JOURNAL OF VIROLOGY, Apr. 1988, p. 1278–1285
0022-538X/88/041278-08$02.00/0

Vol. 62, No. 4

A Deletion Mutant of L-A Double-Stranded RNA Replicates Like M_1 Double-Stranded RNA

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Received 16 October 1987/Accepted 29 December 1987

X double-stranded RNA (dsRNA) is a 0.52-kilobase dsRNA molecule that arose spontaneously in a nonkiller strain of *Saccharomyces cerevisiae* originally containing L-A and L-BC dsRNAs (L-BC is the same size as L-A but shares no homology with it). X hybridized with L-A, and direct RNA sequencing of X showed that the first 5′ 25 base pairs (of the X positive strand) and at least the last 110 base pairs of the 3′ end were identical to the ends of L-A dsRNA. X showed cytoplasmic inheritance and, like M_1, was dependent on L-A for its maintenance. X was encapsidated in viruslike particles whose major coat protein was provided by L-A (as is true for M_1), and X was found in viruslike particles with one to eight X molecules per particle. This finding confirms our "head-full replication" model originally proposed for M_1 and M_2. Like M_1 or M_2, X lowers the copy number of L-A, especially in a *ski* host. Surprisingly, X requires many chromosomal *MAK* genes that are necessary for M_1 but not for L-A.

Up to five different double-stranded RNA (dsRNA) families can be found in strains of the yeast *Saccharomyces cerevisiae*, namely, L-A, L-BC, M, T, and W dsRNAs, three of which (L-A, L-BC, and M) are encapsidated in intracellular viruslike particles (VLPs) about 39 nm in diameter. Genes that code for killer toxin production and resistance (immunity) reside on M dsRNA, the size of which varies around 1.5 (M_2) or 1.8 (M_1) kilobases (kb), depending on the killer determinant. M maintenance or replication requires the presence of L-A dsRNA (4.5 kb), which encodes the major coat protein in which both M and L-A dsRNAs are separately encapsidated. Whereas M also needs the products of at least 32 chromosomal genes (called *MAK* [maintenance of killer] genes), L-A replication requires only three of these genes, namely, *MAK3*, *MAK10*, and *PET18*. Other genes (called *SKI* genes) negatively control dsRNA replication in such a way that *ski* mutants show a superkiller phenotype with an increased M copy number (reviewed in references 21 and 22).

Deletion mutants of M_1 dsRNA lacking the toxin immunity precursor coding region have been described (5, 6, 10, 12). These mutants (called S dsRNAs) interfere with (suppress) the replication of the wild-type M_1 genome (9, 10). S dsRNAs, therefore, are analogous to defective interfering virus particles, the deletion mutants of animal viruses that limit replication of the standard virus during coinfection (for a review, see reference 19). Unlike defective interfering particles, however, S dsRNAs do not depend on the parent M dsRNA for their replication; rather, both M and S are dependent on L-A for maintenance or replication, probably because both are encapsidated in VLPs whose major coat protein is L-A encoded.

All S dsRNAs characterized so far are derived from M_1 dsRNA. Partial sequence analysis (12) and heteroduplex analysis (5) of one of them, S3, shows that 229 base pairs (bp) are from the 5′ end of the M_1 positive strand and the rest (about 550 bp) are from the 3′ end of the positive strand. Another S dsRNA, S14, has been cloned and completely sequenced (6). The break points in S3 and S14 are only 21 bp apart in the 5′ end of the M_1 sequence and 10 bp apart in the 3′ end. All the S dsRNAs preserve a portion of the 5′ end of the M_1 positive strand (a minimum of 44 bp) and a much larger portion (up to 550 bp) of the 3′ end of the positive strand. The fact that S dsRNAs retain both ends of the original molecule implies that these ends probably play an essential role in the processes known to be necessary for these molecules to be maintained: transcription, replication, and encapsidation.

Since L-A produces the capsid protein essential for its own encapsidation, one might expect to find L-A deletion mutants equivalent to the S mutants, but that would not be completely suppressive, since the parental molecule remains essential. Such L-A deletions have not, until now, been observed.

We report here X, the first deletion mutant of L-A dsRNA. We show that X is similar to S mutants in that it originated as a result of a large internal deletion of the parent molecule. Whereas X is derived from L-A, its replication and its relation to host genes resemble those of M more then those of L-A.

MATERIALS AND METHODS

Strains and media. Strains of *S. cerevisiae* used in this study are listed in Table 1. Media were as previously described (20).

Genetic methods. Standard methods of genetic analysis were used (7).

Cytoduction. A cytoplasmic genome can be transferred from one haploid strain to another without diploidization or other change of nuclear genotype by transient heterokaryon formation (cytoplasmic mixing, cytoduction) by using the *kar1-1* mutant, defective in nuclear fusion (2). The procedure was the same as that described by Wesolowski and Wickner (18).

Assay of killing activity. Killing activity was assayed as previously described (4).

VLP preparation and RNA polymerase activity assay. VLP purification was as previously described (4), and the RNA polymerase activity associated with VLPs was assayed by the method of Welsh et al. (17), with slight modifications (4).

