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Genetics

In vitro L-A double-stranded RNA synthesis in virus-like particles from *Saccharomyces cerevisiae**

(killer yeast/minus-strand synthesis)

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ABSTRACT **Most strains of *Saccharomyces cerevisiae* harbor L-A double-stranded RNA (dsRNA), 4.5 kilobases long, contained in virus-like particles (VLPs). These L-A VLPs can be separated by CsCl density gradient centrifugation into a main peak of particles, containing full-length L-A dsRNA, which synthesizes only plus-strand single-stranded RNA (ssRNA), and a lighter fraction of VLPs, containing plus-strand ssRNA, which has L-A dsRNA-synthesizing activity. This dsRNA-synthesizing activity was present in particles from logarithmically growing cells but not from stationary-phase cells. The newly synthesized strand of dsRNA in the lightest particles was full-length minus strand. All or almost all of the new minus strand was synthesized *in vitro*, and the rate of chain elongation was approximately 100 nucleotides per minute. The lightest particles synthesized plus-strand ssRNA only after completion of dsRNA synthesis, indicating that the same particle contains dsRNA- and ssRNA-synthesizing enzyme(s). We also observed dsRNA-synthesizing activity in L-BC dsRNA-containing particles similar to that in L-A VLPs.**

Most strains of *Saccharomyces cerevisiae* harbor virus-like particles (VLPs) which contain linear double-stranded RNA (dsRNA) (1–3). At least three different dsRNA species may be found inside the particles. M dsRNA [1.5 or 1.8 kilobases (kb) long, depending on the killer determinant] encodes a killer toxin lethal to other strains of the same species. M dsRNA also determines immunity to the toxin (1, 2). L-A dsRNA (4.5 kb) encodes the major coat protein in which both M and L-A dsRNAs are separately encapsidated (4–6) and carries the genes [HOK], [NEX], and [EXL], defined by their effects on M replication (5, 7–10). L-BC dsRNA (4.5 kb) has no homology with L-A dsRNA and is encapsidated in particles whose major protein is different from that of L-A and M VLPs (5).

L-A (but not L-BC) dsRNA replication requires the chromosomal genes *MAK3*, *MAK10*, and *PET18* (5, 7, 8, 11). Only a complex chromosomal defect called *clo*$^-$ is known to affect L-BC replication (12).

VLPs prepared from stationary-phase cells have an RNA-dependent RNA polymerase activity producing full-length single-stranded RNAs (ssRNAs) from M and L-A dsRNAs (13–17). These plus-strand ssRNAs are all released from the particles in the case of L-A VLPs (18). M_1 VLPs with two M_1 dsRNAs per particle extrude all of their ssRNA product, whereas those with one M_1 dsRNA per particle often retain the plus-strand ssRNA copies (18). Although a preliminary report of *in vitro* incorporation into L dsRNA exists (19), no *in vitro* L-A minus-strand synthesis has been demonstrated. *In vitro* M dsRNA synthesis in VLPs has recently been reported (18).

Density-transfer experiments indicate that replication of L-A and M dsRNAs is conservative (20, 21), like that of reovirus (22). In contrast, semiconservative replication has been demonstrated in the case of the dsRNA viruses $\phi 6$ of *Pseudomonas phaseolicola* (23) and PsV-S of *Penicillium stoloniferum* (24). *In vivo* pulse–chase experiments suggest that L-A dsRNA replicates by a sequential synthesis of first a plus strand, followed by a minus strand (25). Further, almost all intracellular L-A single strands are plus strands (21). These results predict that minus-strand synthesis occurs using the plus single strand as template.

In this paper we demonstrate *in vitro* L-A dsRNA synthesis. The newly synthesized strand of the dsRNA is shown to be the minus strand. This activity was detected in VLPs prepared from logarithmically growing cells but not from stationary-phase cells. We also observed L-BC dsRNA synthesis in a similar manner to that of L-A. Based on these results, we propose a replication model for L-A and L-BC dsRNAs.

