgl* II digests of *Hin*dIII-digested pB3TP13 (17) were generated, blunt-ended, and digested with *Nsi* I. *Bgl* II–*Nsi* I fragments with and without the intercistronic element were isolated and ligated into *Sna*BI/*Nsi* I-digested pB3RQ39. Plasmid ICR/3′ (pB3RQ53) was constructed by deleting the *Sna*BI–*Pfl*MI and *Bss*HII–*Nsi* I fragments from pB3RQ39.

RESULTS

BMV RdRp Activity from Transgenic Yeast. B3URA3 is a BMV RNA3 derivative with the coat protein gene replaced by the yeast *URA3* uracil biosynthesis gene (14). As reported (14), when *ura3*$^-$ yeast coexpressing BMV replication factors 1a and 2a (B12 cells) are transfected with *in vitro* transcripts of B3URA3 and selected for growth on medium lacking uracil, yeast strains (B123 cells) are obtained in which B3URA3 RNA is maintained and expressed by RNA-dependent replication and transcription, similar to a persistent infection. As first shown by Hall and colleagues (3, 4), BMV-infected plant tissue can be extracted to yield an infection-specific template-selective RdRp that synthesizes complementary (−)-strand RNAs from BMV virion RNA1–4. This RdRp is initially membrane bound but can be solubilized with nonionic detergent (3–6, 18, 19). To test the expectation that B123 yeast should contain a comparable activity, we adapted our RdRp isolation procedures for plants to produce similar detergent-solubilized extracts of yeast membrane preparations.

Fig. 1 compares the [α-^{32}P]CTP-labeled RNA products synthesized when B123 yeast extracts or RdRp from BMV-infected barley were incubated with virion RNAs from several viruses as potential templates. Both extracts recognized added BMV RNAs as templates for (−)-strand RNA synthesis (Fig. 1, lanes 2 and 6). Virion RNAs of the closely related bromovirus cowpea chlorotic mottle virus were also utilized as templates by RdRps from both sources, but at a much lower efficiency than BMV RNAs (Fig. 1, lanes 3 and 7). Surprisingly, tobacco mosaic virus RNA was used, at even lower efficiency, by plant-derived BMV RdRp but not by BMV RdRp extracted from yeast (Fig. 1, lanes 8 and 4). Thus, BMV RdRp from B123 cells is at least as template-specific as its counterpart from BMV-infected plants. In the absence of added RNA templates, both the yeast- and plant-derived enzymes produced low levels of RNA3-sized products (Fig. 1, lanes 1 and 5), apparently due to residual amounts of endogenous templates persisting in both RdRp preparations.

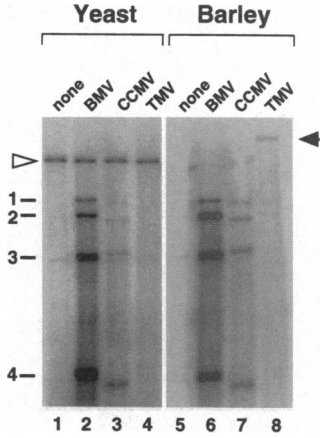

FIG. 1. Comparison of [α-^{32}P]CTP-labeled double-stranded RNA (dsRNA) products synthesized *in vitro* on added templates by BMV RdRp extracts from yeast expressing 1a and 2a and replicating B3URA3 RNA (lanes 1–4) or from wt BMV-infected barley (lanes 5–8). Virion RNAs added as templates are indicated above each lane. CCMV, cowpea chlorotic mottle virus; TMV, tobacco mosaic virus. Each set of templates was added to 200 μg of total RNA per ml of final reaction products. After incubation, the reaction products were extracted with phenol and the RNA products were electrophoresed on a 1% agarose gel that was dried and autoradiographed. BMV dsRNA1–4 are indicated on the left (bands 1–4). The open arrowhead indicates the putative *L-A* RdRp product (see *Results*). The solid arrowhead indicates the TMV RNA-specific product in lane 8.