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TABLE 1. Strains of *S. cerevisiae* used

Designation	Genotype	Source of reference
1368	α *his4 kar1-1* L-A-HNB[a] L-BC M_1	P24-28C→1020; 16
2404	α *his4 kar1-1* L-A-HNB L-BC M-o	1368 ΔK^-
2507	**a** *arg1 ski2-2* L-A-o L-BC M-o	
64-4B	α *ski2-2* L-A-HNB L-BC M-o X	Meiotic segregant from cross 2404 × 2507
1089	**a** *leu1 kar1-1* L-A-o *rho*^{0}	
1101	α *his4 kar1-1* L-A-HN L-BC M_1	A364A→1020
RE57	α *arg1 ski2-2* L-A-o L-BC M-o	Meiotic segregant from cross 1368 × 2507 ΔK^- H^-
RE411	**a** *leu1* L-A-HNB L-BC X	Segregant from cross 64-4B × 1089
RE455	α *arg1 kar1-1 ski2-2* L-A-HNB L-BC X	RE411→RE57
2285	**a** *kar1-1 leu1 clo*$^-$ L-o M-o	
RE327	α *his4 leu1 kar1-1 clo*$^-$ L-A-HNB L-BC-o M_1	This work
RE464	**a** *arg1* L-A-HN L-BC X	This work
RE516	**a** *arg1 ski2-2 clo*$^-$ L-o	This work
RE558	α *his4 leu1 kar1-1 clo*$^-$ L-A-HNB L-BC-o X	RE464→RE327
3950-3B	**a** *ade2 leu2 mak21-1*	
2597	**a** *ura3 trp1 ade1 mak16-1*	
2593	**a** *leu2 ura3 mak18-1*	
2611	**a** *lys1 mak27-1*	
3958-3B	**a** *ura3 mak26-1*	
1868	**a** *leu2 cdc16 mak11-1*	16
TF78-4A	**a** *ura1 leu2 can1 mak10*^{ts}	
2321	**a** *aro7 mak3-1 leu1 his3 ura3*	
2444	**a** *ura3 mak6-1*	
2540	α *tyr1 mak1-2*	
2362	α *lys2 ade1 mkt1 mak4-1*	
1747	**a** *arg9 mak10-1* L-o	
2186	**a** *mkt1 lys1* L-A-o L-B	
1686	α *arg1 thr1* L-A-o L-C	
YHU-1652	**a** *ura3 his5* L-A-HN M-o	

[a] Natural variants of L-A dsRNA carrying various combinations of the [HOK] (or H), [NEX] (or N), [EXL] (or E), and [B] cytoplasmic genes have been identified (reviewed in reference 21; Uemura and Wickner, in press).

dsRNA purification. dsRNAs were purified by cellulose chromatography as previously described (15). To analyze dsRNA directly in cells, the rapid method for extraction described by Fried and Fink (5) was used, except that LiCl precipitation was omitted; extracted dsRNAs were analyzed on 1 to 1.5% agarose gels. When the presence or absence of certain dsRNAs could not be unequivocally established because of the large amount of rRNA and tRNA present in the samples, samples were digested with pancreatic RNase A (10 μg/ml) for 1 h at 37°C in high salt (0.5 M NaCl) before agarose gel electrophoresis. Under these conditions, single-stranded RNA (ssRNA) molecules are completely digested, whereas dsRNAs are resistant to the treatment.

Labeling of the 3′ ends of RNA. The 3′ termini of dsRNA were labeled with cytidine[3′,5′-^{32}P]diphosphate by using T4 RNA ligase under conditions suggested by the supplier (Bethesda Research Laboratories, Inc.).

The strands of X dsRNA bearing 3′-terminal cytidine[3′,5′-^{32}P]diphosphate were separated by electrophoresis in 5% acrylamide gels as previously described (14), with slight modifications. RNA was denatured at 90°C for 1 min in the presence of 7 M urea instead of 30% dimethyl sulfoxide. The samples were quickly chilled on ice and applied to the gel. Positive and negative strands were separated at 8 V/cm for 6 to 8 h.

^{32}P-labeled positive-strand X ssRNA obtained in an in vitro transcription reaction performed by purified X VLPs was used to determine which band was the positive and which was the negative strand. The faster-moving band was the positive strand.

RNA sequencing. The 3′-end-labeled positive- and negative-strand X ssRNAs separated in a 5% polyacrylamide gel as described above were cut and eluted as follows. After completion of electrophoresis, the gel was immersed in TBE buffer (90 mM Tris borate [pH 8.0] with 4 mM EDTA) containing 0.5 μg of ethidium bromide per ml for 15 min. ssRNA bands were visualized and photographed under UV light (approximately 350 nm). The gel then was subjected to autoradiography for 1 min at room temperature, and labeled bands were localized. They were cut out from the gel with an RNase-free razor blade, crushed on a sterile glass plate, and extracted overnight with 0.5 ml of 0.5 M ammonium acetate–1 mM EDTA at 37°C. Extracted RNAs were cleaned from small pieces of contaminating polyacrylamide by filtration through glass wool, and then 2.5 volumes of ethanol was added to precipitate the RNAs. After being washed with 70% ethanol, RNAs were dissolved in 10 to 20 μl of diethylpyrocarbonate-treated H_2O and used to determine the RNA sequence.

The enzymes used to partially digest the RNA and their base specificities were as follows: RNase T_1 (G), RNase U2 (A), RNase Phy M (A + U), RNase *Bacillus cereus* (C + U), and RNase CL3 (C). They were purchased in an RNA sequencing kit from Bethesda Research Laboratories. Conditions used for partial digestions and separation of the products by acrylamide gels were as recommended by the manufacturer.

Transfection with purified VLPs. Transfection experiments with purified X VLPs were carried out by the spheroplast procedure of El-Sherbeini and Bostian (3).

RESULTS

X is a deletion mutant of L-A. In the course of another study, total nucleic acids from two tetrads of the cross 2404 × 2507 (α *his4 kar1* L-A-HNB L-BC × a *arg1 ski2-2* L-BC) were extracted and analyzed on a 1.5% agarose gel (Fig. 1). We noted a band (called X) migrating at 0.52 kb (relative to lambda *Hin*dIII fragments) only in the case of spore clone 64-4B (Fig. 1, lane 6). X was sensitive to RNase A in low salt but not in high salt (data not shown), indicating that it is a dsRNA molecule.

The amount of L dsRNA in strain 64-4B was very low compared with that in the other spore clones from the same tetrad, even lower than in the *SKI* wild-type segregants. Strain 64-4B is a *ski2-2* segregant, and the amount of L dsRNA (mainly L-A) in such strains is about fivefold higher than in wild-type strains (1) (compare lanes 1, 3, 5, and 7 with lanes 2, 4, and 8, Fig. 1). The copy number of X dsRNA (0.52 kb) in strain 64-4B was even greater than that of L-A (4.5 kb) in other *ski2-2* segregants.