MATERIALS AND METHODS

Strains and Growth. Strains RE59 (*a arg1 ski2-2*, L-A-HN, L-BC, M-o), 1774 (*a arg9* L-A-HN, L-BC-o, M-o) (5), and 2506 (α *thr1 ski2-2*, L-BC, L-A-o, M-o) were grown at 30°C in YPAD broth (1% yeast extract/2% peptone/2% glucose containing 0.5 g of adenine·HCl per liter). L-A-HN is the most widely distributed natural variant of L-A dsRNA (5, 7, 10). The *ski* mutation causes high levels of L-A and L-BC dsRNAs (26). Logarithmically growing cells were obtained by growth under the above conditions for 4–5 hr, until the cell density reached 8×10^7 cells per ml. Stationary-phase cells were obtained by growth for 3 days under the same conditions.

VLP Preparations. VLPs were prepared as described elsewhere (27). Briefly, cells were lysed by zymolyase, followed by passage through a French pressure cell. After cell debris was removed, VLPs were precipitated by centrifugation at 100,000 × *g* for 40 min. The precipitate was suspended in buffer A (50 mM Tris Cl, pH 7.6/1 mM dithiothreitol/10 mM Na EDTA/0.15 M NaCl), and the solution was cleared by centrifugation at 9600 × *g* for 15 min. The density of the supernatant was adjusted to 1.35 g/ml by addition of CsCl, and the solution was centrifuged at 130,000 × *g* for 20 hr. All fractions were dialyzed overnight against buffer A containing 20% (vol/vol) glycerol and stored at −70°C before use. Under these conditions, dsRNA-synthesizing activity was stable for at least 4 months.

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Abbreviations: dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; VLP, virus-like particle; kb, kilobase(s).

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RNA Polymerase Reaction and Analysis of the Products. The RNA polymerase reaction mixture (14) contained 50 mM Tris Cl (pH 7.6), 5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM NaCl, 5 mM KCl, 10 mM 2-mercaptoethanol, 1.5 mg of bentonite per ml, 0.5 mM each ATP, CTP, and GTP, and 20 μM [α-^{32}P]UTP (New England Nuclear). The mixture was incubated at 30°C, and the reaction was stopped by addition of 20 mM Na EDTA and 0.3% $NaDodSO_4$. The phenol-extracted products were precipitated with ethanol, washed with 70% ethanol, and dried. Electrophoresis under denaturing conditions was done as described by Thomas (28). Strand separation in a 5% polyacrylamide gel was carried out as described (29).

RESULTS

L-A dsRNA Synthesis in Virus-Like Particles. L-A VLPs were prepared from log-phase cells of strain RE59. After cesium chloride equilibrium density gradient centrifugation, aliquots of each fraction were incubated in an RNA polymerase reaction mixture, and the reaction products were separated by agarose gel electrophoresis (Fig. 1 *A* and *B*). The main peak of L-A dsRNA-containing VLPs (fraction 10) synthesized only material migrating with L-A ssRNA, but lighter fractions also had an activity that incorporated ^{32}P from [α-^{32}P]UTP into material migrating with L-A dsRNA. Fraction 16 synthesized primarily material migrating with L-A dsRNA. When VLPs were prepared from stationary-phase cells of the same strain, almost no dsRNA-synthesizing activity could be detected (Fig. 1 *C* and *D*).

