JOURNAL OF VIROLOGY, Jan. 1991, p. 155–161
0022-538X/91/010155-07$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 65, No. 1

Expression of Yeast L-A Double-Stranded RNA Virus Proteins Produces Derepressed Replication: a *ski*$^-$ Phenocopy

REED B. WICKNER,* TATEO ICHO,† TSUTOMU FUJIMURA, AND WILLIAM R. WIDNER

Section on the Genetics of Simple Eukaryotes, Laboratory of Biochemical Pharmacology, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892

Received 15 August 1990/Accepted 14 October 1990

The plus strand of the L-A double-stranded RNA virus of *Saccharomyces cerevisiae* has two large open reading frames, ORF1, which encodes the major coat protein, and ORF2, which encodes a single-stranded RNA-binding protein having a sequence diagnostic of viral RNA-dependent RNA polymerases. ORF2 is expressed only as a Gag-Pol-type fusion protein with ORF1. We have constructed a plasmid which expresses these proteins from the yeast PGK1 promoter. We show that this plasmid can support the replication of the killer toxin-encoding M_1 satellite virus in the absence of an L-A double-stranded RNA helper virus itself. This requires ORF2 expression, providing a potential in vivo assay for the RNA polymerase and single-stranded RNA-binding activities of the fusion protein determined by ORF2. ORF1 expression, like a host *ski*$^-$ mutation, can suppress the usual requirement of M_1 for the *MAK11*, *MAK18*, and *MAK27* genes and allow a defective L-A (L-A-E) to support M_1 replication. These results suggest that expression of ORF1 from the vector makes the cell a *ski*$^-$ phenocopy. Indeed, expression of ORF1 in a wild-type killer makes it a superkiller, suggesting that a target of the *SKI* antiviral system may be the major coat protein.

Saccharomyces cerevisiae carries three double-stranded RNA (dsRNA) viruses (35, 36), several retroviruses (2), and a single-stranded circular RNA virus (21a). The plus strand of the 4.5-kb L-A dsRNA virus has two open reading frames (19). The 5′ open reading frame ORF1 encodes the 80-kDa major coat protein (5, 18, 19), and a combination of ORF1 and the 3′ ORF2 encodes the 180-kDa Gag-Pol fusion protein (Fig. 1) (13, 19). The C-terminal domain of the fusion protein has single-stranded RNA (ssRNA)-binding activity (13) and an amino acid sequence pattern diagnostic of viral RNA-dependent RNA polymerases (19). Within the 130-bp overlap of ORF1 and ORF2 is a "slippery site" at which ribosomal frameshifting occurs (5a, 19) by a mechanism similar or identical to that described for retroviruses in forming their Gag-Pol fusion proteins (17, 20, 21).

In addition to providing these proteins for its own replication, L-A can support one of several satellite dsRNA viruses, called M_1, M_2, etc., each of which encodes a secreted protein toxin and immunity to that toxin (reviewed in references 3 and 36). L-A can also support the replication of smaller deletion mutants derived either from L-A itself or from M_1 (8, 9). While L-A particles contain only one L-A dsRNA molecule per particle, those containing a satellite genome (M or a deletion mutant of M or L-A) contain one to eight dsRNA molecules per particle. In each case, the plus strand is the species packaged to form new particles and replication occurs until the particle is full ("head-full replication"). Then new plus strands are all extruded from the particles (6, 8, 10–12).

The yeast *SKI* genes are an antiviral system that are essential for host growth at high and low temperatures only in the presence of M dsRNA (7, 23, 24, 26, 27). The SKI proteins repress replication of M_1, M_2, L-A, and L-BC dsRNAs (1, 24, 27) as well as the single-stranded circular 20S RNA virus of yeast (21a).

The interactions of L-A and its natural variants with the M satellite genomes have been studied genetically. A variant of L-A (L-A-E) unable to support M_1 or M_2 in a wild-type host has been previously described (24, 25, 33). L-A-E can, however, support M in a *ski*$^-$ host. This defect can be complemented from a normal L-A, and this activity is called [HOK] (helper of killer) or just H, as in L-A-H (24, 39). [NEX] (or N) is another L-A trait (15, 33, 34).