In addition to BMV RdRp-specific products, extracts of B123 yeast produced a larger labeled RNA band (Fig. 1). This band also occurred in reaction products with equivalent extracts of the starting wt yeast lacking any BMV sequences (Fig. 2*A*, lane 1) and thus must have a non-BMV origin. Yeast strains often contain the virus-like dsRNA agent *L-A* (20). The additional band hybridized with an *L-A*-specific probe and its electrophoretic mobility was consistent with the 4.6-kb size of the *L-A* RNA genome (data not shown). Thus, this band appears to result from coextraction of *L-A* virus-like particles, which contain *L-A* RNA and a polymerase that synthesizes RNA from this template *in vitro* (20).

Formation of Template-Dependent RdRp Requires BMV Proteins Plus BMV RNA. While B123 yeast yielded BMV RdRp activity (Figs. 1 and 2*A*, lane 3), parallel growth and extraction of B12 cells, which express 1a and 2a but lack a replicatable BMV RNA template, showed no RdRp activity on BMV templates added *in vitro* (Fig. 2*A*, lane 2). To determine whether the lack of RdRp activity reflected a failure of 1a and 2a to accumulate in the absence of replicatable BMV RNA, Western blot analysis was performed on RdRp-type extracts from B123 and B12 yeast and from their parent yeast strain YPH500. As shown in Fig. 2 *B* and *C*, equal amounts of 1a and 2a were present in extracts from B12 and B123 cells (lanes 2 and 3), while, as expected, no 1a or 2a was detected in extracts from YPH500 (lane 1). Thus, 1a or 2a accumulated in B12 cells and associated with membranes, since the extracts tested were solubilized from membrane fractions. Nevertheless, extracts from B12 yeast were always as inactive in BMV RdRp assays as extracts from YPH500, which lacks any BMV sequences (Fig. 2*A*, lanes 1 and 2). Thus, formation of stable isolatable RdRp activity in yeast required not only 1a and 2a but also certain viral RNA sequences present in the replicatable RNA3 derivative, B3URA3. Since even the active RdRp extracts from B123 cells were largely template-dependent (Fig. 1, lanes 1 and 2) and RdRp assays were performed on BMV virion RNA templates added *in vitro*, B3URA3 was not required *in vivo* to provide a template in the *in vitro* assay.

Expression of RNA3 Deletion Derivatives *in Vivo*. To determine which RNA3 sequences were required *in vivo* to form

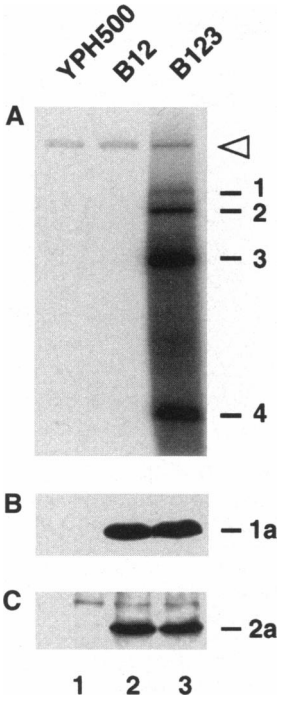

FIG. 2. Dependence of BMV RdRp activity on the *in vivo* presence of viral proteins and RNA. (*A*) Assays for BMV RdRp activity in parallel extracts from untransformed yeast strain YPH500 (lane 1), YPH500 yeast coexpressing BMV proteins 1a and 2a (B12, lane 2), or B12 yeast transfected with RNA3 derivative B3URA3 (B123, lane 3). Each reaction mixture contained purified BMV virion RNA1–4 as templates plus ATP, GTP, UTP, and [α-^{32}P]CTP. After incubation, reaction products were electrophoresed as in Fig. 1 and autoradiographed. Positions of BMV dsRNA1–4 are shown on the right (bands 1–4). The open arrowhead indicates the position of the putative *L-A* RdRp product (see *Results*). (*B* and *C*) Accumulation of BMV proteins 1a and 2a in B12 and B123 yeast. Extracts from YPH500 cells (lane 1), B12 cells (lane 2), and B123 cells (lane 3) were denatured and electrophoresed on an 8% polyacrylamide/SDS gel. After electrophoresis, proteins were transferred to a nylon membrane and probed with antiserum against 1a (*B*) or 2a (*C*).