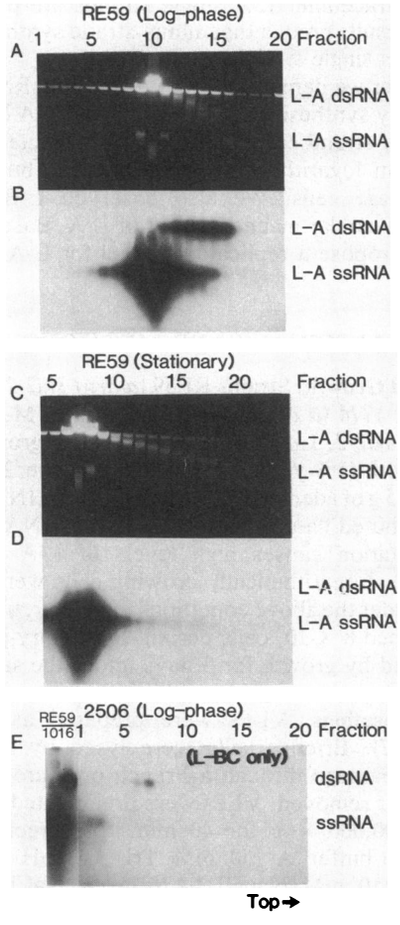

FIG. 1. RNA polymerase reaction products of VLPs in fractions separated by CsCl density gradient centrifugation. VLPs were prepared from logarithmically growing cells of strain RE59 (*ski2* L-A L-BC) (*A* and *B*) or 2506 (*ski2* L-BC) (*E*) or from stationary-phase cells of strain RE59 (*C* and *D*). After dialysis, aliquots of each fraction were incubated with the RNA polymerase reaction mixture for 60 min. Then the products were extracted with phenol and analyzed by electrophoresis in 1.5% agarose gels. (*A* and *C*) Ethidium bromide staining of the gels. (*B*, *D*, and *E*) Autoradiograms of the gels. *D* is overexposed to show the absence of dsRNA synthesis. In *E*, for comparison, the reaction products of fraction-10 and -16 particles prepared from strain RE59 (the same fractions as those in *A* and *B*) were electrophoresed in the same gel.

Strain RE59 also contains a small amount of L-BC dsRNA, which has no homology with L-A dsRNA (5). More than 80% of the labeled products of both fraction 10 and fraction 16 particles hybridize with denatured L-A RNA (Table 1). Further, when VLPs were prepared from log-phase cells of strain 1774, which has no L-BC dsRNA, we found the same pattern of ssRNA- and dsRNA-synthesizing activity as in Fig. 1 *A* and *B* (data not shown). L-A dsRNA is resistant to pancreatic RNase in the presence of 0.6 M NaCl. The product of the fraction-10 particles is fully sensitive to RNase but that of fraction-16 particles is resistant to RNase in the presence of 0.6 M NaCl (Fig. 2). These results indicate that the lighter particles incorporate labeled UTP into L-A dsRNA.

With increasing incubation time, the L-A ssRNA product of fraction 10 grew in length, reaching full length at about 45 min (Fig. 3). The product of the fraction-16 L-A dsRNA-synthesizing particles likewise showed gradual change in migration, starting at slightly larger than the size of L-A ssRNA and reaching full-sized L-A dsRNA after about 45 min. Extrapolation of the migration of the fraction-16 intermediates to zero time shows that the precursor migrates like L-A ssRNA. This indicates that most or all of the second L-A strand is being synthesized *in vitro*. The *in vitro* average chain elongation rate calculated from these data is about 100 nucleotides per min for both dsRNA- and ssRNA-synthesizing activities. Fraction-10 particles incorporate about 0.42 L-A ssRNA molecules per hr per L-A dsRNA molecule. Thus, about 30% of the fraction-10 particles were active in making L-A ssRNA.

If a tiny proportion of particles were semiconservatively making many plus and minus strands, then the time course in Fig. 3 should show full-length material at the earliest times and fraction 7 in Fig. 1*D* should show product incorporated into dsRNA. That this is not the case shows that transcription and replication are conservative. Earlier work (16, 20, 21) produced a similar conclusion, but with the qualification (now eliminated) that a few particles might be rapidly turning out semiconservatively synthesized transcript and dsRNA.

