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Saccharomyces cerevisiae L-BC Double-Stranded RNA Virus Replicase Recognizes the L-A Positive-Strand RNA 3′ End

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L-A and L-BC are two double-stranded RNA viruses present in almost all strains of *Saccharomyces cerevisiae*. L-A, the major species, has been extensively characterized with in vitro systems established, but little is known about L-BC. Here we report in vitro template-dependent transcription, replication, and RNA recognition activities of L-BC. The L-BC replicase activity converts positive, single-stranded RNA to double-stranded RNA by synthesis of the complementary RNA strand. Although L-A and L-BC do not interact in vivo, in vitro L-BC virions can replicate the positive, single-stranded RNA of L-A and its satellite, M_1, with the same 3′ end sequence and stem-loop requirements shown by L-A virions for its own template. However, the L-BC virions do not recognize the internal replication enhancer of the L-A positive strand. In a direct comparison of L-A and L-BC virions, each preferentially recognizes its own RNA for binding, replication, and transcription. These results suggest a close evolutionary relation of these two viruses, consistent with their RNA-dependent RNA polymerase sequence similarities.

Two or more viruses of the same class often share the same host without interference. *Saccharomyces cerevisiae* is a good system for study of this phenomenon, since many available strains contain two different double-stranded (ds) RNA viruses, L-A and L-BC, their satellites and defective interfering RNAs, two different single-stranded (ss) RNA replicons (20S and 23S RNA), and up to five different retrotransposons. Further, the ease of genetic manipulation of *S. cerevisiae* allows study of the cellular interactions of these viruses (reviewed in reference 48).

The L-A genome is a single 4.6-kb segment encoding two proteins, Gag, the major coat protein, and Pol, expressed as a Gag-Pol fusion protein (18, 22) formed by a −1 ribosomal frameshift event (9, 45). L-A, in addition to supporting its own replication, also can propagate M_1 dsRNA, encoding a secreted protein toxin (the killer toxin) and immunity to the toxin (reviewed in references 7 and 48), or X dsRNA, a 530-bp deletion derivative of L-A itself (13). These dsRNAs, which must carry all the signals necessary for propagation by the L-A viral proteins, are separately encapsidated in the L-A coat. Understanding the L-A propagation cycle has depended, in large part, on development of in vitro techniques that reproduce some of the viral functions. Specific in vitro binding of viral positive strands to opened empty viral particles and the template-dependent replication (negative-strand synthesis with a positive-strand template) system have made possible the characterization of RNA sites recognized in each process (Fig. 1) (10, 11, 19, 21). In vitro, template-dependent transcription (positive-strand synthesis with a dsRNA template) was conservative (20), confirming earlier in vivo experiments (29, 36).

Synthetic transcripts of the cDNA clone of X dsRNA were used to define (i) the RNA binding site 400 nucleotides from the 3′ end, (ii) the internal replication enhancer overlapping the RNA binding site, and (iii) the 3′ end sites essential for replication including the terminal three nucleotides and an adjacent stem-loop structure (Fig. 1) (10, 11). The L-A RNA-dependent RNA polymerase binds first to the internal site of the positive strands and then, by a looping mechanism, interacts with the 3′ end where replication begins (21).

Expression of full-length cDNA clones of L-A (22) facilitated study of the functions of the L-A coding sequences in vivo without their *cis* requirement for propagation (49). This system was used to show that part of the Pol domain is responsible for RNA packaging (16, 33), to correlate this site with one of the three in vitro RNA binding sites of Pol (32, 33), and to examine the importance for in vivo replication of the consensus RNA-dependent RNA polymerase sequences that L-A shares with all other dsRNA and positive ssRNA viruses (34). The L-A Gag protein is able to remove the 5′ cap structure from cellular mRNAs both in vitro (3, 4) and in vivo (27), a function which allows the virus to express its information from its own uncapped mRNA in spite of the *SKI1/XRN1*-encoded exonuclease that degrades uncapped mRNAs (27).

L-BC, like L-A, is a family of closely related 4.6-kb dsRNA viruses including L-B and L-C, but with a substantially lower copy number than L-A (38, 51). The viral particles have a transcriptase activity that utilizes an endogenous template (38) and a replicase activity (17), but these activities have not been well characterized. No template-dependent replicase, transcriptase, or RNA binding system has been described for L-BC, nor has an in vivo system to study RNA polymerase, packaging, or capsid assembly. The sequence of L-BC (30) shows close homology of the L-BC Pol consensus RNA-dependent RNA polymerase and surrounding sequences with that of L-A (5, 6, 24).

We report the isolation of L-BC viral particles and the detection of an RNA polymerase activity dependent on added templates. This replicase activity prefers L-BC positive strands as templates and is found only in strains carrying L-BC but, surprisingly, can use L-A or M_1 positive strands and has the same 3′ end sequence requirements when working on L-A or X positive strands as does the L-A-encoded replicase activity. We also describe a specific positive ssRNA binding activity and a transcriptase activity dependent on added dsRNA template.