Replication or maintenance of M dsRNA requires the products of at least 30 host genes, called *MAK* (maintenance of killer) genes (31, 32, 37, 38). Of these genes, only three, *MAK3*, *MAK10*, and *PET18*, are needed for L-A replication (25, 39). Certain variants of L-A can bypass M_1's usual requirement for many of the *MAK* gene products. This trait is called [B] (bypass), and L-As carrying it are designated L-A-B, or L-A-HNB if they have [HOK] and [NEX] activity as well (1, 27, 30). It was from such an L-A-HNB that we isolated our cDNA clone (19). M_1's requirement for most *MAK* genes is also suppressed in a *ski*$^-$ host (29).

In order to dissect the molecular mechanisms of expression of L-A-encoded information, L-A replication, and the L-A genetics, we have constructed an L-A expression vector and studied its biological activities. This work has led us to the hypothesis that one target of the antiviral *SKI* genes is the L-A major coat protein.

MATERIALS AND METHODS

Media and strains. The media used for growth of yeast and assay of the killer phenomenon were as described elsewhere (31). The yeast strains used are listed in Table 1. *Escherichia coli* HB101 was the host for most plasmid manipulations. *E. coli* CJ236 (F$^+$ *dut ung*) and MV1190 (F$^+$ *dut*$^+$ *ung*$^+$), obtained from Bio-Rad, were used for oligonucleotide-directed, site-specific mutagenesis by following the Bio-Rad Mutagene kit instructions.

Construction of mutable L-A expression vector. pTIL05

* Corresponding author.
† Present address: Tokyo Medical and Dental University, Tokyo, Japan.

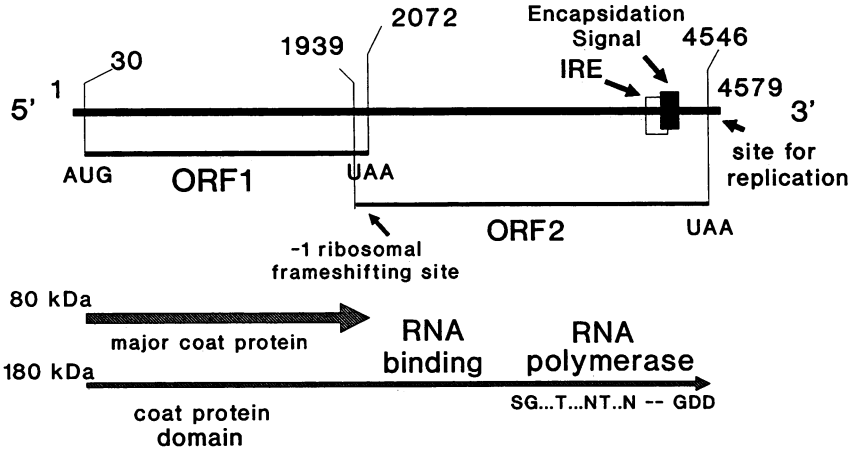

FIG. 1. Expression of coding information from L-A plus strands (modified from reference 19). ORF1 encodes the major coat protein. ORF1 and ORF2 are fused by ribosomal frameshifting to encode the 180-kDa minor viral coat protein. IRE, Internal replication enhancer.

(19) is the Bluescript vector SK$^+$ with the entire L-A sequence (except for 8 bp at the 3′ end that are downstream of the coding region) inserted in the *Sma*I site. p375 is a derivative of YEpIPT (16) into which a multiple cloning site has been inserted in the unique *Eco*RI site (15a). In order to carry out localized mutagenesis, we inserted the f1 *ori* on a 0.52-kb *Sal*I fragment from pDM1 (22) into the unique *Sal*I site in the *tet* gene of p375, forming pI2. The L-A sequence on an *Xho*I-*Sst*I fragment from pTIL05 was inserted between the *Xho*I and *Sst*I sites of p375 and of pI2, forming pTIL131 (pORF1+2) and pI2L2, respectively. From pTIL131 a *Not*I fragment that included nearly all of ORF2 (from base 2580 of