The Newly Synthesized Strand in dsRNA Synthesis Is the Minus Strand. The *in vitro*-synthesized strand of the L-A dsRNA product of fraction-16 particles is full-length, since its

Table 1. Hybridization of RNA polymerase reaction products of various VLPs with L-A dsRNA

L-A dsRNA	RNase treatment	Acid-precipitable product, cpm: VLPs from RE59 Fr. 10	VLPs from RE59 Fr. 16	VLPs from 2506 Fr. 3	VLPs from 2506 Fr. 7
–	–	7980	3750	3330	2660
–	+	0	310	540	330
+	–	8080	3790	3420	2350
+	+	6340	3370	660	390

VLP fractions (Fr.) prepared from logarithmically-growing cells of strain RE59 (containing L-A and L-BC) or 2506 (containing L-BC only) are as defined in Fig. 1. After the RNA polymerase reaction, the products of these particles were denatured and hybridized (27) with denatured L-A dsRNA (1.6 μg) prepared from strain K7. This strain has no L-BC (5). After hybridization, unhybridized ssRNA was digested by pancreatic and T1 RNases under high-NaCl conditions (0.3 M).

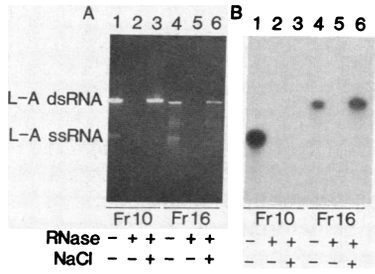

FIG. 2. Effects of RNase treatment on RNA polymerase reaction products of fraction-10 and -16 particles. Phenol-extracted RNA polymerase reaction products of fraction-10 (lanes 1–3) or fraction-16 (lanes 4–6) particles were treated with pancreatic RNase (100 μg/ml, 60 min, 37°C) in the presence (lanes 3 and 6) or absence (lanes 2 and 5) of 0.6 M NaCl. Lanes 1 and 4 are controls (without RNase treatment). After RNase treatment, RNA was separated in a 1.5% agarose gel and detected by ethidium bromide staining (*A*) or autoradiography (*B*).

migration in a denaturing gel was the same as the L-A ssRNA product of fraction-10 particles (Fig. 4*A*) and denatured L-A dsRNA prepared from fraction-10 particles (data not shown).

We proved by two different methods that the newly synthesized strand from the L-A dsRNA made by the fraction-16 particles is the minus strand. The first method was to use a strand-separation gel (29) (Fig. 4 *B* and *C*). Before the RNA polymerase reaction, fraction 10 contained, as expected, both plus and minus strands in roughly equal amounts (Fig. 4*B*, lane 2), but fraction 16 contained mainly plus strands (Fig. 4*B*, lane 1). Indeed, the density of fraction 16 is that expected of particles containing the same protein as fraction 10, but with the L-A dsRNA molecule replaced by a single L-A RNA strand (see *Discussion*). During the RNA polymerase reaction, fraction-10 particles incorporated label only into the lower band—the plus strand—whereas fraction-16 particles labeled mostly the upper band—the minus strand. These results suggest that the fraction-16 particles have