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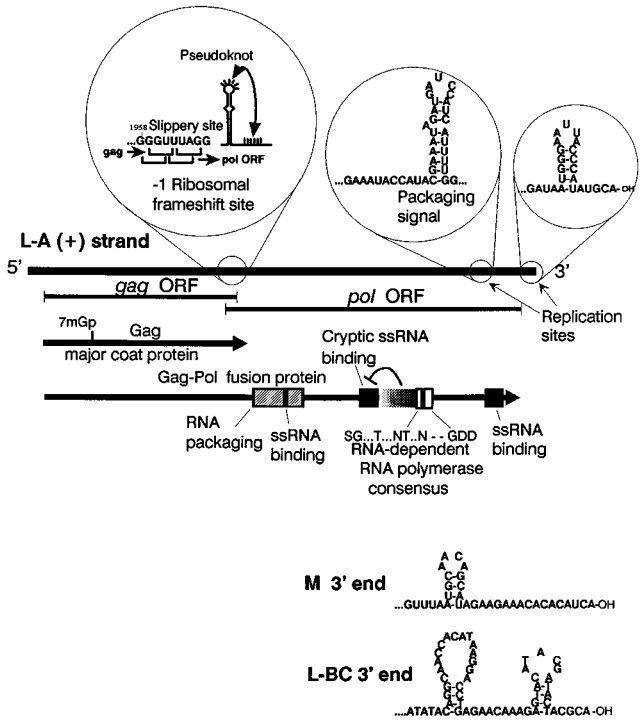

FIG. 1. Sites on the L-A positive strand, domains of the encoded proteins, and comparison with M_1 and L-BC 3′ ends. L-A contains two open reading frames (ORFs), *gag*, which encodes the major coat protein, Gag, and *pol*, which is expressed only as a Gag-Pol fusion protein formed by a −1 ribosomal frameshift event. The packaging signal, a stem-loop and the 10 nucleotides upstream of the stem, directs the specific encapsidation of viral positive ssRNA. Signals for replication (dsRNA synthesis on a positive ssRNA template) are the internal replication enhancer (overlapping the packaging signal) and the 3′ end stem-loop structure and the 3′-most 3 nucleotides. Gag includes an mRNA decapitation activity that covalently attaches the 7mGMP from the cap to His-154. Pol contains the consensus RNA-dependent RNA polymerase sequence pattern conserved in positive ssRNA and dsRNA viruses, and three ssRNA binding domains, all essential for viral propagation. The central RNA binding domain is cryptic and is inhibited by a region C terminal to it. The N-terminal in vitro RNA binding domain is inside the in vivo packaging domain, the latter comprising residues 67 to 213 of Pol. (Below) 3′ end structure of M_1 (42) and L-BC (30) (GenBank accession number U01060) positive strands. The secondary structures in L-BC are hypothetical.

Like the replicase, the L-BC transcriptase can use an L-A template, although it prefers L-BC dsRNA.

MATERIALS AND METHODS

Strains and media. 4.7MB (50), YPAD, YPG, SD, synthetic complete medium lacking specific amino acids (37), and LB and TB media (35) are described elsewhere. *S. cerevisiae* JR1 (*MAT***a** *trp1 ura3 leu2 his3 pep4*::*HIS3 nuc1*::*LEU2* L-A-o L-BC), JR13 (JR1 L-BC-o), 3398 (*MAT***a** *mak10-1 trp1 ade1 ade8 his3 ura3* L-A-o L-BC), JR11 (3398 L-BC-o), JR10 (*MAT***a** *ura3 his3 trp1 ski2-2* L-A-o L-BC), JR2 (JR10 L-BC-o), JR7 (JR2 [*rho*^{0}]), 396 (*MAT*α *arg1 thr1 ura3* L-C T-o W-o), 395 (*MAT***a** *arg9 ura3* L-A-o L-BC-o T W), TF229 [*MAT***a** *his*(*3,4*) *leu2 ski2-2* L-A-HN], TF325 (*MAT*α *arg1 ski2-2 clo*$^-$ *kar1-1* L-A-HN M_1), and JR5 (*MAT*α *kar1-1 ura2 leu2 trp1* L-A-o L-BC) were used. *Escherichia coli* MV1190 (Bio-Rad) and DH5α (Bethesda Research Laboratories) were used.

Plasmids and DNA techniques. pI2L2 is the L-A expression plasmid (*TRP1* selection, *PGK1* promoter) with the entire L-A cDNA sequence (49). pLM1 contains the entire X cDNA sequence except for the 5′ 23 nucleotides cloned into Bluescript SK$^+$ (Stratagene) and has an *Fsp*I site at the 3′ end of the X sequence in order to make an in vitro T7 RNA polymerase transcript with the correct 3′ end (15). pLM19 is identical to pLM1 except that the internal replication enhancer and the viral binding site have been destroyed (21). pRE112 has the entire X cDNA sequence in SK$^+$, but with the X 3′ end sequence AUGC changed to AUAC and a *Sna*BI site in place of the *Fsp*I site to make the T7 transcripts have the desired 3′ end (11). pRE131 lacks bases 13 to 19 from the 3′ end of X and thus lacks the stem-loop structure of which they are a part (11). pRE141, pRE145, pRE148, and pRE149 each lack bases 1 to 23 and 193 to 300 and contain deletions in the X sequence of nucleotides 96 to 135, 145 to 153 (substituted with its complement), 96 to 130, and 96 to 125, respectively. X positive-strand transcripts made by T7 RNA polymerase from pRE145 are replicated normally by L-A opened empty particles, while those from pRE141, pRE148, and pRE149 have a deletion in the internal replication enhancer and have reduced template activity (11).