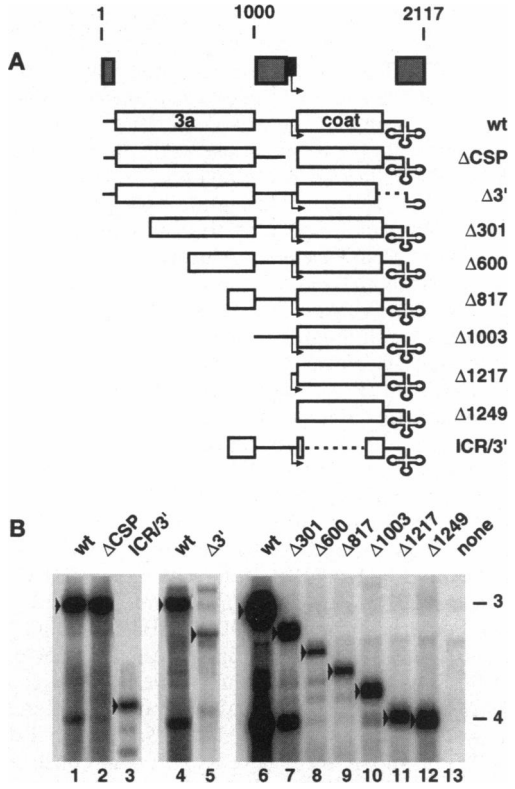

FIG. 3. (*A*) Map of RNA3 deletion derivatives expressed in yeast. Cis-acting sequences required for efficient RNA3 amplification are indicated by shaded boxes above the wt RNA3 map. The core subgenomic promoter (CSP) for transcription of subgenomic RNA4 is indicated by the solid box. The bent arrow indicates the RNA4 transcription start site. Open boxes represent the 3a and coat protein coding sequences; single lines represent NCRs. The cloverleaf structure at right represents the tRNA-like structure of the 3′ NCR. Dashed lines or gaps represent deletions. The numbers at the top show position in nt from the 5′ end of wt RNA3. (*B*) *In vivo* accumulation of (+)-strand RNA3 derivatives. Total RNA extracts from B12 cells containing expression plasmids for the RNA3 derivative indicated above each lane were denatured with glyoxal, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane. Before autoradiography, the membrane was probed with ^{32}P-labeled RNA complementary to the conserved 200 nt at the 3′ end of BMV virion RNAs, except for lanes 4 and 5, which were probed with ^{32}P-labeled RNA complementary to the coat protein gene. Arrowheads to the left of each lane indicate the positions of the expected (+)-strand transcripts. The positions of wt virion RNA3 and -4 are indicated on the right (bands 3 and 4).

functional RdRp, we constructed a series of deletion derivatives of wt RNA3 (Fig. 3*A*). cDNA corresponding to each deletion mutant was inserted in a yeast plasmid under the control of a yeast promoter, and each plasmid was used to transform B12 yeast. For each resulting yeast transformant, a (+)-strand RNA3-derived transcript of the expected size was easily detected in total RNA extracts by Northern blot analysis (Fig. 3*B*). RNA3 derivatives in which the 5′ or 3′ NCR was deleted accumulated to levels lower than wt RNA3 (Fig. 3*B*, lanes 7–12), possibly because such deletions remove cis-acting sequences essential for RNA3 replication (Fig. 3*A*). Transcripts of mutant ΔCSP, which lacked the core subgenomic promoter but retained all signals required for RNA3 replication (Fig. 3*A*), accumulated to the same level as wt RNA3 (Fig. 3*B*, lane 2). Only low levels of RNA4 were detected for Δ600, Δ817, and Δ1003 (Fig. 3*B*, lanes 8–10). Since nt 1–301 (Fig. 3*B*, lane 7) or nt 117–1003 (9) can be deleted without inhibiting RNA4 synthesis, this was unexpected and requires further investigation.