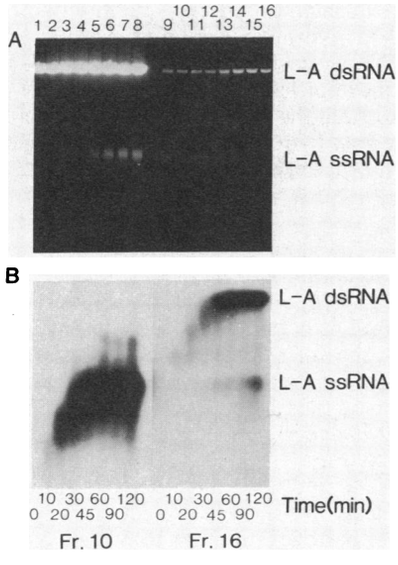

FIG. 3. Time course of RNA polymerase reaction of fraction-10 and -16 particles. Fraction-10 (ssRNA-synthesizing; lanes 1–8) and fraction-16 (dsRNA-synthesizing; lanes 9–16) particles were incubated with the RNA polymerase reaction mixture for the times indicated. After the reaction, products were extracted with phenol, separated in an agarose gel, and detected by ethidium bromide staining (*A*) or autoradiography (*B*). Incubation times are shown below each lane. Note the increase of ethidium bromide-stained L-A dsRNA in fraction-16 particles after 45 min of incubation.

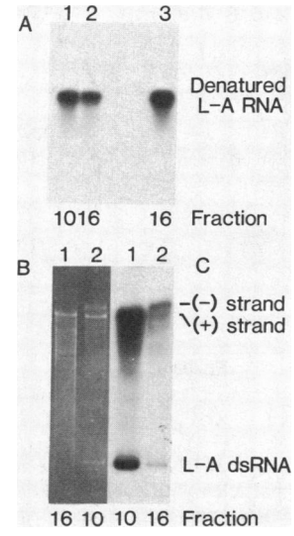

FIG. 4. (*A*) Analysis of denatured RNA polymerase reaction products of fraction-10 and -16 particles. After RNA polymerase reaction for 60 min, the products of fraction-10 (lane 1) and -16 (lane 2) particles were extracted with phenol, denatured by glyoxal and dimethyl sulfoxide, and analyzed in an agarose gel. An autoradiogram of the gel is shown. The dsRNA product of fraction-16 particles purified by agarose gel electrophoresis was processed similarly (lane 3). (*B* and *C*) Strand separation of fraction-10 and -16 particle endogenous RNA and RNA polymerase products in a 5% polyacrylamide gel. (*B*) Nucleic acids from fraction-10 (lane 2) and fraction-16 (lane 1) VLPs were extracted with phenol, denatured with dimethyl sulfoxide, and analyzed by gel electrophoresis (29) followed by staining with ethidium bromide. (*C*) After fraction-10 (lane 1) and fraction-16 (lane 2) VLPs were incubated in RNA polymerase reaction mixtures for 60 min, the products were extracted with phenol, denatured with dimethyl sulfoxide, electrophoresed, and detected by autoradiography.

mainly the plus strand and, using this plus strand as template, synthesize a new minus strand.

We next examined the products of fraction-10 and fraction-16 particles by hybridization with an L-A fragment cloned in phage M13 (Fig. 5). These M13 clones have a 360-base-pair *Pst* I fragment of an L-A cDNA clone (30) in the *Pst* I site of M13 mp9 (21) and were kindly provided by R. Sclafani. Without heat denaturation, almost all of the ssRNA produced by fraction-10 particles hybridized with M13 8w3 ssDNA containing the minus-strand fragment (lane 10), but not with M13 8p1 ssDNA having the plus-strand fragment (lane 9), as judged by the formation of a hybrid moving more slowly than either the L-A dsRNA or the phage DNAs themselves. When the fraction-10 RNA product was heat-denatured before hybridization with the M13 ssDNAs, the extent of hybrid formation was decreased because of competition between the added M13 8w3 ssDNA and the preexisting minus-strand RNA in the L-A dsRNA for hybrid formation with the newly formed plus strands. These results confirm that the product of the fraction-10 particles was the plus-strand ssRNA, as previously shown (16).

In the case of L-A dsRNA produced by fraction-16 particles, more hybrid formation was observed with M13 8p1 ssDNA (plus strand, lane 3) than with M13 8w3 (minus strand, lane 4) (Fig. 5). The hybrid in the latter case seems to come from hybridization of M13 8w3 with L-A ssRNA produced in small quantity by fraction-16 particles because this plus ssRNA disappeared after hybridization with M13 8w3. This confirms that the newly synthesized strand of L-A dsRNA produced by fraction-16 particles is the minus strand.