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description	Reference
Strains		
2928	a *ura3 his3 trp1 GAL*$^+$ L-A-HN M_1	This work
2604	a *trp1 leu2 his3 pho3 pho5* L-A-HN M_1	This work
2966 (2604 H^-K^-)	a *trp1 leu2 his3 pho3 pho5* L-A-o M-o	This work
4219-2A	a *trp1 leu2 ski2-2* L-A-E M_1	This work
4219-2C	a *trp1 his3 ski2-2* L-A-E M_1	This work
2955rho^0	a *trp1 his3 ade1 mak10-1* L-A-o M-o[*rho*^{0}]	This work
2128	a *ura3-52 trp1 cdc16-1 mak11-1* L-A-HN M-o	30
1074	a *leu kar1-1* L-A-HN M_1	30
1101	α *his4-15 kar1-1* L-A-HN M_1	30
1368	α *his4-15 kar1-1* L-A-HNB M_1	30
2629	α *leu1 kar1-1* L-A-HNB M_1	30
3012	α *his met14 trp1 ura3 mak18-1* L-A-HN M-o	32
2057	a *ura3 trp1 leu2 rna1 mak27-1* L-A-HN M-o	38
1717	α *aro7 leu2 trp1 ura1 mak3-1* L-o M-o	37
1720	α *aro7 leu2 trp1 lys2 mak3-1* L-o M-o	37
Plasmids		
pTIL05	Entire L-A-HNB cDNA (except 8 bases at 3′ end) in the *Sma*I site of Bluescript vector SK$^+$	19
p375	Yeast expression plasmid with *PGK1* promoter, *TRP1*, 2μm DNA origin, pBR322, and a multiple cloning site	16
pTIL131 (pORF1+2)	*Xho*I-*Sst*I fragment from pTIL05 carrying entire L-A insert ligated into *Xho*I-*Sst*I-cut p375; L-A's ORF1 and ORF2 are intact	This work
pDM1	pAT153 with a 0.52-kb fragment carrying the f1 *ori* in the *Sal*I site	22
pI2	p375 with f1 *ori* on a *Sal*I fragment from pDM1	This work
pI2L2	*Xho*I-*Sst*I fragment from pTIL05 carrying entire L-A insert ligated into *Xho*I-*Sst*I-cut I2; L-A's ORF1 and ORF2 are intact	This work
pTIL141 (pORF1)	pTIL131 from which the 2.0-kb *Not*I fragment (including most of ORF2) has been removed	This work
M10 (pORF1oc+2)	Ochre mutation at amino acid 20 of ORF1; from pI2L2	This work
M11 (pORF1+2oc)	Ochre mutation in ORF2 at third codon after the end of ORF1; derived from pI2L2	This work
M2 (pORF1fus2)	ORF1 and ORF2 placed in frame by addition of an A residue at the site of the ribosomal frameshift (5a); derived from pI2L2	This work

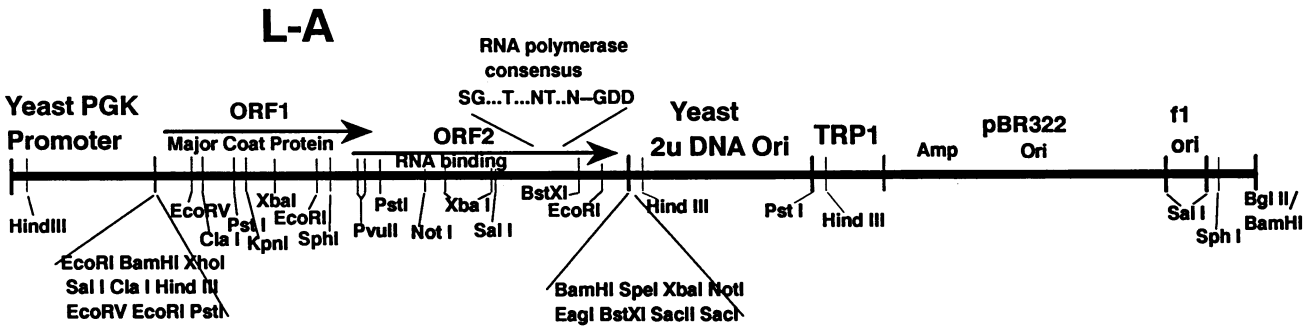

FIG. 2. Plasmid for expressing L-A-encoded proteins in *S. cerevisiae*.

the L-A plus strand to the 3′ end) was removed. The remaining plasmid was ligated to form pTIL141 (pORF1). M2, M10, and M11 were constructed by oligonucleotide-directed localized mutagenesis of pI2L2 (Table 1).