Effect of RNA3 Deletions on *in Vitro* RdRp Activity. For each of the yeast strains shown in Fig. 3*B* (which express 1a, 2a, and a selected RNA3 derivative), solubilized membrane extracts were prepared and tested in parallel for RdRp activity on BMV RNA templates added *in vitro*. Similar to results with B123 cells (Figs. 1 and 2), cells expressing wt RNA3 along with 1a and 2a yielded significant levels of template-dependent RdRp activity (Fig. 4*A*, lane 1). Intriguingly, in repeated experiments, deletion of the core subgenomic promoter (mutant ΔCSP) increased the RdRp yield per cell by 2- to 3-fold relative to cells expressing wt RNA3 (Fig. 4*A*, lane 2). However, no RdRp activity was detected in extracts of cells expressing RNA3 mutant Δ3′, which lacks all but the last 47 nt of the 3′ NCR, including most of the 3′-terminal element required in cis for RNA3 replication (Fig. 4*A*, lane 3).

To determine whether other RNA3 sequences beyond the 3′ NCR might be required for RdRp formation, we tested progressive 5′-terminal deletions, designated Δ301, Δ600, Δ817, Δ1003, Δ1217, and Δ1249 (Fig. 3*A*), where the number after Δ indicates the number of 5′ nt deleted. Yeast expressing 5′ deletions that retained the intercistronic NCR (i.e., Δ301, Δ600, Δ817, and Δ1003) yielded similar levels of template-dependent RdRp activity (Fig. 4*A*, lanes 4–7). Thus, neither the 5′ NCR nor the 3a gene was required to form RdRp. The lower RdRp yield for these mutants might be related to the lower level to which their (+)-strand transcripts accumulated *in vivo* (Fig. 3*B*, lanes 6–10).

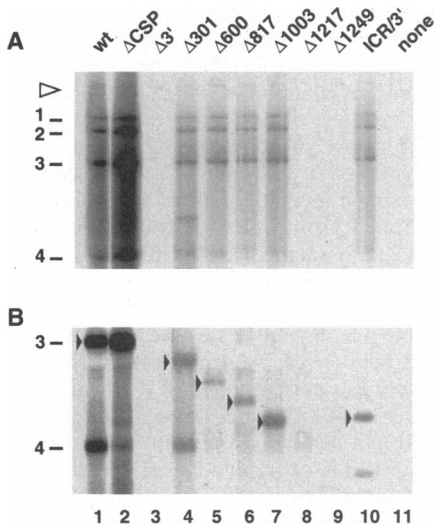

FIG. 4. Effect of RNA3 deletions on (−)-strand synthesis *in vitro* and *in vivo*. (*A*) Assays for BMV RdRp activity in parallel extracts from yeast expressing 1a, 2a, and the RNA3 derivatives indicated above each lane. Each reaction mixture contained purified BMV virion RNA1–4 as templates plus ATP, GTP, UTP, and [α-^{32}P]CTP. After incubation, reaction products were electrophoresed as in Fig. 1 and autoradiographed. Positions of BMV dsRNA1–4 are shown at left (bands 1–4). The open arrowhead indicates the position of the putative *L-A* RdRp product (see *Results*). (*B*) Northern blot analysis of *in vivo* accumulation of (−)-strands. Total RNA extracts from the yeast strains of *A* were denatured with glyoxal, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane. The membrane was probed with ^{32}P-labeled RNA complementary to the 5′-proximal 200 nt of BMV (−)-strand RNA3, except for lane 3, which was probed with coat protein sequences. Arrowheads to the left of individual lanes indicate (−)-strand RNA bands with mobilities corresponding to the (+)-strand RNA3 derivative expressed in that yeast strain (Fig. 3*B*). The position of wt RNA3 (−)-strand is shown at left.