Yeast transformation was as described previously (23) with minor modifications (46). RNA transcripts were made with T7 or T3 RNA polymerase (for positive and negative strands, respectively), [α-^{32}P]UTP, and restriction enzyme-digested DNA template, as suggested by the supplier (U.S. Biochemical-Amersham).

Genetic manipulations. To cure L-A, strains were first cured of mitochondrial DNA with ethidium bromide (30 μg/ml) and then grown on YPAD plates at 39°C. Screening for loss of L-A was done by cytoducing M_1 from JR5pI2L2K$^+$ (a strain in which M_1 is maintained by an L-A cDNA clone without the L-A virus) and checking cytoductants for the killer phenotype. Nonkiller cytoductants (L-A-o clones) were checked by extraction of dsRNA (14) and in some cases by Northern (RNA) blot hybridization with an X negative-strand probe made with T3 RNA polymerase from pLM1.

L-BC was cured by successive growth on YPAD plates at the limiting growth temperature for the individual strain (40 to 42°C), isolation of single colonies at 30°C, and extraction of dsRNA (14). Although the strains used here were cured by this method, some other strains could not be cured.

Introduction of L-BC was by cytoduction (transfer of cytoplasm from donor strain to recipient without changing the nuclear genotype [8]) from strain JR5. The [*rho*$^+$] donor and [*rho*^{0}] recipient were mixed for 6 h, and the mating mixture was plated on media selecting against the donor. Presence of L-BC in [*rho*$^+$] colonies with the recipient genotype was confirmed by dsRNA extraction (14).

Virus particle preparation. L-A and M_1 viral particles were prepared from stationary-phase cells of strains TF229 and TF325, respectively, grown in 1 liter of YPAD broth as described previously (12, 21) except that smaller CsCl gradients were run at 130,000 × *g* (42,000 rpm in a Beckman 50Ti rotor) for 20 h at 4°C. Fractions of 0.5 ml were collected, and those containing viral particles were dialyzed against 50 mM Tris Cl (pH 7.6)–150 mM NaCl–1 mM EDTA–1 mM dithiothreitol–20% glycerol (dialysis buffer) for 4 h and kept at −70°C. Fractions containing M_1 viral particles were detected by RNA extraction and gel electrophoresis of an aliquot of each fraction. Heavy and light fractions containing M_1 (12) particles were dialyzed and stored as for the L-A particles.

L-BC viral particles were prepared from stationary-phase cells of strain JR1 grown in 1 liter of YPAD broth at 30°C for 3 days. Cells (~13 g) were harvested, washed with water, and suspended in 1.2 ml (per g of cells) of 100 mM Tris Cl (pH 7.6)–20 mM 2-mercaptoethanol–1.4 M sorbitol–5 mg of Zymolyase 20T per ml and incubated for 50 to 60 min at 37°C. The cells were collected by centrifugation at 1,500 × *g* for 10 min, suspended in 35 ml of buffer A (50 mM Tris Cl [pH 7.6], 150 mM NaCl, 10 mM EDTA) by vortexing, and lysed by passage through a French pressure cell (14,000 lb/in^2). Cell debris was removed by centrifugation at 13,000 × *g*. Viral particles were collected by centrifugation at 100,000 × *g* for 1 h. The pellet was resuspended in 35 ml of buffer A and centrifuged at 10,000 × *g* for 20 min. The supernatant was adjusted to a density of 1.35 g/ml by addition of CsCl in a 40-ml total volume. The suspension was centrifuged for 20 h at 130,000 × *g* at 4°C. The first 9 ml of the bottom of the gradient was collected (ρ = 1.36 to 1.45 g/ml), and the density was adjusted to 1.35 g/ml by addition of CsCl and buffer A in a total volume of 13 ml. After 20 h of centrifugation at 130,000 × *g*, 0.5-ml fractions were collected. Fractions 1 to 5 from the bottom of the gradient were pooled and concentrated with a Centricon-100 (Amicon) to 100 to 200 μl or centrifuged (as described below for empty particles) and resuspended in 100 to 200 μl of buffer A. After 4 h of dialysis against dialysis buffer, particles were stored at −70°C.