We showed by Northern (RNA) blot hybridization that X dsRNA had homology with L-A. L-A VLPs were prepared from a *clo* L-A L-BC-o M_1 strain (RE327) (*clo* strains lose L-BC), and the VLP transcriptase was used to make an L-A

positive ssRNA probe. Strong hybridization with X dsRNA (lanes 1 and 3, Fig. 2) and L-A from a control strain (lane 4) was observed, but no hybridization was seen with several L-BC preparations (lanes 5, 6, and 7, Fig. 2). Thus, X is a deletion mutant of L-A. Further proof of this conclusion is presented below. A small amount of full-length L-A was detected in the strain carrying X (lane 3). Its copy number was only about 1/20 that of X in the same strain. This and other data (see below) show that X lowers the copy number of L-A. Strain 64-4B (carrying X and L-A) was subcloned three times in succession, and nucleic acids from six subclones were analyzed by Northern blot hybridization as for Fig. 2. All clones gave the same result as in lane 3, Fig. 2 (data not shown). Thus, X and L-A are stably maintained together.

X depends on L-A for its maintenance and is independent of L-BC. The dependence of X on L-A for its maintenance, replication, or both was tested by two methods. (i) Using the *mak3* and *mak10*^{ts} mutant strains 2321 and TF78-4A (Table 1), which are unable to maintain L-A dsRNA, we showed that neither L-A nor X was found after introduction of both molecules by cytoduction into such strains and that by meiotic crosses the segregation pattern obtained was 2L-A X:2L-A-o X-o. When X was introduced into the *MAK3* or *MAK10* wild-type strains, all cytoductants acquired X, and in meiotic crosses, all tetrads were 4X:0X-o. This also means that X, like L-A, shows nonchromosomal inheritance. (ii) Growth of strain RE455 (α *arg1 ski2-2* L-A L-BC X) at 39°C for 3 days resulted in loss of both X and L-A from most clones. Whereas loss of X without loss of L-A was often observed, there was no case in which X dsRNA was found in the absence of L-A, indicating that X depends on L-A (data not shown). As we show below, X is encapsidated in the L-A-encoded major coat protein, and this is presumably why X requires L-A. By Northern blot hybridization, we found that when X was cured by growth at high temperature, the copy number of L-A increased to the level of the parental X-o strain. This again shows that X lowers the copy number of L-A dsRNA.

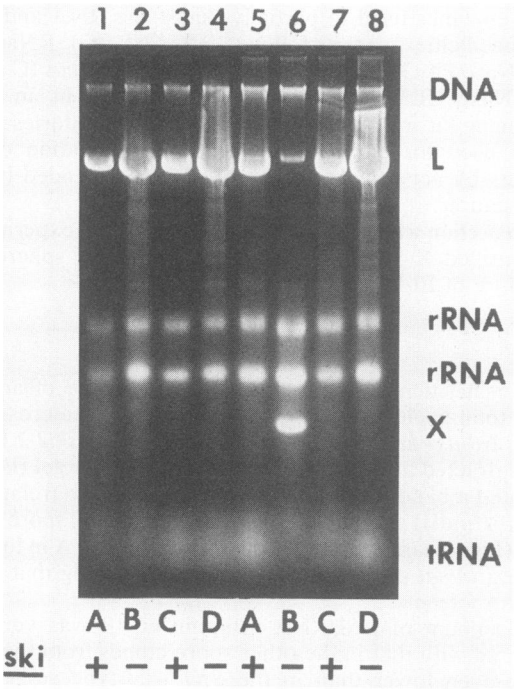

FIG. 1. Initial detection of X dsRNA. Total nucleic acids were extracted from clones of two tetrads dissected from the diploid strain 2507 × 2404 (a *arg1 ski2-2* L-BC × α *his4-15 kar1-1* L-A L-BC) and analyzed on a 1.5% agarose gel. Lanes 1 to 4 are clones from tetrad 1, and lanes 5 to 8 are clones from tetrad 4. A, B, C, and D indicate the four spore clones of each tetrad. The symbols − and + represent *ski2-2* and wild-type *SKI*$^{+}$ meiotic segregants, respectively. L means mainly L-A dsRNA; only a minor amount of L-BC is present in these preparations and is indistinguishable from L-A by size on this type of gel. X is the spontaneously deleted L dsRNA molecule present only in spore clone 64-4B (lane 6).

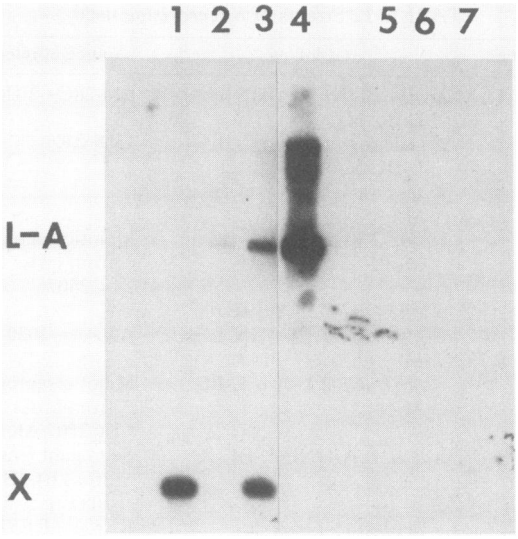

FIG. 2. Northern blot hybridization of X and different L dsRNA preparations with a ^{32}P-labeled L-A positive ssRNA probe obtained as an in vitro transcript of purified L-A VLP-associated RNA polymerase from strain RE327 (α *his4 leu1 kar1-1 clo* L-A M_1 L-BC-o). Lanes: 1, X dsRNA purified from clone 64-4B (Fig. 1, lane 6) by electroelution from an agarose gel; 2, L dsRNA from the same clone, also electroeluted from the gel; 3, total nucleic acids from clone 64-4B (the same sample as in lane 6 in Fig. 1); 4, crude extract of clone 64-4D (lane 8 in Fig. 1) that does not contain X dsRNA; 5, 6, and 7, total nucleic acids extracted from strains 2186 (L-A-o L-B), 1686 (L-A-o L-C), and 2507 (L-A-o L-BC), respectively. These were included to confirm that the L-A probe was not contaminated with L-BC.