In contrast, yeast expressing RNA3 mutants with 5′ deletions extending into the intercistronic region (i.e., mutants Δ1217 and Δ1249) yielded little or no RdRp activity above background (Fig. 4*A*, lanes 8, 9, and 11). The inability of the Δ1217 and Δ1249 RNA3 mutants to support RdRp formation was highlighted by the fact that their (+)-strand transcripts accumulated *in vivo* to levels higher than other RNA3 derivatives that yielded significant levels of RdRp, such as Δ600 and Δ817 (Fig. 3*B*, lanes 11 and 12 vs. lanes 8 and 9). Thus, in addition to the 3′ NCR, RNA3 sequences between nt 1003 and 1217 strongly influence RdRp formation.

Since B3URA3 supported RdRp formation despite replacement of the wt coat gene by URA3 and deletion or other replacements of the coat gene do not inhibit RNA3 replication *in vivo* (14, 21), it appeared that the 3′ NCR and intercistronic region should be sufficient to support RdRp formation. To explore this possibility, we used convenient restriction sites in RNA3 cDNA to construct ICR/3′ (Fig. 3*A*), which retained the full intercistronic region and 3′ NCR but deleted the 5′ NCR and most of the 3a and coat genes. As anticipated, cells expressing ICR/3′ yielded RdRp levels similar to Δ1003 and other active 5′ truncations (Fig. 4*A*, lane 10).

***In Vitro* RdRp Activity Correlates with *in Vivo* (−)-Strand Accumulation.** To complement the *in vitro* RdRp assays of Fig. 4*A*, we probed blots of total RNA from the same yeast strains with strand-specific probes to test whether the RNA3 derivatives in Fig. 3 served as templates for BMV-directed (−)-strand RNA synthesis *in vivo*. For the entire set of RNA3 derivatives, (−)-strand accumulation *in vivo* (Fig. 4*B*) paralleled the yield of RdRp activity detected *in vitro* (Fig. 4*A*): ΔCSP, whose expression yielded higher template-dependent RdRp activity than wt RNA3, showed 2- to 3-fold higher (−)-strand accumulation *in vivo* than wt RNA3, as determined by quantifying Fig. 4, lanes 1 and 2, and similar experiments. Deleting the 3′ or intercistronic NCRs, which abolished RdRp activity *in vitro*, abolished (−)-strand accumulation *in vivo* (Fig. 4, lanes 3, 8, and 9). Δ301, Δ600, Δ817, Δ1003, and ICR/3′, which lacked the 5′ NCR but retained the intercistronic and 3′ NCRs, served as templates for *in vivo* (−)-strand accumulation and supported the formation of RdRp active on other templates *in vitro*, and all showed similar reduction from wt in both activities (Fig. 4, lanes 4–7 and 10).

While RNA4 sequences alone were insufficient to direct (−)-strand accumulation *in vivo* (Fig. 4*B*, lanes 8 and 9), both (−)-strand RNA3 and a (−)-strand band at or near RNA4 appeared in cells expressing wt RNA3 and Δ301 (Fig. 4*B*, lanes 1 and 4). Single-cycle BMV infections of plant protoplasts show that RNA4-sized (−)-strands accumulate much more slowly than (−)-strand RNA3 (refs. 15 and 17 and unpublished results). However, Fig. 4*B* reflects steady-state RNA accumulation over the entire span of yeast growth since transformation with BMV components. The origin of RNA4-sized (−)-strands is not known and might include (−)-strand RNA3 degradation or partial ability of the intercistronic region to function in trans (see *Discussion*).