Empty viral particle preparation. L-A opened empty viral particles were prepared on 13-ml CsCl gradients as described previously (10) and processed as described above for L-A viral particles. L-BC empty viral particles were prepared from stationary-phase cells grown in 1 liter of YPAD or H-Trp or H-Ura media at 30°C for 3 days. Cells were collected, lysed, and processed as for L-BC viral particles (above), but the particle suspension was adjusted to 1.32 g/ml by addition of CsCl (40-ml total volume) and centrifuged for 20 h at 130,000 × *g* at 4°C, and 1.5-ml fractions were collected. Fractions with a density of 1.295 to 1.33 were pooled, diluted with 2 mM Tris Cl (pH 7.6)–1 mM EDTA, and centrifuged (130,000 × *g*, 1.5 h). These fractions correspond in density to those having L-A empty particles in a similar gradient. Empty particles were resuspended in 150 to 200 μl of 2 mM Tris Cl (pH 7.6)–1 mM EDTA and dialyzed against the same buffer. Samples were clarified by centrifugation at 4°C for 5 min in a microcentrifuge and stored at −70°C without glycerol. Dialysis against low-ionic-strength buffer is not sufficient to open the particles as it is in the case of L-A viral particles (19), and a cycle of freeze-thaw in the absence of glycerol is critical to open them and detect or enhance their RNA polymerase and RNA binding activities. After thawing of the particles, they were made 20% glycerol to stabilize their activities, and they were stored at −70°C.

ssRNAs. L-A, L-BC, or M_1 ssRNAs were made with L-A, L-BC, or M_1 dsRNA containing viral particles. The in vitro transcriptase reaction mixture (150 μl) contained 50 mM Tris Cl (pH 7.6), 10 mM $MgCl_2$, 20 mM NaCl, 5 mM KCl, 10 mM 2-mercaptoethanol, 0.5 mM (each) nucleoside triphosphates, 0.04% bentonite, 240 U of RNasin (Promega), and different amounts of viral particles (50 to 100 μg of protein). For synthesis of ^{32}P-RNA, the UTP concentration was 20

μM [α-^{32}P]UTP and total volume was 25 μl. Mixtures were incubated at 30°C for 3 h, and the reaction was stopped by extraction with phenol, phenol-chloroform, and chloroform. Products were precipitated with ethanol, washed with 70% ethanol, and dried.

dsRNAs. L-A dsRNA was extracted from mature viral particles prepared from strain TF229, ethanol precipitated, washed with 70% ethanol, and dried. L-BC dsRNA was obtained by extracting dsRNA as described previously (14) from strain JR1 grown in 250 ml of YPAD broth for 3 days at 30°C. The RNA was treated with DNase I, phenol extracted, ethanol precipitated, dried, and dissolved in diethylpyrocarbonate-treated water. The RNA was adjusted to 1× SSC (15 mM Na citrate, 150 mM NaCl) and 1 M LiCl, kept at −20°C for 1 h, and centrifuged for 20 min. These steps were repeated with serial precipitations at increasing concentrations of 2, 3, 4, and 5 M LiCl. L-BC dsRNA was highly enriched in the 4 M LiCl precipitate. Finally, L-BC dsRNA was purified by agarose gel electrophoresis, and the dsRNA was recovered.

Site-specific RNA binding assay. The RNA binding reaction was carried out as described previously (32) except that L-A and L-BC empty particles (2 and 100 μg of protein, respectively) were used. Larger amounts of L-BC particles were required to observe comparable binding, replication, and transcription, probably because they were less pure and were not opened as efficiently as were the L-A particles.

In vitro replication reaction. Minor modifications were made to the method previously described (18). The 25-μl reaction mixture contained 50 mM Tris Cl (pH 7.6); 10 mM $MgCl_2$; 20 mM NaCl; 5 mM KCl; 10 mM 2-mercaptoethanol; 0.5 mM (each) ATP, CTP, and GTP; 20 μM [α-^{32}P]UTP; 0.04% bentonite; 40 U of RNasin (Promega); 5% polyethylene glycol 6000; one of the unlabeled ssRNAs (L-A ssRNA [100 ng], M_1 ssRNA [100 ng], L-BC ssRNA [less than 10 ng] or T7 RNA polymerase transcript [100 ng]); and empty particles (2 μg of protein for L-A and ≈100 μg for the L-BC fraction). The reaction mixture was incubated for 3 h at 30°C; phenol extracted, ethanol precipitated, and analyzed on a 1% agarose gel (L-A, L-BC, and M_1 products) or a 1.5% NuSieve 3:1 agarose (FMC BioProducts) gel (T7 RNA products); and detected by autoradiography.

In vitro transcription reaction. The reaction was carried out as described previously (20) with slight modifications. The 25-μl reaction mixture contained 50 mM Tris Cl (pH 7.6); 10 mM $MgCl_2$; 20 mM NaCl; 5 mM KCl; 10 mM 2-mercaptoethanol; 0.5 mM (each) ATP, CTP, and GTP; 20 μM [α-^{32}P]UTP; 0.04% bentonite, 40 U of RNasin (Promega); 20% polyethylene glycol 1000; dsRNA (0.6 to 1.2 μg); and empty particles (2 μg of protein from L-A and ≈150 μg of protein from the L-BC fraction). The reaction mixture was incubated for 3 h at 30°C, phenol extracted, and analyzed on a 1% agarose gel.