In the case of L-BC, the same type of experiments were carried out by cytoducing X into strain 2285 (a *kar1-1 leu1 clo*$^{-}$ L-o). X was maintained in all cytoductants even though L-BC was absent. That L-BC was absent in those cytoductants was proved by cytoducing their cytoplasm again into the *mak10-1* L-o strain 1747. When analyzed on agarose gels, those cytoductants were shown to be L-o, and since the *mak10* mutation only affects L-A maintenance, not L-BC, the donors must have lacked L-BC.

X is encapsidated in L-A-encoded VLPs. Stationary-phase cells of a 4-day culture were used to prepare VLPs from strain RE455. This strain contains L-A, minor amounts of L-BC, and X dsRNAs (Table 1). The last step in the purification is a cesium chloride density gradient equilibration sedimentation (4), and particle bands are located in the gradient according to their density, which depends on the RNA/protein ratio of the particles.

Figure 3 shows an ethidium bromide-stained agarose gel of the dsRNAs of the VLPs present in the fractions collected from a cesium chloride gradient (A) and an autoradiogram of the products of the RNA polymerase activity associated with them (B).

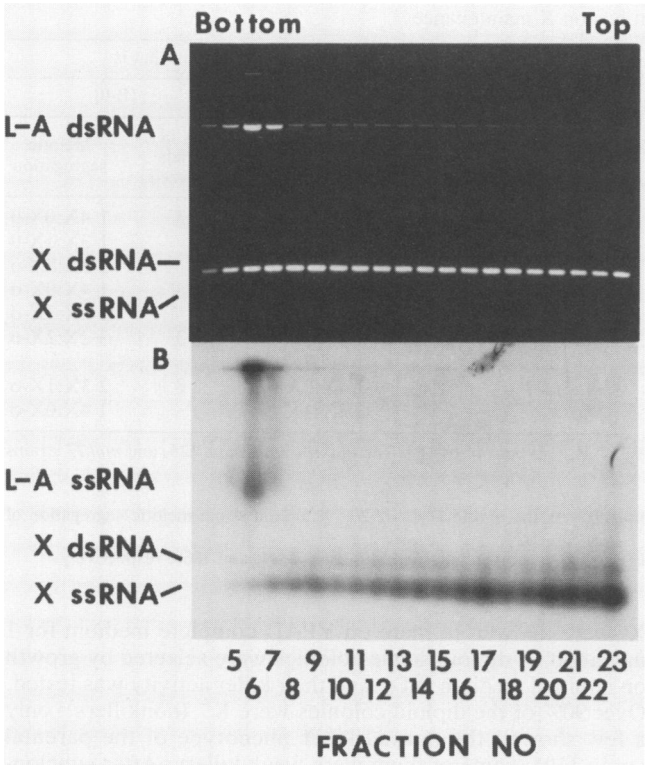

FIG. 3. Ethidium bromide-stained agarose gel (A) and autoradiogram (B) of the dsRNAs contained in purified VLPs from strain RE455 by cesium chloride density gradient centrifugation and the products of the RNA polymerase activity associated with the VLPs. The bottom shows the denser fractions, and the top shows the lighter ones. A sample of each fraction was incubated in an RNA polymerase reaction mixture that contained [^{32}P]UTP, and the other three ribonucleotides for 2 h at 30°C. After completion of the reaction, RNAs were phenol extracted and separated on a 1.5% agarose gel, and the gel was subjected to autoradiography. In panel A, a band of dsRNA is seen, peaking in fraction 14, whose molecular size is 2.3 kb, consistent with that of W dsRNA (18), which is present in this strain. Stains lacking L-A and L-BC do not show a W-size band at this place in the gradient even though the strain carries W dsRNA. We do not have direct evidence that this band is W dsRNA.

There was only one type of L-A-containing VLP; it banded around fraction 6, and its density was, as previously reported, 1.4076 (4). However, X was found throughout the cesium chloride density gradient. That X was encapsidated inside VLPs and was not naked dsRNA was proved by the RNA polymerase activity present in each fraction that synthesized X dsRNA in addition to X positive ssRNA (Fig. 3B). Furthermore, the density of naked dsRNA is higher than that of the particles, and in this type of cesium chloride gradient it would pellet at the bottom of the tube. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis, we found that X VLPs had the same protein composition as L-A VLPs, with a major coat protein subunit of about 81 kilodaltons.

"Head-full replication" of X dsRNA. M_1 dsRNA, which is less than half the size of L-A, is found in VLPs with either one or two M_1 dsRNA molecules per particle. These arise because M_1 positive ssRNA transcripts made in VLPs carrying only one M_1 dsRNA molecule are often retained within the VLP, where they are then copied to make a second M_1 dsRNA molecule. VLPs with two M_1 dsRNA molecules per particle are full, however, and all new positive strands are extruded from the particles (4). This head-full replication mechanism predicts that any small genome like X dsRNA replicating in these VLPs with an L-A coat should generate multiple VLP species, in this case, the largest integer less than (molecular weight of L-A dsRNA)/(molecular weight of X dsRNA). The results of Fig. 3 and 4 show that this prediction was fulfilled. The banding of X VLPs throughout the gradient was due to the presence of multiple discrete species with different densities. Rebanding of three different fractions from the initial cesium chloride gradient of Fig. 3 in three new cesium chloride gradients (Fig. 4) showed that they banded true and all three had RNA polymerase activity. Equal aliquots of gradient fractions were used for measurement of dsRNA (Fig. 4, left), RNA polymerase (Fig. 4, right), and VLP proteins (Fig. 4, right, insets). These data show that the differences in the banding densities of different VLP fractions are due to differences in the ratio of RNA to protein in the VLPs. Six peaks of X positive ssRNA-synthesizing activity were observed in the gradient shown in Fig. 3 (five peaks can be seen in the figure, and a sixth peak was present in fraction 26 [not shown]). The density of particles peaking in fraction 9 corresponds to seven X dsRNA molecules per particle. A small amount of X-containing particles was present at the same density as the L-A VLPs, although it did not form a discrete peak.