***In Vitro* Maturation of the Low-Density Particles.** Fraction-16 particles produce mainly dsRNA when incubated for 60 min in an RNA polymerase reaction mixture (Fig. 1*B*), but

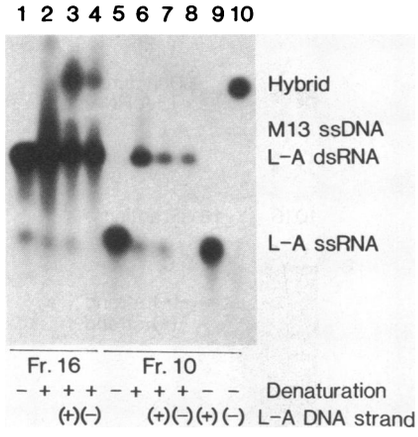

FIG. 5. Hybridization of RNA polymerase reaction products of fraction-10 and -16 particles with each strand of an L-A fragment cloned in phage M13. RNA polymerase reaction products of fraction-10 particles (60-min incubation; lanes 5–10) and fraction-16 particles (50-min incubation; lanes 1–4) were prepared using [α-^{32}P]UTP at 50 Ci/mmol. They were extracted with phenol, ethanol-precipitated, dried, and dissolved in H_2O containing 3 mg of *Torula* RNA per ml. To 1.5 μl of the RNA polymerase products described above, 1 μl of M13 8p1 or M13 8w3 at 2 mg/ml or a buffer (10 mM Tris Cl, pH 7.6/1 mM EDTA) as control and 10 μl of formamide were added. Then the mixture was incubated at 87°C for 1 min. Ten microliters of 10× SSC (1× SSC is 15 mM sodium citrate, pH 7.0/0.15 M NaCl) was added, and the mixture was incubated at 46°C for 10 min to allow annealing. The hybrids formed were precipitated with ethanol, washed once with 80% ethanol, and analyzed in a 1.5% agarose gel. An autoradiogram of the gel is shown. Lanes 1 and 5: RNA polymerase products as control. Lanes 2 and 6: heat-denatured products, but annealed in the absence of M13 ssDNA. Lanes 3 and 7: heat-denatured products hybridized with M13 8p1 (plus strand). Lanes 4 and 8: heat-denatured products hybridized with M13 8w3 (minus strand). Lanes 9 and 10: nondenatured fraction-10 particle product hybridized with M13 8p1 (plus strand) and M13 8w3 (minus strand), respectively.

after completion of dsRNA synthesis, fraction-16 particles apparently start to make ssRNA (Fig. 3). To prove this, fraction-16 particles were incubated in an RNA polymerase reaction mixture from which [α-^{32}P]UTP was omitted; then, at 60 min, [α-^{32}P]UTP was added to the reaction mixture (Fig. 6). The particles now incorporated the label mainly into ssRNA and only slightly into dsRNA. The rate of incorporation of the label into ssRNA was almost the same as the incorporation into total RNA when label was added to the same particles at zero time. In this experiment, the UTP incorporated into dsRNA and ssRNA was only about 5% of the total present in the assay mixture, and breakdown of UTP was negligible. Thus, the specific radioactivity of UTP was almost the same whether the labeled UTP was added at the beginning of the reaction or after 60 min. We conclude that most fraction-16 particles can make ssRNA only after completion of dsRNA synthesis and that the same particle has an enzyme or enzymes that make ssRNA and dsRNA. Thus, fraction-16 particles functionally matured *in vitro*. It is possible that the determinant of products might be just the RNA itself inside of the particle; that is, when a particle has a plus ssRNA, it makes dsRNA, and if dsRNA is inside of a particle, this particle makes plus ssRNA.