Testing for suppression of *mak* mutations. Plasmids were introduced into *trp1 mak* hosts, and then L-A and M_1 were introduced by cytoduction using the *kar1* mutant defective in nuclear fusion (4). When a *kar1* strain mates, binucleate cells carrying the cytoplasmic elements (viruses, mitochondria, etc.) of both parents are formed. The nuclei fail to fuse and separate at the next cell division. When a *kar1* strain carrying L-A, M_1, and mitochondrial DNA ([*rho*$^+$]) is used to mate with a *mak* M-o [*rho*^{0}] strain, the net effect is to transfer cytoplasmic elements from the *kar1* parent (called the donor) to the *mak* parent (called the recipient). Cytoductions were performed as described previously (39), except that cytoduction mixtures were plated on media that simultaneously selected for retention of the plasmid and against the cytoduction donor.

RESULTS

L-A expression vector. The expression vector, p375, has the PGK1 promoter, a multiple cloning site, the 2μm DNA replication origin, the yeast *TRP1* gene, and pBR322 sequences. In order to be able to do site-directed mutagenesis and express the mutated L-A sequence in yeast cells, we inserted the f1 origin of replication into the yeast expression vector p375 (Fig. 2). The L-A sequence was inserted between the PGK1 promoter and the 2μm DNA terminator to make pORF1+2 (pTIL131).

[HOK] activity requires only ORF1. To test whether the L-A information was being expressed, pORF1+2 was put into a *SKI*$^+$ L-o strain, and the transformants were crossed with a *ski2-2* L-A-E M_1 strain (Table 2). The diploids formed were *SKI*$^+$ and would have lost M_1 if L-A information had not been expressed from the clone, since L-A-E is defective in its ability to maintain M_1. However, the diploids were all stable killers as long as selection for the expression plasmid pORF1+2 was maintained. Allowing the plasmid to be lost by growth on nonselective medium showed that loss of the L-A expression plasmid resulted in the loss of M_1. Thus, pORF1+2 has [HOK] activity, the ability to provide M_1 with the helper function that is deficient in the natural variant L-A-E. The same result was obtained when pORF1 was used instead of pORF1+2, indicating that the ssRNA-binding RNA polymerase domain (ORF2) is not necessary for [HOK]. When we introduced an ochre mutation in place of amino acid 20 of ORF1, the resulting plasmid (pORF1oc+2) had no [HOK] activity, indicating that the protein encoded by ORF1 and not just the RNA was essential for [HOK]. As expected, introducing an ochre mutation in ORF2 in place of the third amino acid after the end of ORF1 (pORF1+2oc) did not interfere with [HOK] activity.

[B] activity requires only pORF1. Certain isolates of L-A, called L-A-HNB and including that from which our cDNA clone was isolated, make several *MAK* genes, such as *MAK11*, dispensable for M_1 replication or maintenance (30). When pORF1+2, pORF1, and p375 (vector alone) were transformed into a *mak11-1* strain and L-A-HN and M_1 were then introduced by cytoduction, the same pattern as for [HOK] activity was seen, namely, only ORF1 was needed for [B] activity and ORF1 RNA was not sufficient; an ochre mutation early in ORF1 prevented expression of [B] from the vector (Table 3).

pORF1 suppresses *mak18* and *mak27* mutations. While many *mak* mutations are suppressed by L-A-HNB (1), mutations in *mak18* and *mak27* are among those which are not suppressed. We found, however, that expression of L-A proteins from pORF1+2 or from pORF1 suppress both *mak18* and *mak27* mutations (Table 4). These results could suggest that the *MAK18* and *MAK27* products are concerned with the expression of L-A proteins, particularly the major coat protein encoded by ORF1. Alternatively, since *mak18* and *mak27* mutations are suppressed by *ski* mutations, these results could mean that expression of pORF1 makes the cells a *ski*$^-$ phenocopy.

