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Thermolabile L-A Virus-Like Particles from *pet18* Mutants of *Saccharomyces cerevisiae*

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***pet18* mutations in *Saccharomyces cerevisiae* confer on the cell the inability to maintain either L-A or M double-stranded RNAs (dsRNAs) at the nonpermissive temperature. In in vitro experiments, we examined the effects of *pet18* mutations on the RNA-dependent RNA polymerase activity associated with virus-like particles (VLPs). *pet18* mutations caused thermolabile RNA polymerase activity of L-A VLPs, and this thermolability was found to be due to the instability of the L-A VLP structure. The *pet18* mutations did not affect RNA polymerase activity of M VLPs. Furthermore, the temperature sensitivity of wild-type L-A RNA polymerase differed substantially from that of M RNA polymerase. From these results, and from other genetic and biochemical lines of evidence which suggest that replication of M dsRNA requires the presence of L-A dsRNA, we propose that the primary effect of the *pet18* mutation is on the L-A VLP structure and that the inability of *pet18* mutants to maintain M dsRNA comes from the loss of L-A dsRNA.**

Saccharomyces cerevisiae has five nonhomologous families of double-stranded RNA (dsRNA), called L-A, L-(BC), T, W, and M (2, 17, 18, 25, 28, 34). Each variant of M (M_1, M_2, etc.) encodes a secreted protein toxin and resistance to that toxin, so that strains carrying M are called killers (2, 3, 25). L-A is the major dsRNA in most killer strains (18, 30) and encodes the major protein of the intracellular noninfectious virus-like particles (VLPs) in which both L-A and M are found (1, 6, 10–12, 18). M, in fact, depends on L-A for its maintenance (17), and this is presumably because it must be encapsulated in VLPs. For its maintenance and replication, M_1 requires the products of at least 30 chromosomal genes, including *MAK1* through *MAK28* (29, 32; for a review see reference 31) *PET18* (2, 4, 13, 32), and *SPE2* (4). In several cases, the role of these genes for the host is known; namely, *MAK8* encodes ribosomal protein L3 (33), *SPE2* encodes adenosylmethionine decarboxylase (spermine and spermidine biosynthesis) (4), and *MAK1* encodes DNA topoisomerase I (20). In no case, however, has the role of these genes in M_1 dsRNA metabolism been defined.

While all of these genes are needed for M_1 replication, only *MAK3*, *MAK10*, and *PET18* are needed for L-A replication (18, 34); and none are known to be involved in L-(BC), T, or W replication, although most have been tested. *PET18* is, in addition, needed for replication of mitochondrial DNA and for cell growth (13, 32).

To investigate the role of these genes in dsRNA replication, we chose to begin with *pet18* mutants since they are temperature-sensitive for M_1 replication (32) and, as we show here, for L-A replication. This enabled us to isolate M- and L-A-containing VLPs from these temperature-sensitive mutants grown at the permissive temperature and compare them with VLPs from wild-type strains at various temperatures. Our results indicate that the *PET18* gene is involved in maintaining the structure of the L-A-containing VLPs. Based on the mutants studied, *PET18* does not appear to affect M VLP structure. Other differences between M- and L-A-containing VLPs are also described here.

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MATERIALS AND METHODS

Strains and media. Strains used in this study are listed in Table 1. Media were as described previously (29).

Genetic analysis and cytoduction. Genetic analysis was done by the usual methods (15). Cytoplasmic genomes were transferred by cytoduction from one haploid strain to another with the karyogamy-defective *kar* mutation (5). The procedure was essentially the same as that described previously (16) but was slightly modified as follows. First, the temperature was kept at 20°C during cytoduction, and the following steps were performed to select cytoductants if the recipient strain was *pet18*. Second, in all cases, cytoduction was carried out between [*rho*^{0}] donor and recipient strains. When L-A-HN was transferred, donor cells were counterselected by plating the mating mixture for single colonies on the appropriate media. Usually 50 to 100 colonies were randomly picked, and their [HOK] activity was assayed by crossing them with a [KIL-sd] strain (22, 34). If cytoductants received L-A-HN, the resultant diploids could maintain M dsRNA and therefore show the normal killer character. Transfer of L-A-HN was also confirmed by a concomitant increase of total L dsRNA (18, 30).