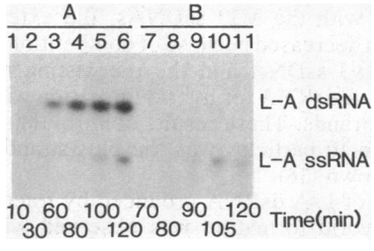

FIG. 6. Delayed labeling of RNA polymerase reaction products of fraction-16 particles. Fraction-16 particles were incubated in two RNA polymerase reaction mixtures. That represented in *A* contained [α-^{32}P]UTP from time zero. In that represented in *B*, only nonradioactive UTP was present until 60 min; then [α-^{32}P]UTP was added and the incubation was continued. As discussed in the text, the specific activities of UTP in *A* and *B* were essentially the same (1 Ci/mmol). Aliquots were withdrawn at the times indicated, and the products were extracted with phenol and separated in an agarose gel. An autoradiogram of the gel is shown.

The "Staircase" Molecules. Lighter fractions than the main peak of L-A dsRNA-containing VLPs also contained a "staircase" of L-A dsRNA migrating faster than full-length L-A dsRNA, but with the fraction-10 particles as one extremity (Fig. 1 *A* and *C*) (18). The migration of the staircase molecules does not change after RNase treatment in high salt (Fig. 2), indicating that these are partial-length, fully dsRNA molecules, presumably a spectrum of deleted molecules derived from L-A rather than replication intermediates, which would be expected to have one full-length (plus) strand and a partial-length (minus) strand. Further, the staircase is present in similar amounts in VLPs from log-phase and late stationary-phase cells, but stationary-phase cells make little dsRNA *in vitro* (or, presumably, *in vivo*). The staircase also extends to fractions lighter than the lightest of those synthesizing dsRNA (namely, fraction 16). This suggests that there are staircase particles containing less than one full strand's weight of L-A RNA. This again suggests dsRNA deletion molecules derived from L-A.

dsRNA Synthesis in L-BC-Containing Particles. We similarly observed *in vitro* dsRNA synthesis in L-BC-containing particles (Fig. 1*E*). Strain 2506 has L-BC dsRNA but not L-A dsRNA. The main peak particles (fraction 3) made a product that moved at the same rate as L-A ssRNA. Again, particles lighter than the main peak particles made a product whose migration rate was the same as L-BC and L-A dsRNAs. These products were confirmed not to be related to L-A dsRNA, by hybridization experiments (Table 1). Products of fraction 3 (the main peak particles) and fraction 7 (the lighter particles) did not hybridize with an excess of L-A dsRNA, as judged by RNase sensitivity in high salt. (Compare these results with those of L-A-containing particles.)

We conclude that the main peak particles containing L-BC dsRNA made only L-BC ssRNA and that the lighter particles had an activity that synthesizes L-BC dsRNA.

DISCUSSION

Four chromosomal genes, *PET18* [= *MAK31* + *MAK32* (11)], *MAK3*, and *MAK10*, are necessary for L-A dsRNA replication. While *PET18* is known to be necessary for L-A VLP stability (27), the roles of the other chromosomal genes in dsRNA replication have not been assigned because of a lack of information on the replication processes and also a lack of proper *in vitro* experimental systems for examining each process.

In the present study, we have demonstrated *in vitro* L-A dsRNA synthesis. We showed this activity in particles lighter than the major particles (fraction 10) which synthesized only plus L-A ssRNA. The density of the major particle fraction was 1.410 g/ml, and it has been calculated that these particles have one L-A dsRNA molecule per particle (18, 31). The density of the lightest particles (fraction 16) that have dsRNA-synthesizing activity was 1.359 g/ml. Assuming that the fraction-16 particles have the same amount of protein per particle as those of the major particles, fraction-16 particles have about 1.30×10^6 daltons of RNA per particle. Since the molecular mass of L-A dsRNA is 3.0×10^6 daltons, the