The ratio of RNA polymerase activity producing X ssRNA and dsRNA (Fig. 3B) to X dsRNA (Fig. 3A) was highest in the upper part of the gradient and decreased with increasing density. This is the expected result of particles with several X dsRNA template molecules per particle are limited in the RNA synthesized by having only one RNA polymerase molecule.

Also as predicted by the head-full replication mechanism, X dsRNA synthesis occurred in particles of many distinct densities in the cesium chloride gradient of Fig. 3. Thus,

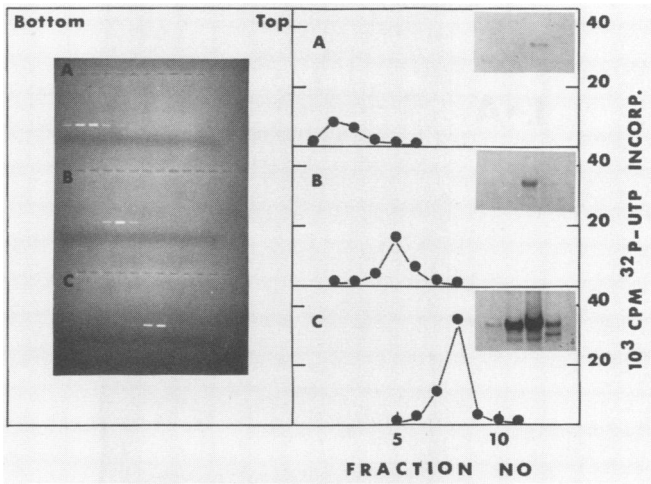

FIG. 4. X VLPs of fractions 9 (panels A), 17 (panels B), and 26 (panels C) from the cesium chloride gradient shown in Fig. 3 were subjected to three independent cesium chloride density gradient equilibrium sedimentations. Panels: left, one sample of each of the fractions collected was phenol extracted and separated on a 1.5% agarose gel; right, RNA polymerase activity associated with the X VLPs as measured by incorporation of [^{32}P]UTP into acid-insoluble material. The insets show protein content in main peak fractions analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The same volumes in samples A, B, and C were used for all measurements (dsRNA, protein, and RNA polymerase activity).

TABLE 2. Effect of *mak* mutations on X maintenance

mak mutant X	X-containing [B] strain that is:			
	SKI$^+$		*ski*	
	No. of tetrads analyzed	Meiotic segregation	No. of tetrads analyzed	Meiotic segregation
mak18-1[a]	2	2 2X:2X-o	2	1 3X:1X-o 1 4X:0X-o
mak21-1[a]	2	2 2X:2X-o	2	2 3X:1X-o
mak26-1[a]	2	2 2X:2X-o		ND[b]
mak27-1[a]	2	2 2X:2X-o		ND
mak16-1[c]	3	3 2X:2X-o	3	3 3X:1X-o

mak mutant X	X-containing *SKI*$^+$ strain that is:			
	[B]		[B-0]	
	No. of tetrads analyzed	Meiotic segregation	No. of tetrads analyzed	Meiotic segregation
mak1-2[d]	3	3 4X:0X-o	3	1 4X:0X-0 1 3X:1X-0 1 2X:2X-o
mak4-1	3	3 4X:0X-o	3	1 4X:0X-o 2 2X:2X-o
mak6-1	2	1 4X:0X-o 1 2X:2X-o	3	3 2X:2X-o
mak11-1	2	1 4X:0X-o 1 3X:1X-o	3	2 3X:1X-o 1 4X:0X-o

[a] *mak* mutations belonging to this group are bypassed by a *ski* mutation in the case of M_1 (16) but not by [B]. The *mak18*, *mak21*, *mak26*, and *mak27* strains used were strains 2593, 3950-3B, 3958-3B, and 2611, respectively.

[b] ND, Not done.

[c] *mak16-1* in the case of M_1 was bypassed by neither *ski* nor [B]. The result obtained with the *mak16-1*/+ *ski2-2*/+ X diploid strain meiotic segregation of 3X:1X-o is thus different from that of M_1. The *mak16* strain 2597 was used.

[d] *mak* mutations bypassed by either *ski* or [B]. The *mak1*, *mak4*, *mak6*, and *mak11* strains used were strains 2540, 2362, 2444, and 1868, respectively.

rather than preformed X dsRNA molecules being packaged at more than one molecule per particle, we believe that a single X positive ssRNA is packaged and that this molecule replicates until the package is full.

X and M_1 are incompatible. We studied the behavior of M_1 and X when present together in the same strain. Strains 1101 (α *his4 kar1-1* L-A L-BC M_1) and RE411 (a *leu1* L-A L-BC X) were allowed to mate on YPAD complete medium for 1 day at 30°C; diploid single colonies were selected by growth on minimal medium (SD), and their killer activity was tested. Over 90% of the diploid colonies were K^- (nonkillers); only a few showed the normal killer phenotype of the parental strain 1101; some of them were weak killers. After subcloning, nonkillers and normal killers were stable, but weak killers segregated mainly nonkillers and a few normal killers.