pORF1 makes cells superkillers. Introduction of pORF1 into the wild-type killer strains 2604 and 2928 made them

TABLE 2. [HOK] requires only ORF1 protein[a]

Plasmid	Insert	Characteristics of diploids formed by crossing with:	
		4219-2A (*ski2-2* L-A-E M_1)	4219-2C (*ski2-2* L-A-E M_1)
None	None	All K$^-$	All K$^-$
p375	None	All K$^-$	All K$^-$
pTIL131	ORF1 + ORF2	All K$^+$	All K$^+$
pTIL141	ORF1	All K$^+$	All K$^+$
M10	ORF1oc + ORF2	All K$^-$	All K$^-$
M11	ORF1 + ORF2oc	All K$^+$	All K$^+$

[a] Plasmids were transformed into strain 2966 (L-A-o M-o), and transformants were mated with the *ski2-2* L-A-E M_1 strains. Finding that all diploids were killers (K$^+$) indicated that the plasmid was expressing [HOK] activity. At least 10 diploid single colonies were isolated on plates lacking Trp (−Trp) to ensure retention of the plasmid and tested for K_1. Subcloning on YPAD resulted in frequent loss of the expression plasmid and concomitant loss of M_1. Subcloning on −Trp plates of diploids carrying pTIL131 or pTIL141 yielded only K$^+$ single colonies. K$^-$, Nonkiller.

TABLE 3. [B] requires ORF1 only

Donor characteristics[a]	Recipient characteristics[b]	Characteristics of cytoductants	No. of cytoductants
L-A-HN M_1	*mak11-1* L-A-HN	All K^-	>500
L-A-HNB M_1	*mak11-1* L-A-HN	All K^+	15
L-A-HN M_1	*mak11-1* L-A-HN p375 (vector)	All K^-	>30
L-A-HN M_1	*mak11-1* L-A-HN pORF1+ORF2	All K^{+c}	20
L-A-HNB M_1	*mak11-1* L-A-HN pORF1+ORF2	All K^+	>15
L-A-HN M_1	*mak11-1* L-A-HN pORF1	All K^{+c}	8
L-A-HNB M_1	*mak11-1* L-A-HN pORF1	All K^+	>15
L-A-HN M_1	*mak11-1* L-A-HN pORF1+2oc	All K^+	>20
L-A-HN M_1	*mak11-1* L-A-HN pORF1oc+2	All K^-	>20

[a] Cytoduction donors were strains 1101 (L-A-HN M_1) and 1368 (L-A-HNB M_1).
[b] Cytoduction recipients were strain 2128 (*mak11-1* L-A-HN M-o) carrying one of the L-A expression plasmids. Cytoductions were done on SD agar containing His and uracil to keep in the L-A expression plasmid and plated on SD agar containing uracil to select against donor cells and retain the plasmid.
[c] Subcloning these cytoductants on −Trp plates to retain the plasmid produced only K^+ subclones. Subcloning on YPAD to allow loss of the L-A expression plasmid produced only K^- and a few weak killer (K^w) subclones.

superkillers, as judged by their zone of killing of a sensitive strain at 30°C. In contrast, the vector p375 or the vector pORF1oc+2, which has an ochre mutation early in ORF1, had no such effect.

***mak10-1*, but not *mak3-1*, is suppressed by pORF1+2.** The *MAK3*, *MAK10*, and *PET18* genes are needed for L-A replication (25), although Toh-e and Sahashi (28) have shown that *PET18* is needed only at temperatures above 30°C. The loss of M_1 by *mak10-1* strains was suppressed by pORF1+2, as shown by both meiotic crosses (Table 5) and cytoduction experiments (Table 6). However, pORF1 did not detectably suppress *mak10-1*. To determine whether pORF1+2 allowed replication in the *mak10-1* host of the L-A virus itself, the *mak10-1* pORF1+2 M_1 strains (Table 6) were mated with either of two *trp1 mak3* L-A-o strains (1717 and 1720) and the diploids were examined to determine whether they were killers before and after mitotic loss of pORF1+2. Since the *mak3* and *mak10* mutations complement each other, and since *mak3* mutants lack L-A, the diploids, after loss of pORF1+2, are expected to be killers only if pORF1+2 allows replication of L-A in the *mak10* host. In each case, all diploids were stable killers while pORF1+2 was present and nonkillers when pORF1+2 was lost. The same result was obtained by examination of isolated viral particles for L-A dsRNA by Northern (RNA) blot hybridization (Fig. 3). This shows that pORF1+2 did not allow the replication of L-A-HN in the the *mak10* background and suggests that *MAK10* is needed for the replication or maintenance of L-A independent of the supply of L-A-encoded proteins. Even though the proteins are being supplied in amounts sufficient for M_1, L-A cannot replicate. This result also indicates that M_1 does not need the L-A genome itself for its replication but needs only the products of L-A. It also suggests that the *MAK10* product is not necessary for the translation of L-A products. These results also show that pORF1+2 can maintain M_1 in a wild-type host. In contrast, crosses carried out with *mak3-1* and pORF1+2 showed no evidence of suppression (Table 5).