RNase A protection assay. The reaction mixture (50 μl) contained 20 mM Tris Cl (pH 7.5), 2.5 × 10^4 cpm of the RNA polymerase reaction product (phenol extracted and ethanol precipitated), 10 μg of tRNA, 400 ng or 1 μg of pancreatic RNase A, and 0 or 0.5 M NaCl. The reaction mixture was incubated for 30 min at 30°C, and the products were phenol extracted, ethanol precipitated, separated in a 1.5% agarose gel, and detected by autoradiography.

Strand separation gel. Strand separation gels (5% polyacrylamide gels) were prepared as described previously (42, 43) with slight modifications. M_1 RNA was denatured for 1 min at 90% in the presence of 7 M urea. The samples were quickly chilled and applied to the gel. The positive and negative strands of M_1 RNA were separated for 10 min at 25 V/cm and then at 8 V/cm for 20 h. ^{32}P-M_1 positive strands made from M_1 viral particles served as positive-strand marker. Cold M_1 positive strands were used as template for L-A empty viral particles to make ^{32}P-labeled negative-strand marker.

RESULTS

Extracts of L-A-o strains replicate L-A positive-strand fragments only if L-BC is present. Using as template X positive ssRNA in vitro T7 RNA polymerase transcripts (having the normal L-A 3′ end), we tested L-A viral particles made from L-A cDNA clones in vivo for replicase activity. The activity we found proved not to be due to L-A viral particles (data not shown), although it required the same 3′ end structures for template activity as does the L-A replicase (see below).

Several L-A-o strains were heat cured of L-BC and examined for replicase activity with the X positive-strand template (Fig. 2). All three strains lacking L-A but carrying L-BC had the activity, but the isogenic L-BC-o derivatives lacked it. The presence of the activity in the *ski2-2* L-BC strain JR10, constructed by cytoduction from strain JR5 (L-A-o L-BC) into the *ski2-2* L-A-o L-BC-o derivative (JR2), shows that the activity was due to a cytoplasmic genetic element. Strain 396, lacking 20S RNA (W dsRNA) and 23S RNA (T dsRNA) but carrying L-C, had activity, while strain 395, carrying both 20S and 23S RNAs but lacking L-BC, showed no activity (Fig. 2). Thus, the activity is not encoded by 20S or 23S RNAs, and the presence

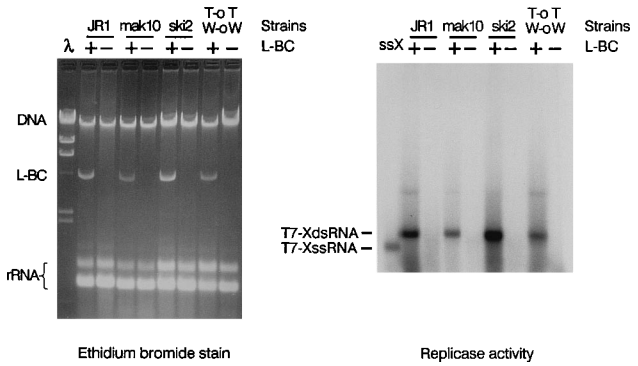

FIG. 2. Replication of X positive strands depends on the presence of L-BC particles. (Left) Nucleic acids were extracted (14) from isogenic pairs of strains (all L-A-o) with and without L-BC and T (20S RNA) and W (23S RNA) dsRNAs, analyzed by electrophoresis on a 1% agarose gel, and stained with ethidium bromide. λ = bacteriophage λ DNA digested with *Hin*dIII. (Right) Extracts were made of the strains in the left panel, and particles with the density of L-A empty particles were purified by CsCl equilibrium density gradients, precipitated, and used in replication reactions. The template was unlabeled T7 RNA polymerase transcripts of pLM1 (X positive-strand sequence). The reaction product was phenol extracted, ethanol precipitated, and analyzed on a 1.5% NuSieve 3:1 agarose gel. An autoradiograph of the dried gel is shown. ssX, ^{32}P-labeled X ssRNA template as a size marker.

and absence of the activity correlated well with that of L-BC. We chose strain JR1 as the source of L-BC particles for further study because of the high level and stability of activity in this strain.

L-BC particles replicate M_1, L-A, and X positive ssRNAs with 3′ end specificity. When M_1 and L-A positive ssRNAs, synthesized by isolated viral particles, were used as templates for the purified L-BC opened empty particles (Fig. 3), each was converted to its double-stranded form.