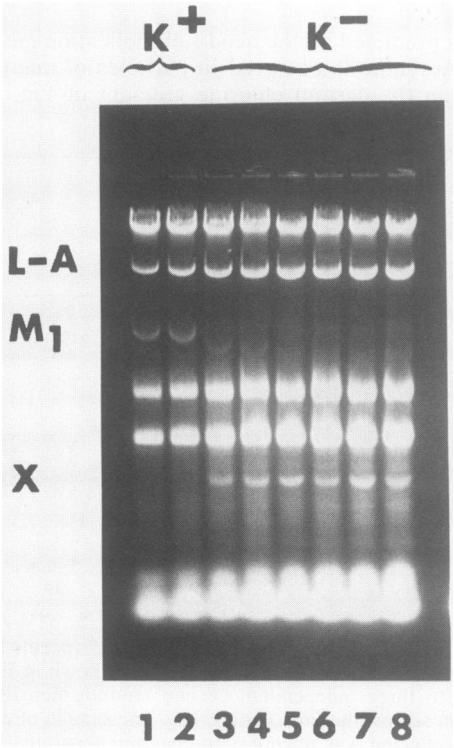

FIG. 5. X and M_1 incompatibility. Total nucleic acids were extracted from K^+ (samples 1 and 2) and K^- (samples 3 to 8) diploid colonies obtained by mating the X-containing strain RE411 and the killer strain 1101 and separated on a 1.5% agarose gel. X was present only in the K^- derivatives, and these contained no M_1. All K^+ colonies were X-o.

We purified total nucleic acids from a number of K^+ and K^- subclones picked randomly from the SD plates and analyzed them on 1.5% agarose gels (Fig. 5). Samples 1 and 2 were K^+, and samples 3 to 6 were K^-. As expected, M_1 dsRNA was only present in samples 1 and 2, but they did not contain X dsRNA. X dsRNA was present in the K^- samples (samples 3 to 6), and M_1 was lost. Thus, X and M_1 are incompatible.

We further studied this phenomenon of incompatibility by crossing other X- and M_1-containing strains, some of which were *SKI*$^+$ and some of which were *ski*$^-$. Although we found some differences in the ratio of nonkiller diploids to killer ones, no strain that could stably maintain X and M_1 together was found. In all crosses, most diploid cells lost M_1 and retained X, with only a minority losing X and retaining M_1. This effect was even more dramatic when X was introduced by cytoduction into an M_1-containing strain. Practically all cytoductants became K^- because of the acquisition of X.

X requires many *MAK* gene products needed by M_1 but not by L-A. M_1 requires at least 30 chromosomal gene products for its maintenance or replication, only 3 of which are needed by L-A (*MAK3*, *MAK10*, and *PET18*). As shown above, X could not be maintained when introduced into *mak3* and *mak10* mutant strains 2321 and TF78-4A by cytoduction. The discovery that X also excluded M_1 from the cytoplasm of a killer strain led us to study the role of the other *MAK* genes, needed only by M_1, on X maintenance.

The *MAK* genes tested are listed in Table 2. They belong to three different categories, according to their properties in M_1 maintenance. Group 1 includes *MAK18*, *MAK21*, *MAK26*, and *MAK27*, among others; mutations in such genes show clear $2M_1$:2M-o meiotic segregation when both parental strains are wild type for *SKI* genes; they are bypassed, however, by *ski*$^-$ recessive mutations so that a double mutant (e.g., *mak18-1 ski2-2*) can then maintain M_1.

Mutations in this group are not bypassed or suppressed by the cytoplasmic genetic element [B] (16). *mak1*, *mak4*, *mak6*, *mak11*, and several other *mak* mutations belong to group 2, which is bypassed by either a *ski*$^-$ mutation or the extrachromosomal element [B], known to be on L-A (H. Uemura and R. B. Wickner, Mol. Cell. Biol., in press). Only the *mak16-1* mutation is suppressed by neither *ski*$^-$ mutations nor [B] (group 3).

We crossed strains belonging to these three categories with appropriate X-containing strains, some of which were *SKI*$^+$ and some of which were *ski*$^-$. After tetrad dissection, total nucleic acids were extracted from spore clones and analyzed on agarose gels. The results obtained are summarized in Table 2. Briefly, *mak* mutant group 1 gave the meiotic segregation 2X:2X-o in all tetrads examined when both parents were *SKI* (two or three in each cross); when one of the parental strains was a *ski*$^-$ mutant, the most frequent type of tetrad formed was the tetratype 3X:1X-o, as expected for segregation of mutations in two unlinked loci, one of which bypasses the other. The second group of *mak* mutations was presumably bypassed by the extrachromosomal element [B], and most of the tetrads showed 4X:0X-o segregation. We suspected that all of our X-containing strains carried [B], because the deletion of L-A that produced X occurred in a meiotic segregant of the [B]-containing strain 2404 × 2507 (Table 1; Fig. 1). To confirm that the result found was due to the [B] present in the X-containing strain and not to the fact that X did not require the *MAK* gene products, we carried out the following transfection experiment to introduce X into a [B-o] strain.

X VLPs purified from strain RE455 (fraction 26 in the cesium chloride gradient shown in Fig. 3A) were used to transfect spheroplasts prepared from the L-A-containing, [B-o], X-o strain YHU1652 (Uemura and Wickner, in press) by the method of El-Sherbeini and Bostian (3). Among the URA3$^+$ transformants obtained, 34 were tested for acquisition of X VLPs by growth on YPAD plates for 2 days and analysis of their nucleic acids on agarose gels. We found that 12 (35% of URA3$^+$ transformants) had acquired X VLPs. We then introduced by cytoduction the X and L-A VLPs from one X-containing transfectant into standard tester strains that were L-A-o and X-o and used those cytoductants to study the effect of the *mak* mutations belonging to group 2 on X maintenance when [B] was not present. The results obtained with *mak1-2*, *mak4-1*, *mak6-1*, and *mak11-1* strains are summarized in Table 2. When [B] was present, most of the tetrads (8 of 10 analyzed) were 4X:0X-o, and when [B] was absent, only a few (3 of 13 analyzed) gave the same segregation pattern. We also saw in this case an increase in the number of tetrads that gave 2X:2X-o meiotic segregation. Thus, these results confirmed that the *MAK* genes belonging to group 2 are required for X maintenance, as well as for M_1, and that in both cases these requirements are bypassed by the cytoplasmic element [B] carried on some L-A molecules (Uemura and Wickner, in press).