VLP preparation. Cells were cultured in YPAD broth (1% yeast extract, 2% peptone, 2% glucose, 0.5 g of adenine sulfate per liter) at 20°C for 3 days, harvested, and washed once with water. Washed cells were treated with 0.1 M Tris hydrochloride (pH 7.6)–1 M sorbitol–20 mM 2-mercaptoethanol–0.5 mg of zymolyase 60,000 (Kirin Brewery Co., Tokyo, Japan) per ml at 20°C for 1 h. The resultant spheroplasts were collected, suspended in buffer A (50 mM Tris hydrochloride [pH 7.6], 10 mM sodium EDTA, 0.15 M NaCl, 1 mM dithiothreitol), and lysed by passing them through a French pressure cell (14,000 lb/in^2). Bentonite was added (1 to 2 mg/ml), and cell debris was removed by low-speed centrifugation (9,600 × *g* for 30 min). VLPs were collected by high-speed centrifugation (100,000 × *g* for 40 min), suspended in buffer A, and precipitated again by high-speed centrifugation. The final precipitate was suspended in a small volume of buffer A containing 20% glycerol and served as the source of enzyme and template for the RNA polymerase reaction.

TABLE 1. Strains of *S. cerevisiae* used in this study

Designation	Genotype[a]	Source or reference
1067	a *ade2-1 trp1 pet18-1* [HOK] [NEX] [KIL-o] [*rho*^{0}]	7
1089	a *leu1 kar1-1* [HOK-o] [NEX-o] [KIL-o] [*rho*^{0}]	5
1090	α *his4-15 kar1-1* [HOK-o] [NEX-o] [KIL-o] (*rho*^{0}]	
1538	α *ade(2,3) his1 trp1 pet18-1* [HOK] [NEX] [KIL-k_2] [*rho*^{0}]	7
1197	α *pet18-2 lys1 his5 ura5 ura3 ade* [HOK-o] [NEX-o] [KIL-o] [*rho*^{0}]	14
1889	α *lys2 pet18-3* [HOK-o] [NEX-o] [KIL-o] [*rho*^{0}]	13
TF87	α *his4-15 kar1-1* [HOK] [NEX] [KIL-o] [*rho*^{0}]	Cytoductant from 1067 into 1090
TF116	a *leu1 kar1-1* [HOK] [NEX] [KIL-o] [*rho*^{0}]	Cytoductant from TF87 into 1089
TF138	α *lys2 pet18-3* [HOK] [NEX] [KIL-o] [*rho*^{0}]	Cytoductant from TF116 into 1889
TF141	α *pet18-2 lys1 his5 ura5 ura3 ade* [HOK] [NEX] [*rho*^{0}]	Cytoductant from TF116 into 1197
TF100	a *leu1 kar1-1* [HOK] [NEX] [KIL-k_2] [*rho*^{0}]	Cytoductant from 1538 into 1089
1686	α *arg1 thr1* [HOK-o] [NEX-o] [KIL-o] [*rho*$^+$]	18
K7	a *arg9* [HOK] [NEX] [KIL-k_1] [*rho*$^+$] L-BC-o	18

[a] [KIL-k_2], wild-type K_2 killer cytoplasmic genome (its molecular determinant is the 1.5-kilobase M_2 linear dsRNA) carrying killing and resistance functions; [HOK], helper of killer; the [HOK] activity enables M_1 dsRNA to replicate in a *SKI*$^+$ nuclear background; [NEX] makes [KIL-k_2] nonexcludable by [EXL]; [EXL] excludes [KIL-k_2] in the absence of the [NEX] activity; [HOK], [NEX], and [EXL] reside on certain kinds of L-A dsRNA, such as L-A-HN and L-A-E; [KIL-o], [HOK-o], and [NEX-o], absence of [KIL-k_2], [HOK], or [NEX].

For separation of L-A VLPs from M VLPs, the final precipitate was suspended in buffer A, the density was adjusted to 1.35 by adding CsCl, and the solution was centrifuged (130,000 × *g*) for 20 h at 4°C. Fractions were collected, dialyzed against buffer A containing 20% glycerol, and used for the RNA polymerase assay.

RNA polymerase assay. The RNA polymerase assay was carried out as described previously (27), with slight modifications. VLPs were incubated in an assay mixture containing 50 mM Tris hydrochloride (pH 7.6); 5 mM $MgCl_2$; 0.1 mM EDTA; 20 mM NaCl; 5 mM KCl; 10 mM 2-mercaptoethanol; 0.5 mM each of ATP, GTP, and CTP; 20 μM [α-^{32}P]UTP (New England Nuclear Corp., Boston, Mass.); and 1.5 mg of bentonite per ml. After incubation at 20°C for 1 h, the reaction products were precipitated by the addition of 0.3% *Torula* RNA (Sigma Chemical Co., St. Louis, Mo.) as carrier and 10 mM sodium PP_i, followed by the addition of 10% trichloroacetic acid. After incubation on ice for 10 min, the precipitate was collected on a Whatman GF/B glass fiber filter, and its radioactivity was measured. Bentonite was prepared as described previously (8). All the assays were done under conditions in which the activity was linear with time and with the amount of VLPs. All the experiments were carried out in duplicate.

dsRNA. L-A dsRNA was purified from strain K7 by cellulose chromatography as described previously (21). The purified L-A dsRNA did not hybridize with L-(BC), T, or W and served for hybridization experiments.