The sequence specificity of the L-BC particles was examined by using as templates T7 transcripts of derivatives of the X cDNA clone previously used to characterize the sites recognized by the L-A replicase (11). Plus-strand transcripts terminating at the normal 3′ end (Fig. 4A, lanes 1 and 2, *Fsp*I site) were replicated, but minus-strand transcripts (Fig. 4A, lane 5) and plus-strand transcripts terminating at the upstream *Eco*RI site were not (Fig. 4A, lanes 3 and 4). Alteration of the pen-

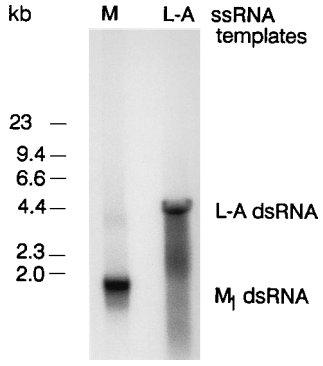

FIG. 3. Replication of M_1 and L-A positive ssRNAs by L-BC empty viral particles. Unlabeled M_1 and L-A positive ssRNAs, obtained by endogenous transcription of mature M_1 or L-A dsRNA-containing viral particles, were used as templates for a replication reaction with [α-^{32}P]UTP and L-BC empty viral particles obtained from strain JR1. The reaction products were phenol extracted, ethanol precipitated, and analyzed by electrophoresis on a 1% agarose gel, and an autoradiogram of the dried gel is shown. The migration of λ *Hin*dIII markers is shown.

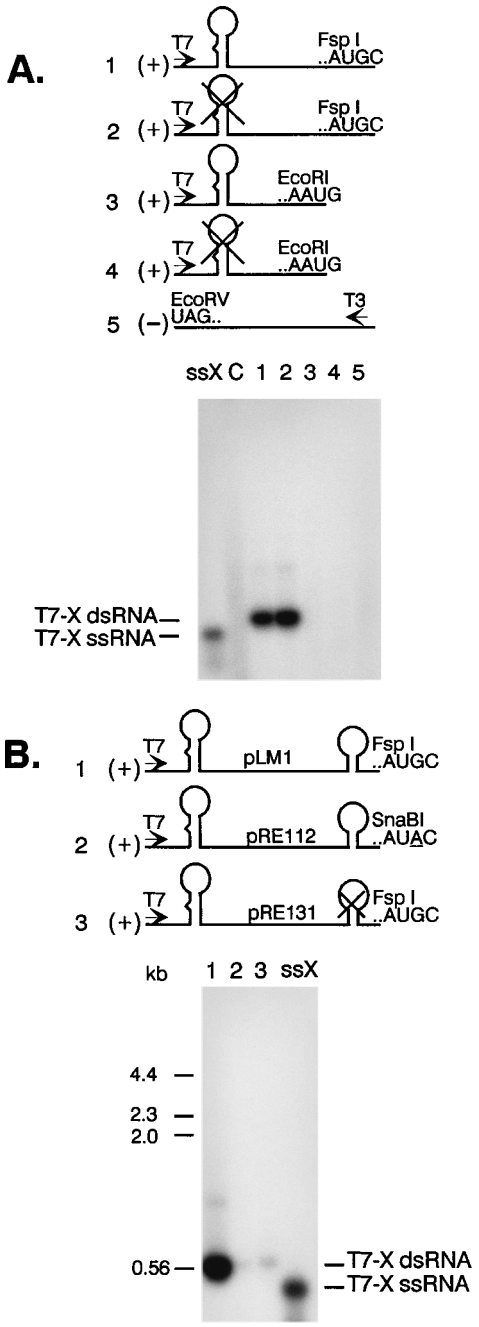

FIG. 4. Template specificity of L-BC empty particles with X positive ssRNA variants. (A) T7 or T3 RNA polymerase transcripts were made from pLM1 (lanes 1, 3, and 5, with internal replication enhancer) or pLM19 (lanes 2 and 4, no internal replication enhancer) cut with *Fsp*I (lanes 1 and 2, leaves correct 3′ end), *Eco*RI (lanes 3 and 4, leaves wrong 3′ end), or *Eco*RV (lane 5, to make X negative strand). Lane C, no template added. The procedure was as for Fig. 2. (B) Effect of 3′ end changes in X positive-strand template on replication by L-BC empty particles. T7 RNA polymerase transcripts were made from pLM1 cut with *Fsp*I (lane 1, normal 3′ end), pRE112 cut with *Sna*BI (lane 2, G-to-A change at second nucleotide from the 3′ end), or pRE131 cut with *Fsp*I (lane 3, deletion of nucleotides 13 to 19 from 3′ end removing stem-loop [Fig. 1]). The procedure was as for Fig. 2.

ultimate nucleotide G to A (pRE112) or deletion of the stem-loop near the 3′ end (pRE131) eliminated activity with L-BC viral particles (Fig. 4B) just as had been found for L-A viral particles (11).

In addition to the critical 3′ end structures, the L-A replicase is stimulated about 10-fold by a stem-loop structure located about 400 nucleotides from the 3′ end of L-A or M_1 (11). This internal replication enhancer is not, however, necessary for template activity for L-BC replicase (Fig. 4A, compare lanes 1 and 2). This was further confirmed with transcripts of pRE141, pRE145, pRE148, and pRE149 (reference 11 and data not shown) that contain different modifications in this structure.