Effect of *ski* mutations on X copy number. *SKI* genes are repressors of L-A, L-BC, and M_1 dsRNA replication so that recessive *ski*$^-$ mutants show an increase in copy number compared with *SKI*$^+$ strains. The copy number of individual dsRNA species also depends on the other dsRNAs present in the same cell. For example, M_1 lowers the copy number of L-A, and this effect is even stronger in *ski*$^-$ strains.

The presence of X in strain 64-4B (α *ski2-2*) lowered the copy number of L-A (Fig. 1); however, this strain contained small amounts of L-BC as well. To better study the effect of a *ski* mutation on X and L-A copy number, we used the *clo*$^-$ chromosomal defect that makes strains L-BC-o, and so the only L dsRNA present is L-A. The cross RE558 (α *his4 leu1 clo kar1-1* L-A L-BC-o X) × RE516 (a *arg1 ski2-2 clo*$^-$ L-o) was carried out at 20°C because L-BC dependence on *clo* is temperature dependent (18). Total nucleic acids were extracted from a number of tetrads and analyzed on agarose gels (Fig. 6). We found that X dsRNA is favored in *ski2-2* meiotic segregants, and its high copy number results in an extra decrease in L-A dsRNA as compared with *SKI*$^+$ clones. This is similar to the results with M_1 and L-A (1).

When meiotic segregants of the same cross were incubated at low temperature (8°C) for 15 days, we observed no difference in the pattern of growth between the *ski*$^-$ and *SKI*$^+$ clones of each tetrad, although M_1-containing *ski*$^-$ mutants showed cold sensitivity for growth at the same temperature (8).

Sequence of the ends of X dsRNA. X dsRNA extracted from purified X VLPs of strain RE455 (Fig. 3) was 3′ end labeled with cytidine[3′,5′-^{32}P]diphosphate as described in Materials and Methods. Positive and negative strands were separated in a polyacrylamide gel, recovered from the gel, and used to determine the terminal nucleotide sequence by partial digestions with base-specific RNases.

Figure 7 shows the sequences of both 5′ and 3′ ends of the positive X ssRNA. For comparison, the 5′- and 3′-end sequences of positive L-A ssRNA are also included (13). For convenience, the 5′-end sequence of the X positive strand is shown, although direct RNA sequencing data gave us the 3′-end sequence of the complementary negative strand.

Both the 5′ and 3′ ends of L-A are present in X. In the case of the 5′ end, only the first 25 bp of L-A are the same as the first 25 bp of X. The breakpoint occurred only 5 bp upstream of the first putative open reading frame initiation codon AUG at position 30 in L-A (underlined in Fig. 7). Beginning with

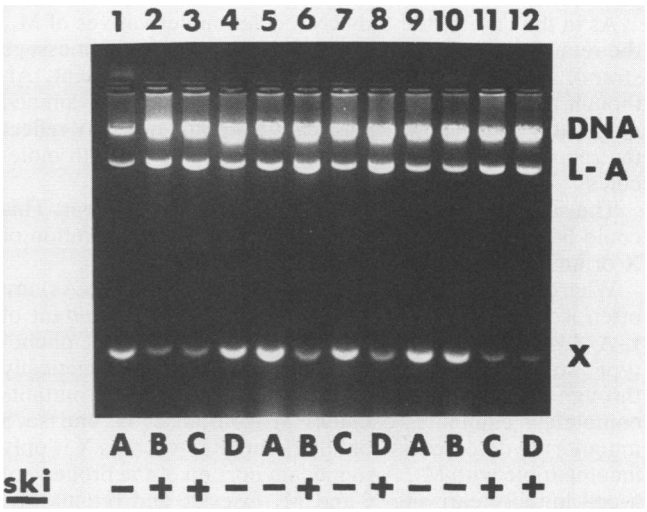

FIG. 6. Effect of the *ski2-2* mutation on X and L-A copy number. Clones from three different tetrads, dissected from the diploid strain RE558 × RE516 (a/α *ski2-2*/+ *clo*$^-$/*clo*$^-$ L-BC-o L-A X), were grown on YPAD medium plates at 20°C for 4 days, and their nucleic acids were extracted, treated with RNase A in the presence of 0.5 M NaCl as described in Materials and Methods, and analyzed on a 1.5% agarose gel. In other agarose gels (not shown), we analyzed the samples without RNase digestion and used the 26S rRNA as an internal standard to normalize the relative concentration of dsRNA in each sample. A, B, C, and D are the four spore clones of each tetrad. The symbols − and + represent *ski2-2* and *SKI* wild-type segregants, respectively.