To analyze dsRNAs in cells, the rapid method for extraction described by Fried and Fink (9) was done, and the extracted dsRNAs were analyzed on 1% agarose gels.

Hybridization. The hybridization procedure was essentially the same as that described by Welsh and Leibowitz (26).

RNA polymerase reaction products, labeled with ^{32}P as described above, were extracted with phenol in the presence of 0.2% sodium dodecyl sulfate (SDS)–7 mM EDTA, precipitated with 70% ethanol, and washed once with 70% ethanol. The precipitate was dried and suspended in 8× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), containing 0.2% SDS and 200 μg of *Torula* RNA per ml as carrier.

One microgram of purified, cold L-A dsRNA (or water alone, as control) was denatured and heat sheared at 83°C for 3 min in the presence of 90% formamide. Then, the same volume of ^{32}P-labeled RNA polymerase reaction products dissolved in 8× SSC was added. After another 1-min incubation at 83°C, annealing was begun at 46°C. After a 30-min incubation at 46°C, annealing was stopped by the addition of 50 volumes of 2× SSC alone or 2× SSC containing RNase A (40 μg/ml) and T1 (160 U/ml), and unhybridized single-stranded RNA was digested for 15 min at 37°C. Hybridized RNA was precipitated with 10% trichloroacetic acid in the presence of 1 mg of *Torula* RNA and 10 mM sodium PP_i and collected onto a Whatman GF/B filter, and its radioactivity was counted.

Structural stability of VLPs. Fifty microliters of the VLP fraction prepared by high-speed centrifugation was incubated at 35°C for 10 or 60 min. After incubation, 450 μl of buffer A was added, and VLPs were precipitated by centrifugation (110,000 × *g*) for 1 h at 4°C. Then, the dsRNA content in the precipitate and in the supernatant was measured. To 200 μl of the supernatant was added 200 μl of phenol in the presence of 0.3% SDS. To the precipitate, 500 μl of buffer A containing 0.3% SDS was added. After solubilization, 200 μl of phenol was added to 200 μl of the solubilized precipitate. Thereafter, dsRNAs extracted from the precipitate and from the supernatant with phenol were treated similarly. After phenol extraction, 1.2 ml of ethanol was added to 200 μl of the aqueous solution. dsRNAs were precipitated by incubation in dry ice-ethanol for 30 min, followed by centrifugation for 10 min at 4°C in an Eppendorf centrifuge. The precipitate was dried and analyzed on 1% agarose gels. After electrophoresis, dsRNAs were stained with 0.5 μg of ethidium bromide per ml and photographed with Polaroid 55 P/N film. The film negative was scanned by Quick Quant II (Helena Laboratories Corp.), and relative amounts of dsRNA were obtained.

RESULTS

L-A replication is thermolabile in *pet18* strains. *pet18* mutations confer on the cell temperature sensitivity for maintenance of M dsRNA; that is, *pet18* mutants can replicate and maintain M dsRNA at 20°C (permissive temperature) but they lose it at 30°C (nonpermissive temperature) (32), whereas wild-type strains can maintain M dsRNA at both of these temperatures. Maintenance of L-A dsRNA is also affected by *pet18* mutations (17, 18, 34). We find that this effect is also temperature dependent.

Spores from a diploid heterozygous for *pet18* and carrying L-A-HN were germinated and grown at 20°C. The *pet18* spore clones all maintained [HOK], a marker on L-A, if they were maintained at 20°C and had the same amounts of L dsRNA as the L-A-carrying wild-type spore clones if they