Characterization of L-BC replicase. The L-BC empty particle replicase activity requires all four nucleoside triphosphates, with essentially complete loss of activity on elimination of even a single nucleoside triphosphate (data not shown). The labeled products of L-BC replicase with the X T7 transcript, authentic M_1 positive ssRNA, or L-A positive ssRNA as templates were examined by RNase A digestion. The single-stranded templates were digested at both high (0.5 M NaCl and 20 mM Tris Cl) and low (20 mM Tris Cl) salt concentrations while the products were RNase A sensitive only at low salt, as expected for dsRNA (data not shown). Using a strand separation gel and M_1 RNAs, we confirmed that the newly synthesized strand was the negative strand (data not shown).

L-BC and L-A viral particles prefer their homologous templates in binding, replication, and transcription. The ability of L-BC opened empty viral particles to utilize L-A and M_1 positive ssRNAs is paradoxical in view of the apparent independence of these systems in vivo. We thus directly compared L-A and L-BC positive ssRNA and dsRNAs as substrates for L-A and L-BC opened empty particles in the in vitro RNA binding, replication, and transcription reactions.

^{32}P-labeled L-A or L-BC positive ssRNAs, synthesized with L-A or L-BC mature (dsRNA-containing) particles, were tested for binding to L-A and L-BC empty particles by a gel retardation assay (Fig. 5). As expected, L-A particles specifically bind L-A positive ssRNA while L-BC particles preferentially bind L-BC positive ssRNA. Addition of up to 20 μg of tRNA, 10 μg of *Torula* RNA, 2 μg of T7 transcripts of plasmids SK^+ or KS^+, or 20 μg of denatured DNA did not diminish binding.

Unlabeled L-A and L-BC positive ssRNAs were prepared to measure their relative utilization as replication templates. The L-BC particles have relatively poor transcriptase activity, making the synthesis of large amounts of positive ssRNA impossible. Equal amounts of L-BC positive ssRNA were supplied to the L-A and L-BC empty particles, but six times the amount of L-A positive ssRNA was supplied to the L-BC particles as was supplied to the L-A particles. Although only small amounts of L-BC positive ssRNA template were available, it was better utilized by L-BC particles than the larger amounts of L-A positive ssRNA (Fig. 5). Similarly, the lower amount of L-A template was nonetheless much better utilized by the L-A empty particles.

Transcriptase activity (positive-strand synthesis on a dsRNA template) was compared by supplying L-A and L-BC empty particles with L-A and L-BC dsRNA in comparable amounts. Although each showed some synthesis with the nonhomologous template, each clearly preferred its own template (Fig. 5).

DISCUSSION

Although we initially detected the L-BC RNA-dependent RNA polymerase activity with an L-A template (X positive strands), we found that its presence was not connected with the L-A virus. We found the activity was transmitted by a nonchromosomal genetic element, was present only in strains carrying L-BC, and was unrelated to 20S RNA or 23S RNA. Although template specificity of this enzyme for the 3′ end of X positive

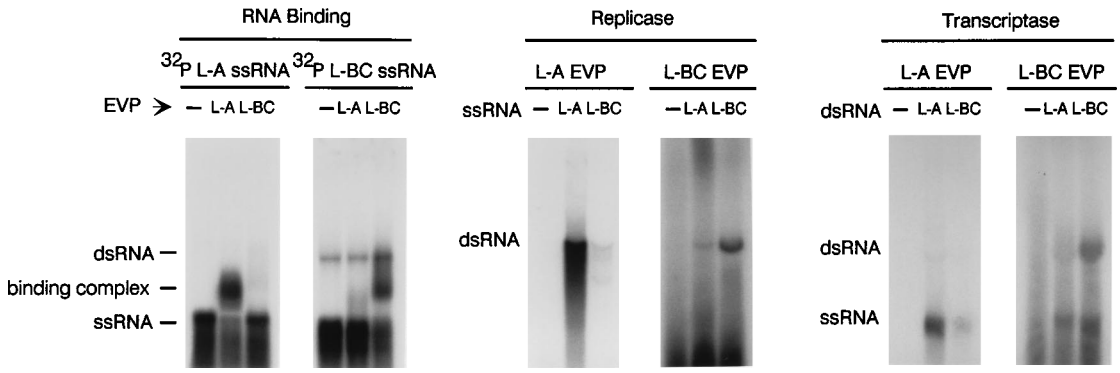

FIG. 5. Comparison of L-A and L-BC viral particles in RNA binding, replication, and transcription specificity. (Left) Binding complexes between L-A or L-BC empty particles and L-A or L-BC positive ssRNAs. ^{32}P-labeled positive ssRNAs were made by L-A or L-BC dsRNA-containing viral particles. The binding reaction is described in Materials and Methods. Binding complexes were separated on a 1% agarose gel. Labeled L-BC ssRNA was partially contaminated with labeled L-BC dsRNA (see control with no empty particles) because of contamination of the mature L-BC viral particles (used to make the positive ssRNA) with immature viral particles containing incompletely double stranded RNA. (Center) Replication by L-A or L-BC empty viral particles of L-A or L-BC ssRNA. Unlabeled L-A or L-BC ssRNAs were made with L-A or L-BC dsRNA-containing viral particles. A constant amount of L-BC ssRNA (<10 ng) was used as template. A total of 6.6 and 1.1 ng of L-A ssRNA were used as template for L-BC and L-A empty particles, respectively. (Right) Transcription by L-A or L-BC empty viral particles of L-A or L-BC dsRNA. Unlabeled L-A and L-BC dsRNAs were from particles from strains TF229 and JR1, respectively. A total of 600 ng of dsRNA template was used for L-A viral particles, and 1.2 μg was used for L-BC particles. Procedures were as for Fig. 3.

ssRNA was the same as that of the L-A replicase, L-BC enzyme was unaffected by the internal replication enhancer, a stem-loop structure 400 nucleotides from the 3′ end which increases template activity for the L-A enzyme 5- to 10-fold.