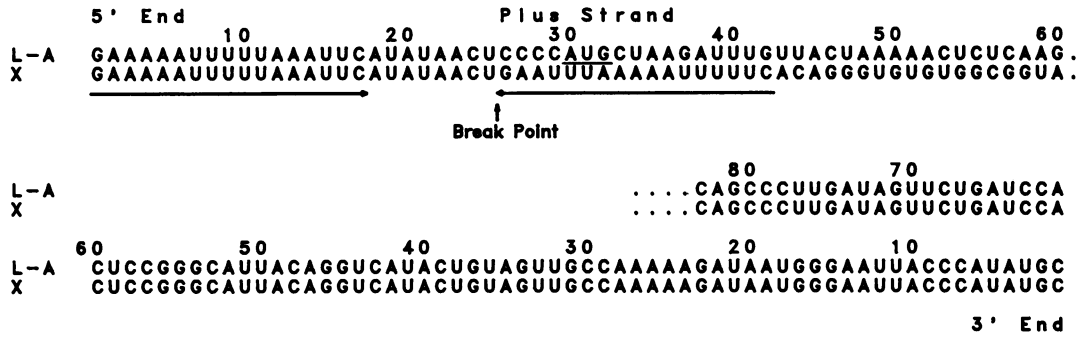

FIG. 7. Comparison of the 5'- and 3'-end sequences of L-A and X positive strands. The L-A sequence was taken from the data published by Thiele et al. (13). The sequence of the X 5'-end positive strand was deduced from the 3'-end sequence of the negative strand. The 3'-end sequence of X was directly determined. The break point indicates the beginning of different sequences for L-A and X 5' ends. The two 17-bp inverted repeats in the X 5' end (between bases 1 to 17 and 26 to 42) are underlined and marked with arrows. The underlined AUG in the 5' end of the L-A sequence is the first AUG and is thus presumed to be the initiation codon of the coat protein gene.

the breakpoint at bp 25, there is a stretch of 17 bp that is a perfect inverted repeat of the first 17 bp of the RNA molecule (both inverted repeats are underlined and marked with arrows). The second one ends at bp 42. We do not know whether the second inverted repeat found in X was already present somewhere in L-A or whether this type of structure was generated during the process of deletion. In the case of the 3' ends, both L-A and X are identical through the 110 bp sequenced (Fig. 7).

DISCUSSION

We describe X dsRNA, a 0.52-kb molecule that has lost most of the internal regions of L-A and retains the ends. Since it is encapsidated, transcribed, and replicated, X delimits the sequences of L-A needed in *cis* for these processes.

As in the case of the S dsRNA deletion derivatives of M_1, the retained 5' segment of X (5' with respect to the message strand) is much shorter than the retained 3' segment. Although the small number of independent isolates examined makes this observation statistically tenuous, this may reflect the requirement for some 3' subterminal site on both molecules.

The significance of the 5' inverted repeat is not clear. This could be an accident of the mechanism of the generation of X or an essential feature.

Whereas deletion mutants of M_1 dsRNA (S dsRNAs) are often isolated, this is the first described deletion mutant of L-A. L-A dsRNA does not give cells a recognizable phenotype, so mutation of L-A can only be detected genetically through its effect on M. Given sufficient time, S mutants completely eliminate (exclude) M from all cells, and so S mutants are detected as nonkiller mutants, whereas X is only incompatible with M_1. A significant portion of the progeny of a cell initially carrying X and M_1 loses X and retains M_1. Thus, the phenotype of an X mutant would not be as clear-cut as that of an S mutant. Moreover, S mutants can completely exclude their parent M_1 and still survive to be found, since they depend only on L-A, not on M_1. In contrast, if an X mutant completely excludes L-A, it will be lost itself. This may set some limits on the type of L-A deletion mutant that can be isolated and thus make X mutants rarer than S mutants.

The finding that X is in VLPs and that X VLPs can have from one to eight X dsRNA molecules per VLP shows that an L-A replicon, like an M_1 replicon, can have multiple genomes per particle if only it is small. X dsRNA synthesis in vitro was observed in particles with various densities. This confirms our head-full replication model originally postulated for M_1. This model is, of course, distinct from Streisinger's original head-full packaging model, in which T4 DNA replicates outside the particle and is stuffed into the head until the head is full (11). In the case of X and M_1 dsRNAs, one positive single strand is encapsidated in a VLP, and this replicates inside the particle until the particle is full. Once the particles are full, all of the newly synthesized positive strands are extruded from the particles. The capacity limit observed for X particles (eight per particle) is just the value expected (4.5/0.52 = 8.6) if one L-A dsRNA (4.5 kb) per particle is all the RNA that these particles can hold.

Although X is an L-A replicon, X surprisingly replicates like M_1. X requires all of the nine *MAK* genes tested that are dispensable for L-A but required for M_1. In addition, the requirements of X for several *MAK* genes is apparently suppressed by *ski*$^-$ mutations or by [B], as is the case for M_1 (16). X and M_1 are incompatible, unlike X and L-A or M_1 and L-A. These facts indicate that X and M_1 share many aspects of their replication and packaging mechanisms.

Why does a deletion mutant of L-A need many extra *MAK* genes like M_1? X and M_1 have in common their small size and their need to borrow coat protein from L-A. Their small size may impose special requirements at the packaging stage, while their need to borrow coat protein from L-A might require some help. Clearly, an in vitro packaging system would be useful in examining these problems further. The finding that [B], a suppressor of mutations in one class of *MAK* genes, is a variant of L-A (and thus presumably a variant of the coat protein) (Uemura and Wickner, in press) also points to the packaging process or the VLPs themselves as the site of action of this class of *MAK* gene products.

X arose in a strain carrying only L-A-HNB (Uemura and Wickner, in press); that is, X is derived from an L-A carrying [B]. Nevertheless, in the absence of an intact L-A-HNB, X apparently requires the *MAK* genes that are bypassed by [B]. This indicates that the [B] function has been deleted from X. X carries all of the sites for transcription, replication, and packaging. Thus, [B] is probably a property of a protein product of L-A.

X, like M_1 or M_2, lowers L-A copy number, especially in *ski*$^-$ strains. Thus, whereas both X and *SKI* products lower L-A, removing a *SKI* product by a *ski*$^-$ mutation actually lowers L-A copy number if X is present because X copy

number is raised. The same effect is seen with M_1 (1). Whereas *ski*$^-$ M_1 strains are cold sensitive for growth (8) or even grow slowly at normal growth temperatures if another cytoplasmic element called [D] is also present (Esteban and Wickner, Genetics, in press), *ski*$^-$ X [D] strains do not show this effect. The explanation of these phenomena will await further insight into the mechanism of action of *SKI* gene products and the details of X and M replication.

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