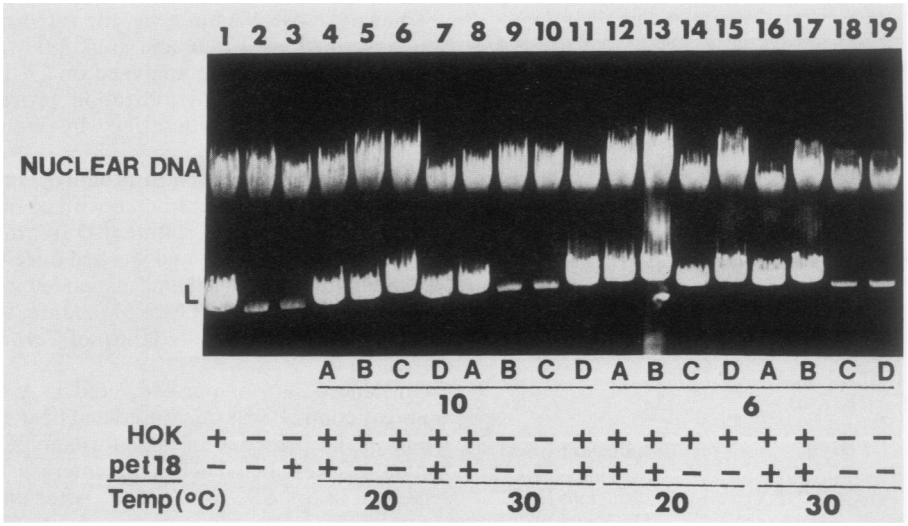

FIG. 1. Maintenance of L-A is temperature sensitive in *pet18* strains. dsRNAs were extracted from two tetrad clones (asci 10 and 6) and from their parental strains, 1067 (*pet18-1*) and 1686 (*PET*$^+$), and analyzed on a 1% agarose gel. The same amount of cells was used for each extraction. Positions of L dsRNAs and nuclear DNA are shown. Segregation of *PET18* alleles and maintenance of [HOK] activity are also shown in the lower part of the figure. All strains were grown at 20°C for the dsRNA preparation, but some were first grown to single colonies at 30°C to eliminate L-A. Lanes: 1, 1067; 2, 1067, but with L-A-HN cured by growth at 30°C; 3, 1686; 4 to 7, tetrad clones from ascus 10; 8 to 11, same as in lanes 4 to 7, but subcloned at 30°C; 12 to 15, tetrad clones from ascus 6; 16 to 19, same as in lanes 12 to 15, but subcloned at 30°C.

were grown only at 20°C (Fig. 1). Subcloning at 30°C resulted in the loss of [HOK] from all *pet18* spore clones but not from wild-type spore clones (10 tetrads). When such *pet18* [HOK-o] subclones were then grown at 20°C, they had only a much lower amount of L dsRNA (Fig. 1), which we have previously shown is L-(BC) (17), a completely unrelated dsRNA. Thus, the maintenance of L-A is temperature sensitive in *pet18* strains, and *pet18* L-A strains grown at 20°C have normal amounts of L dsRNA, most of which is L-A.

We partially purified L-A-containing VLPs (L-A VLPs) from *pet18* cells grown at the permissive temperature and compared the thermostability of their RNA-dependent RNA polymerase activity with that of particles from *PET*$^+$ cells (Fig. 2). The strains that were compared contained the same L-A which had been introduced into the *PET*$^+$ strain by cytoduction from the *pet18* strain. L-A VLPs from *pet18* cells and from *PET*$^+$ cells were preincubated at various temperatures for 10 min, and then the remaining activity was assayed at 20°C (Fig. 2A). L-A RNA polymerase from *pet18* cells was more thermolabile than that from *PET*$^+$ cells. This difference was more pronounced when preincubation was carried out for various times at 35°C, followed by assay at 20°C (Fig. 2B).

To determine if the difference was due to the *pet18* mutation, similar experiments were carried out with L-A VLPs from strains carrying two other independent *pet18* mutant alleles, one of which was isolated by Wickner and Leibowitz (32) (Fig. 2C) and one of which was isolated by R. K. Mortimer (personal communication; data not shown). The results were similar in each case, although the difference

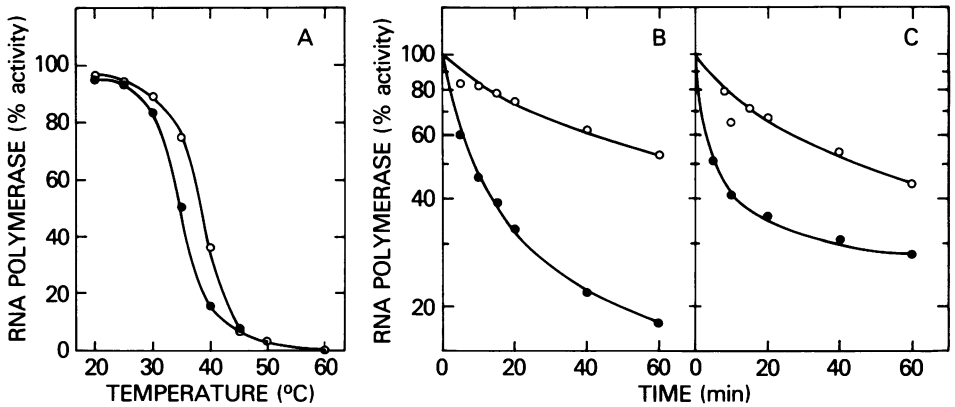

FIG. 2. Thermolabile RNA polymerase activity of L-A VLPs from *pet18* mutants. (A) Partially purified L-A VLPs from *PET*$^+$ strain TF87 (○) or *pet18* strain 1067 (●) were preincubated for 10 min at the indicated temperatures, and then the remaining RNA polymerase activity was assayed at 20°C. Data were normalized by using the values obtained without preincubation. (B) L-A VLPs were preincubated at 35°C for the indicated times, and then the remaining activity was assayed at 20°C. Symbols: ○, L-A VLPs from TF87 (*PET*$^+$); ●, L-A VLPs from 1067 (*pet18-1*). (C) Experimental protocol was the same as in panel B. Symbols: ○, L-A VLPs from TF116 (*PET*$^+$); ●, L-A VLPs from TF138 (*pet18-3*).

from wild-type particles was not as great in the case of the mutant from Mortimer. In addition, the thermolability cosegregated in two tetrads with the *pet18* mutation. The results for one tetrad are shown in Fig. 3. In each case, the L-A VLPs from a *pet18* strain, which became [*rho*^{0}] because of the *pet18* defect, was compared with L-A VLPs from a wild-type strain made [*rho*^{0}] by treatment with ethidium. A mixture of L-A VLPs from wild-type and *pet18* cells gave the expected average of the two samples assayed separately (Fig. 4).