These results posed two problems. (i) How does L-BC maintain its genetic identity in vivo? If it were to replicate L-A positive strands, it would be quickly lost. (ii) Why does L-BC work on L-A positive strands at all? Is there some structural similarity?

The binding of L-A and M_1 positive ssRNA by L-A opened empty particles has been shown to be a measure of the RNA packaging reaction since the site recognized by the particles in binding RNA (10) serves as a portable RNA packaging sequence in vivo (15). It is likely that the binding of L-BC positive ssRNA by L-BC empty particles described here represents part of the L-BC packaging reaction, although this remains to be directly demonstrated. We find that this reaction is highly specific, with L-A and L-BC empty particles each preferring their cognate positive ssRNAs in the binding. The replication reaction also is quite specific, with each virus preferring its own template, even though each empty particle can utilize the nonhomologous template at some lower efficiency. Finally, the L-A and L-BC transcriptase activities each prefer the homologous templates. Thus, there is the expected specificity at each step of the viral propagation cycle.

The explanation of the partial recognition of L-A positive strands by the L-BC replicase may lie in a similarity of structure of L-A and L-BC RNAs and RNA-dependent RNA polymerases. Recently, Park et al. (30) (GenBank accession number U01060) have sequenced L-BC. Its overall architecture is similar to that of L-A, with two open reading frames, the 5′ open reading frame encoding the major coat protein and the 3′ open reading frame encoding the RNA-dependent RNA polymerase. This is the typical *Totivirus* architecture, other examples include the *Leishmania* dsRNA virus and *Giardia* dsRNA virus. There is remarkable similarity throughout between the L-A Pol and the L-BC Pol, beyond what is expected from their both encoding RNA-dependent RNA polymerases (6, 22, 30). While the location of signals for L-BC replication has not yet been determined, this homology makes it likely that there is similarity with the L-A system. The L-BC positive-strand 3′ end is . . .ACGCA-OH, while that of L-A is . . .AUGCA-OH (41) (Fig. 1). While the terminal untemplated "A" is dispensable for the L-A replicase, the preceding three bases are each important (11). We find these terminal bases are also important for illegitimate recognition of the L-A 3′ end by the L-BC replicase. The stem-loop sequence near the L-A 3′ end is also necessary for L-BC replicase activity, and L-BC itself may have such a structure, although not in exactly the same location (Fig. 1). A precedent for this is the L-A replicase requiring the stem-loop at the 3′ end of the L-A positive strand, but M_1 lacking this structure and yet being able to substitute for the L-A positive strand (11). These results suggest that either there are two (or more) different structures that these replicases can recognize or we have not yet identified the common feature(s) that makes them recognizable.

While the L-BC replicase recognizes the L-A 3′ end with requirements similar to those of the L-A replicase itself, the L-BC replicase is indifferent to the presence or absence of the L-A internal replication enhancer, a sequence 400 nucleotides from the 3′ end that increases the template activity of L-A positive strands 5- to 10-fold for the L-A replicase. The quantitative differences in template activity of the L-A and L-BC positive ssRNAs can be roughly accounted for by assuming that L-BC has its own distinct internal replication enhancer with a 5- to 10-fold effect (data not shown).

Other RNA viruses, including Qβ (2, 28), the coronavirus mouse hepatitis virus (26), and brome mosaic virus (31), have internal sites involved in replication. The Qβ replicase binds first to the internal M site of the positive strand before it interacts with the 3′ end (2, 28), much like the mechanism proposed for L-A (21). In brome mosaic virus segment 3 replicating in *S. cerevisiae*, the intercistronic region is necessary for assembly of the replicase (31).

There is no evidence that the L-A and L-BC viruses interact. Their stable compatibility implies that they do not compete for common limiting replication components. Both L-A and L-BC respond to the *SKI2 SKI3 SKI8* antiviral system that acts by limiting translation of non-poly(A) mRNA (such as viral mRNAs) (1, 27, 52). However, while L-A needs the *MAK3* *N*-acetyltransferase to acetylate the N terminus of its Gag (38–40) and the *MAK10* and *PET18* products (25, 38, 44) (functions unknown), L-BC needs neither of these but does require the *CLO* product (47) (function unknown).

Thus, different host factors and significantly different specificities at each step of their replication cycles allow these viruses to peacefully coexist without either helping or interfering with each other.

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