DISCUSSION

The results presented here show that BMV RdRp can be isolated from suitably transformed yeast, reveal a nontemplate role for BMV RNA in RdRp activity, and clarify the relation between cis-acting sequences of BMV RNA3 and (−)-strand RNA3 accumulation *in vivo*.

Isolation of BMV RdRp from Transgenic Yeast. Figs. 1 and 2 show that yeast expressing BMV RNA replication factors 1a and 2a and maintaining a BMV RNA replicon contain an RdRp activity that is not present in wt yeast and will copy BMV RNA templates *in vitro*. These results complement prior genetic evidence that maintenance and expression of BMV RNA derivatives in yeast is due to RNA-dependent replication and transcription (14). The ability to extract and solubilize BMV RdRp from yeast by straightforward adaptation of procedures developed for plant cells, similar template selectivities and product profiles of the yeast- and plant-derived extracts (Fig. 1), *in vivo* dependence on the same cis-acting signals (Figs. 3 and 4 and refs. 9 and 14), comparable (+)- to (−)-strand RNA ratios *in vivo* (14), and other similarities suggest that BMV RNA replication in plant cells and yeast are fundamentally similar. *In vitro* studies with BMV RdRp from yeast should be a useful adjunct to yeast genetics to study the role of host factors in BMV replication.

BMV RNA Is Required *in Vivo* To Form Template-Dependent BMV RdRp Activity. Equivalent levels of 1a and 2a were present in parallel extracts of yeast expressing 1a and 2a that lacked or possessed RNA3 sequences (B12 and B123 cells, respectively; Fig. 2 *B* and *C*). However, even though RdRp activity was assayed on BMV RNA templates added *in vitro*, only the extracts from B123 cells had RdRp activity (Fig. 2*A*). Thus, 1a and 2a were insufficient to direct formation of functional stable RdRp in yeast and, in addition to its template function, BMV RNA must have a role in the assembly, activity, or stability of the RdRp complex.

RdRp isolation from yeast required expression of 1a, 2a, and RNA3 derivatives containing both 3′ and intercistronic NCRs. For the entire set of RNA3 deletions tested, there was good correlation between the levels of (−)-strand RNA accumulation *in vivo* and RdRp activity assayed *in vitro* (Fig. 4). Thus, the influence of RNA3 cis-acting sequences on RdRp activity was not simply an *in vitro* artifact but reflected *in vivo* effects on (−)-strand RNA synthesis. This correlation suggests that RdRp activity characterized by the *in vitro* (−)-strand synthesis assay may actually reflect the formation and isolation of

complexes that were engaged in (−)-strand RNA synthesis *in vivo*. In keeping with this, deleting the core subgenomic promoter in mutant ΔCSP increased RdRp activity over wt RNA3 (Fig. 4*A*), possibly by blocking diversion of (−)-strand RNA synthesis complexes into RNA4 synthesis.

As noted above, BMV RNA was not required *in vivo* for normal accumulation or membrane association of 1a and 2a. BMV RNA might directly contribute some function to the RdRp. However, since adding BMV virion RNAs (including RNA3) *in vitro* does not make extracts from B12 cells active (Fig. 2*A*), lack of a function contributed directly by RNA3 appears unlikely to be the sole reason for the lack of RdRp activity in B12 yeast extracts. Alternatively, since active BMV RdRp extracts contain an immunoprecipitable complex of 1a, 2a, and multiple host factors (2, 5, 6), BMV RNA may be required to recruit and/or retain essential host factors in the RdRp. The role of BMV RNA in RdRp assembly might be similar to the role of RNA in ribosome or small nuclear ribonucleoprotein assembly or to the way that transcription complexes assemble on DNA templates with the assistance of multiple protein–DNA interactions. Consistent with these possibilities, the 3′-terminal 135–200 bases of RNA3 interact with a variety of tRNA-specific host enzymes (22), while the intercistronic region is suspected to interact with host factors through a functionally important copy of the "box B" motif of RNA polymerase III promoters, which also corresponds to the invariant residues of the TψC loop of tRNAs (9–11). It remains unknown whether RNA is only required transiently in RdRp assembly or is an essential component of active RdRp.