That we actually measured L-A RNA polymerase activity and not L-(BC) activity, for example, can be shown by three lines of evidence. First, after L-A dsRNA was cured by growth at 30°C, we could not detect RNA polymerase activity in such VLP fractions that were prepared similarly, although these cells still had a small amount of L-(BC) (Fig. 1). Second, more than 70% of the RNA polymerase reaction product hybridized to L-A dsRNA in hybridization experiments (see below and Table 2). Thus, most of the reaction product was the L-A transcript because L-BC has no homology with L-A. Third, the *pet18* L-A strains grown at the permissive temperature had normal amounts of L, most of which appeared to be L-A (Fig. 1).

From the results presented above, we conclude that the RNA polymerase activity of L-A VLPs from *pet18* strains is thermolabile when compared with that of the wild type and that this difference is due to the *pet18* mutation itself and not

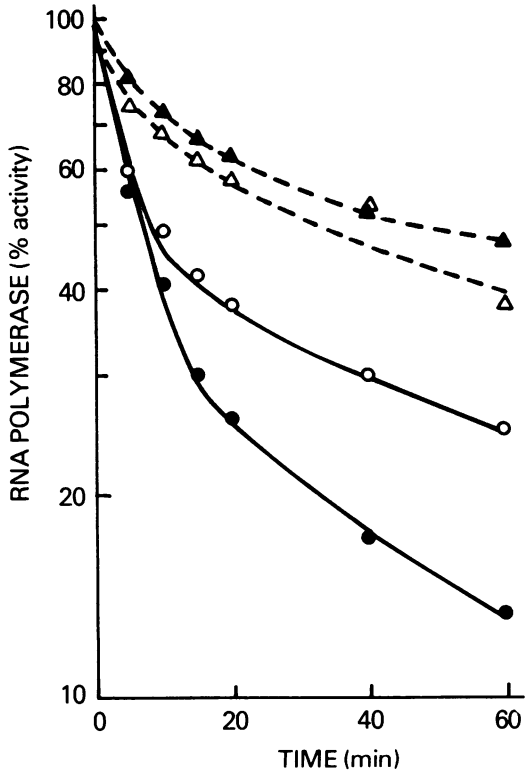

FIG. 3. Cosegregation of *pet18* mutation with thermolabile RNA polymerase activity of L-A VLPs. L-A VLPs were prepared from spore clones of ascus 10 (Fig. 1), which was grown at 20°C and which came from a cross of 1067 (*pet18*) with 1686 (*PET*$^+$). L-A VLPs were preincubated at 35°C for the indicated times, and then the remaining RNA polymerase activity was assayed at 20°C. Symbols: ▲, 10A (*PET*$^+$); ○, 10B (*pet18*); ●, 10C (*pet18*); △, 10D *PET*$^+$).

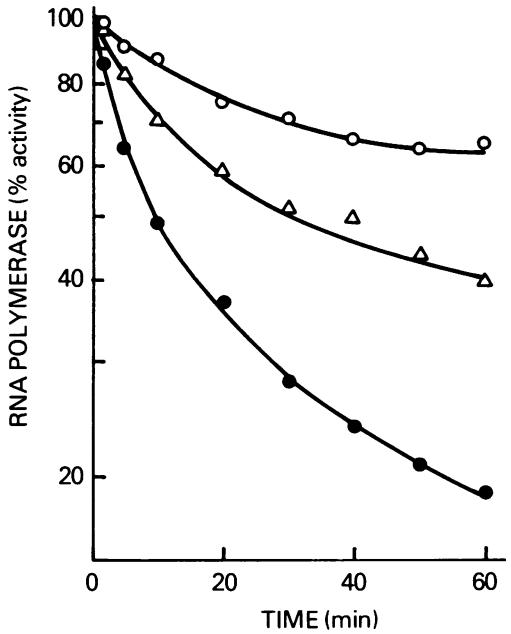

FIG. 4. Mixing experiments. L-A VLPs from *pet18* cells (1067) were mixed with those from *PET*$^+$ cells (TF87) that had the same RNA polymerase activity. The mixture was preincubated at 35°C for the indicated times. The remaining activity was assayed at 20°C (△). Data were normalized with the values obtained without preincubation. As controls, L-A VLPs from 1067 (●) and those from TF87 (○) were incubated separately at 35°C as described above, and their remaining activities were assayed separately at 20°C.

to either the absence of mitochondrial DNA or an abnormally low level of such particles.