fraction-16 particles have about one full-length L-A ssRNA. The result of the calculation is consistent with three lines of evidence. First, the majority RNA species of the fraction-16 particles was plus-strand L-A ssRNA (Fig. 4*B*). Second, time-course experiments (Fig. 3) showed that the precursor of dsRNA replication of the fraction-16 particles seems to be L-A ssRNA. Third, the replication product of the fraction-16 particles was full-length L-A dsRNA. This means that the precursor is a full-length plus L-A ssRNA or a partial dsRNA with a full-length plus L-A strand. We do not know whether the fraction-16 particles can initiate minus-strand synthesis *in vitro*, although we were able to observe incorporation of [γ-^{32}P]GTP into L-A dsRNA of the fraction-16 particles in an RNA polymerase reaction (data not shown).

Two lines of evidence suggest that the demonstrated *in vitro* dsRNA synthesis in the particles is really one of the processes of replication. First, dsRNA-synthesizing activity was detected when the particles were prepared from logarithmically growing cells but not from stationary-phase cells. Second, *in vivo* pulse–chase experiments have suggested a sequential L-A dsRNA replication, first plus-strand synthesis, followed by minus-strand synthesis (25). This *in vivo* replication sequence is consistent with the *in vitro* L-A dsRNA synthesis presented here.

Fig. 7 shows a model of L-A and also L-BC dsRNA replication, since we observed the same pattern of L-BC dsRNA synthesis as of L-A dsRNA. A similar model has been proposed for M dsRNA replication, except for the formation of a particle with two M dsRNA molecules inside (18). In the model of Fig. 7, synthesis of the particles with plus ssRNA has not yet been demonstrated.

The fraction-16 particles can make ssRNA after completion of L-A dsRNA synthesis. This indicates that fraction-16 particles have undergone *in vitro* maturation, at least functionally. This also means that the same particles have the enzyme or enzymes that catalyze transcription and replication. In the case of reovirus, core protein λ-3, encoded by the L1 genome segment, is thought to catalyze both reactions (22). Time-course experiments (Fig. 3) show that the chain-elongation rate of replication (dsRNA synthesis) is the same as that of transcription (ssRNA synthesis). This may indicate that the transcriptase is identical to the replicase of L-A VLPs.

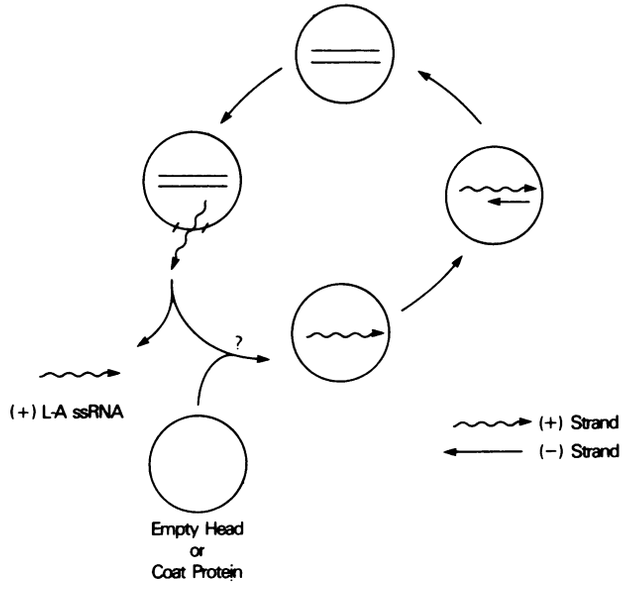

FIG. 7. A model of L-A and L-BC dsRNA replication. A particle with dsRNA (top of figure) makes plus (message)-strand ssRNA, all of which is extruded from the particle. Some of the plus-strand ssRNA molecules are encapsidated by coat protein or preformed empty heads, so that a particle with plus-strand ssRNA is formed. This particle performs minus-strand synthesis using plus-strand ssRNA as template to again produce a particle with dsRNA.

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