While the experiments reported here studied the ability of RNA3 sequences to support RdRp formation, equivalent signals must also exist in wt BMV RNA1 and/or RNA2, which can replicate in cells lacking RNA3 (9, 23). B12 yeast presumably fail to assemble functional RdRp because the plasmids that express 1a and 2a in these cells contain only the 1a and 2a open reading frames, with most of the 5′ and 3′ NCRs of RNA1 and RNA2 deleted (14). The 200 bases at the 3′ ends of RNA1 and RNA2 are highly conserved with the 3′ sequence of RNA3, and the 5′ NCRs of RNA1 and RNA2 each contain the "box B" sequence present in the RNA3 intercistronic region and implicated in RNA3 replication *in vivo* (9, 10).

Role of RNA3 Intercistronic Region in (−)-Strand Accumulation. *In vivo* (−)-strand RNA3 synthesis did not require the 5′ sequences of RNA3. However, the 3′ cis-acting sequences, which contain the (−)-strand initiation site, were essential but insufficient to direct significant (−)-strand RNA3 accumulation *in vivo* (Fig. 4*B*, lanes 8 and 9). In addition, intercistronic sequences 1 kb away from the 3′ initiation site were required. Previous studies (9, 10) showed that intercistronic deletions severely inhibit RNA3 replication, although it was unclear whether the primary defect was in (−)- or (+)-strand RNA synthesis. The present results indicate that intercistronic nt 1003–1217 have an important effect on (−)-strand accumulation, although they do not rule out that these sequences might also influence (+)-strand synthesis.

In plant cells, intercistronic deletions suppress RNA3 replication ≈100-fold but do not abolish it (9). Similarly, long exposures of the blot in Fig. 4*B* and similar experiments suggest that, in B12 yeast, small amounts of (−)-strand RNA (<1% of wt) were produced from Δ1217 and Δ1249 (+)-strand RNAs. Thus, the intercistronic region may not be absolutely required for (−)-strand initiation but may greatly increase the frequency of initiation. The possibility that some factor(s) interacts with intercistronic sequences to facilitate initiation at the 3′ end of BMV RNA3 is similar to proposals that Qβ RNA replicase binds an internal M site on Qβ RNA and remains attached there while initiating (−)-strand synthesis from the 3′ end (24) and to the role of an internal enhancer in *L-A* virus (−)-strand synthesis (25). Similarly, template RNA sequences distal to coronavirus transcription initiation sites may recruit cell and viral factors into a functioning transcription complex (26). Finally, influenza virus RNA polymerase binds both the 3′ initiation site of its template and sequences at the 5′ end (27).

Despite the strong influence of intercistronic sequences on (−)-strand synthesis *in vivo* (Fig. 4), most BMV RdRp reactions yielded similar levels of *in vitro* (−)-strand synthesis from RNA3 and from RNA4, which lacks intercistronic sequences (Figs. 1, lane 2, and 2, lane 3). This might reflect an ability of the intercistronic region to function in trans; i.e., once formed *in vivo* in the presence of RNA3 containing intercistronic sequences, active RdRp might be able to initiate on templates lacking these sequences. Alternatively, the present *in vitro* (−)-strand synthesis system may not duplicate all features of the *in vivo* reaction. As long recognized, the efficiency of (−)-strand initiation *in vitro* by current BMV RdRp extracts is low: typically, <0.1% of templates are copied. Thus, perhaps due to loss of some factor(s) in extraction, the present *in vitro* reaction may not be responsive to the pathway by which intercistronic sequences stimulate (−)-strand synthesis *in vivo* and may reflect only the basal (−)-strand initiation frequency directed by 3′ RNA3 sequences alone.

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