The *pet18* mutation does not affect M VLP RNA polymerase activity. Since *pet18* mutants lose M dsRNA at 30°C, we examined the effect of *pet18* on M VLP RNA polymerase activity. M_2-containing VLPs were purified from *pet18* and *PET*$^+$ cells and separated from L-A particles on CsCl gradients. L-A and M_2 VLPs were preincubated at various temperatures, and then the remaining activity was assayed at 20°C (Fig. 5). The RNA polymerase activity of M_2 VLPs from *PET*$^+$ cells was not only more thermostable than that of L-A VLPs from the same cells, but the former was significantly heat activated. We also observed similar thermostability and heat activation of the RNA polymerase activity of M_1 VLPs from *PET*$^+$ cells.

M_2 VLP RNA polymerase activity from *pet18* cells showed similar thermostability to that from *PET*$^+$ cells (Fig. 5), and experiments from several preparations showed that

TABLE 2. Hybridization of RNA polymerase reaction product(s) to L-A dsRNA

L-A dsRNA	RNase treatment	L-A VLPs[a]		M_2 VLPs[a]	
		cpm	%	cpm	%
−	−	14,900	100	16,500	100
−	+	910	6.1	1,270	7.7
+	−	15,600	105	15,400	93
+	+	11,600	78	770	4.7

[a] L-A and M_2 VLPs were prepared from a K_2 killer strain TF100 (*PET*$^+$) by using CsCl density gradient centrifugation, and RNA polymerase reaction products of these particles were analyzed by hybridization with L-A dsRNA as described in the text.

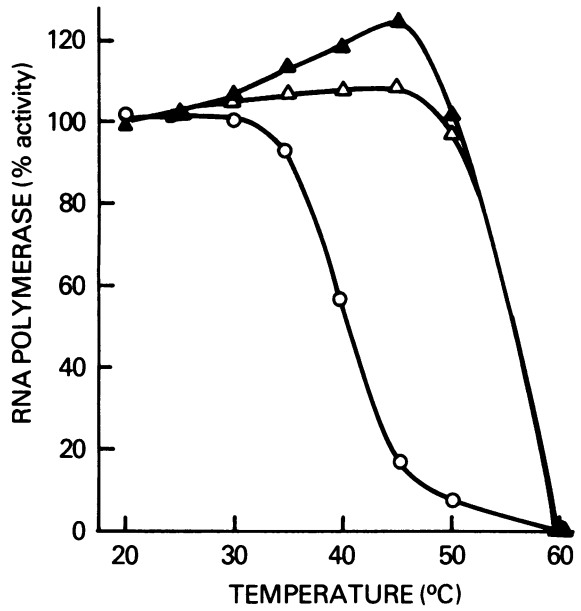

FIG. 5. Thermolability of L-A RNA polymerase differs from that of M_2 RNA polymerase. L-A VLPs and M_2 VLPs were prepared from strain 1538 (*pet18*) or TF100 (*PET*$^+$) and separated by CsCl density gradient centrifugation as described in the text. VLPs were preincubated for 10 min at the indicated temperatures, and then the remaining RNA polymerase activity was assayed at 20°C. Symbols: ○, L-A from *PET*$^+$ cells; △, M_2 from *PET*$^+$ cells; ▲, M_2 from *pet18* cells.

there was no significant effect of the *pet18* mutation on this thermostability.

From these results, we conclude that M_1 and M_2 VLP RNA polymerase activities are more thermostable than L-A VLP RNA polymerase activity and that *pet18* mutations do not affect the M_2 VLP RNA polymerase activity.

We were unable to obtain L-A VLP RNA polymerase activity from *pet18* strains (such as 1538) by CsCl gradient equilibrium centrifugation, probably because of the instability of such particles. That this strain 1538 had thermolabile L-A VLP RNA polymerase was proven as follows. Both L-A and M_2 were eliminated by growth at the nonpermissive temperature (30°C), and L-A alone was reintroduced by cytoduction at 20°C (permissive temperature). This obviated the need for the CsCl gradient, the purpose of which was to separate L-A and M_2 VLPs. L-A VLPs were purified from the *pet18* cytoductant by high-speed centrifugation, and the thermolability of their RNA polymerases was compared with L-A VLPs from a wild type. We got essentially the same results as those shown in Fig. 2; that is, L-A VLP RNA polymerase activity from the *pet18* cytoductant was more thermolabile than that from *PET*$^+$ cells (data not shown) and thus is far more thermolabile than the M_2 VLP RNA polymerase activity obtained from essentially the same strain (isogenic for nuclear genes and carrying the same L-A).

To confirm that we were indeed measuring L-A and M_2 transcription in the respective fractions, the products were hybridized with excess denatured L-A dsRNA and then treated with RNase A and T1 under conditions in which only single-stranded RNA was digested. About 80% of the trichloroacetic acid-insoluble RNA polymerase reaction product of the L-A VLP fraction became RNase resistant after hybridization with L-A dsRNA (Table 2), but in the case of the M_2 VLP fraction, the product was completely digested by the RNase treatment after hybridization with L-A. This confirms the identification of the templates that were used in the L-A and M_2 VLP fractions.

Instability of L-A VLPs from *pet18* cells. To study the basis of the thermolability of the L-A VLP RNA polymerase activity in *pet18* strains, L-A and M_2 VLPs from *pet18* cells and from *PET*$^+$ cells were incubated at 35°C, and VLPs were precipitated by centrifugation. Then, the amounts of dsRNAs in the precipitate and supernatant were analyzed by agarose gel electrophoresis and by scanning of the photographs (Table 3). L-A dsRNA from VLPs from *pet18* cells was released into the supernatant more rapidly than that from *PET*$^+$ cells. This effect paralleled the loss of RNA polymerase activity in the same experiment. Total amounts of L-A dsRNA in the precipitate and supernatant (total recovery) did not change during the incubation in either case. The amount of M_2 in the precipitate of particles from *PET*$^+$ cells was also constant during the incubation.

From these data, we conclude that the apparent thermolability of RNA polymerase activity of L-A VLPs from *pet18* cells is due to an instability of these particles relative to that of *PET*$^+$ L-A VLPs, resulting in the loss of the template RNA from the particle.

Polyamines are known to stabilize virus particles (for a review, see reference 19) and are necessary for maintenance of M_1, M_2, and L-A-E, although not for L-A-HN or L-(BC). The addition of spermidine or spermine stabilized RNA polymerase activity of L-A VLPs and magnified the difference of the temperature sensitivity between L-A VLPs from *PET*$^+$ and *pet18* cells (Fig. 6). For example, the temperature required for 50% inhibition was 4°C different between wild-type and *pet18* L-A VLPs if no polyamines were added, but in the presence of spermidine or spermine, the difference was 9°C.

This stabilization seems to be due to the effect of polyamine on the L-A VLP structure. In the presence of 5 mM spermine, incubation at 45°C for 10 min caused only a 10% release of L-A dsRNA from *PET*$^+$ VLPs, but a 70% release of that from *pet18* VLPs. These values show good correlation with degrees of apparent RNA polymerase inactivation (Table 4 and Fig. 6).

DISCUSSION

Our data show that the *PET18* product is necessary for the in vitro stability of L-A-containing VLPs. This is probably

TABLE 3. Instability of L-A VLPs from *pet18* cells

Source of particles	VLP	dsRNA in particles (% of that at time zero) at the following times (min)[a]:		RNA polymerase activity (% of that at time zero) at the following times (min)[b]:	
		10	60	10	60
PET$^+$	L-A	106	64	83	58
pet18	L-A	61	17	46	19
PET$^+$	M_2	105	110	108	116
pet18	M_2	ND[c]	ND	117	124

[a] VLPs were incubated at 35°C for the times indicated, and then the dsRNA remaining within the particles was determined as described in the text.

[b] VLPs were preincubated at 35°C for the times indicated, and then the remaining RNA polymerase activity was assayed at 20°C.

[c] ND, Not determined.

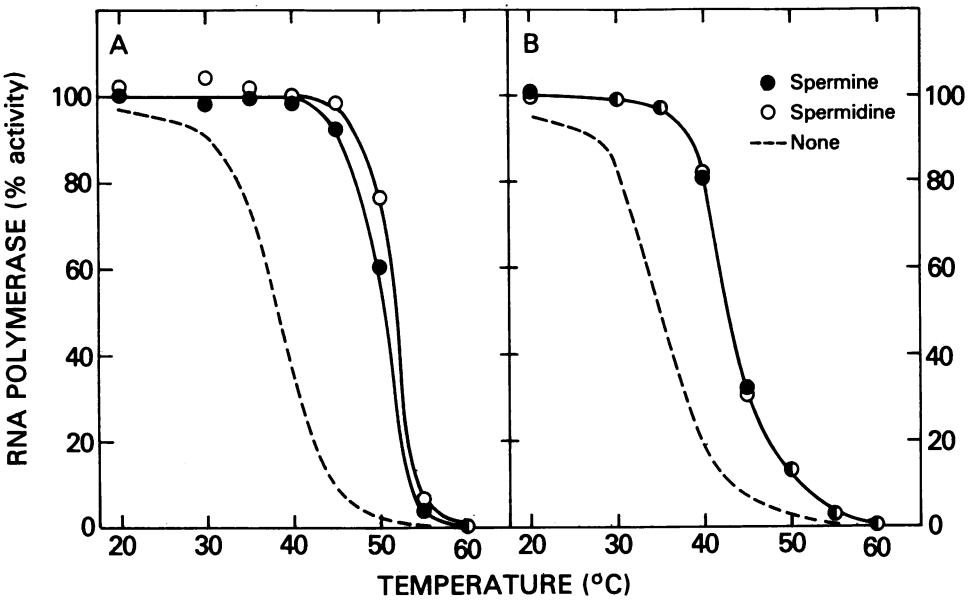

FIG. 6. Effects of polyamines on the stability of L-A RNA polymerase activity. L-A VLPs from TF116 (*PET*$^+$) cells (A) or 1067 (*pet18*) cells (B) were preincubated at the indicated temperatures for 10 min in the presence of 1 mM spermidine · 3HCl (○) or 5 mM spermine · 4HCl (●). Then, the remaining RNA polymerase activity was assayed at 20°C. Data were normalized by using the values obtained without preincubation. Dotted lines show RNA polymerase activity obtained in the absence of polyamines and are replotted from Fig. 2A.

the explanation for the temperature-dependent loss of L-A VLPs in vivo in *pet18* mutants. In the absence of the *PET18* product, L-A is released from the particles. M_1- and M_2-containing particles are not so affected. Moreover, wild-type M VLPs are much more stable than L-A VLPs. These results are of interest because they give the first insight into the role in dsRNA replication of any of the many chromosomal genes known to have such a role and because they demonstrate important differences between L-A VLPs and M VLPs.

The following three lines of evidence indicate that M dsRNA requires L-A dsRNA for its replication or maintenance: (i) no M L-A-o strains have been reported; (ii) L-A encodes the major coat protein in which both L-A and M are encapsidated (6, 12, 18); and (iii) under submaximal curing conditions, all colonies that retain M_1 also retain L-A (17). Our results indicate that *pet18* affects the L-A VLP structural stability but not that of M VLPs. This suggests that the *PET18* product(s) is directly involved in L-A replication, but is only indirectly needed for M through M's requirement for L-A.

The *pet18* mutations caused thermolability of the L-A VLP structure in a temperature-sensitive mutant. Such a result normally suggests that the *PET18* gene product is present in the VLPs and that its thermolability in the mutants is responsible for the particle structure thermolability. However, A. Toh-e (personal communication) has recently cloned and sequenced the *PET18* locus and found that the *pet18* mutants (including two of those used in our study) each comprise a deletion of the entire locus. The results of Toh-e imply that the thermolability of L-A VLPs that we have documented must be due to the absence of the *PET18* gene product(s). We thus attempted to examine purified L-A VLPs by SDS-polyacrylamide gel electrophoresis for differences in protein. Because of the instability in CsCl gradients of L-A VLPs from *pet18* strains, we were unable to purify the mutant L-A VLPs sufficiently to make a meaningful comparison. Even the addition of polyamines to stabilize the VLPs, as we have described here, was insufficient to allow the isolation of *pet18* L-A VLPs of sufficient purity. Not surprisingly, comparison of M_2 VLP preparations from *pet18* and *PET*$^+$ strains did not show protein differences which could be attributed to the VLPs.

The manner in which the *PET18* product(s) stabilizes the L-A VLP structure will require further study. The cloned *PET18* locus (A. Toh-e, personal communication) might be used to identify the protein product and then to test whether the *PET18* product is located in L-A VLPs.

The *mak16-1* mutation causes the inability to maintain M_1 dsRNA but does not affect L-A maintenance (29). We purified M_1 VLPs by CsCl density gradient centrifugation from a temperature-sensitive *mak16* mutant grown at the permissive temperature. When we examined the stability of the RNA polymerase of these particles at various temperatures, we found that the RNA polymerase of M_1 VLPs from the mutant had the same heat stability and heat activation profile as that from M_1 VLPs isolated from wild-type strains (unpublished data). This is an indication of the specificity of the findings reported here for *pet18*.

The approach used here should be applicable to the study of the role of other chromosomal genes in dsRNA replica-

TABLE 4. Effect of *pet18* on stability of L-A VLPs in the presence of spermine[a]

VLP	Particle source	dsRNA (%) in particles under the following conditions:		
		Control	45°C	55°C
L-A	*PET*$^+$	100	88	14
L-A	*pet18*	100	27	13

[a] L-A VLPs were incubated at 45°C or 55°C for 10 min in the presence of 5 mM spermine · 4HCl, and then the remaining L-A dsRNA in the particles was determined as described in the text. Data were normalized by using the values obtained without incubation.

tion. It is essential, however, to have mutants which are conditional (e.g., temperature sensitive) for their maintenance or replication of the dsRNA in question.

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