TABLE 4. Suppression of *mak27-1* and *mak18-1* by pORF1 or pORF1+2

Donor characteristics (*kar1-1*)[a]	Recipient characteristics[b]	Cytoductant characteristics
L-A-HN M_1	*mak27-1*	All K^-
L-A-HNB M_1	*mak27-1*	All K^-
L-A-HN M_1	*mak27-1* pORF1+2	All K^{+c}
L-A-HNB M_1	*mak27-1* pORF1+2	All K^+
L-A-HN M_1	*mak27-1* pORF1	All K^{+c}
L-A-HNB M_1	*mak27-1* pORF1	All K^+
L-A-HN M_1	*mak18-1*	All K^-
L-A-HN M_1	*mak18-1*	All K^-
L-A-HN M_1	*mak18-1* p375	All K^-
L-A-HN M_1	*mak18-1* pORF1	All K^{+c}
L-A-HN M_1	*mak18-1* pORF1+2	All K^{+c}

[a] Cytoduction donors were strains 1101 and 1074 (L-A-HN M_1) and strains 1368 and 2629 (L-A-HNB M_1).
[b] The *mak27-1* recipient was strain 2057, and the *mak18-1* recipient was strain 3012.
[c] Four cytoductants from each of these cytoductions were subcloned on −Trp plates, and all were stably K^+. Subcloning the same cytoductants on YPAD plates showed that all Trp^- mitotic segregants had become K^-, showing that the suppression of *mak27-1* or of *mak18-1* depended on the L-A expression plasmid.

DISCUSSION

We report the establishment of an expression system for the information encoded by the L-A dsRNA virus of yeast. That both open reading frames are in fact expressed from this clone is shown by its maintenance of M_1 in a *mak10* strain (or in a wild-type strain) in the absence of the L-A dsRNA genome, an activity dependent on both ORF1 and ORF2. This activity proves that our clone of L-A encodes biologically active proteins. That our sequences of three complete independent cDNA clones of L-A were, except for a single nucleotide, in complete agreement with each other also supports this conclusion (19).

Using this expression system, we have begun to examine the molecular basis of several phenomena we have previously described. L-A-E can support M_1 only in a *ski*$^-$ host defective in the antiviral system. [HOK] is the ability of many L-A isolates to supplement L-A-E in supporting M_1 in a wild-type host. That [HOK] activity is expressed from pORF1+2 and requires only ORF1, which encodes the major coat protein, indicates that the defect in L-A-E is in the major coat protein and is either qualitative or quantitative. L-A-E must, however, be supplying the fusion protein. Since [HOK] is needed only in a *SKI*$^+$ host, this suggests that one function of the major coat protein is to protect M_1 (and presumably L-A as well) from the *SKI* antiviral system.

[B] is the ability of certain L-A natural variants (called L-A-HNB) to support M_1 in certain *mak*$^-$ hosts from which it would otherwise be lost. [B] suppresses many *mak* muta-

TABLE 5. pORF1+2 suppresses *mak10-1* but not *mak3-1*

Parents	Segregation[a]	No. of tetrads
1717 (*mak3-1* L-oM-o) × 4224-8B (pORF1+2 L-A-E M_1)	$2K^-:2K^+$	10
2956 (*mak10-1* L-oM-o) × 4224-8B (pORF1+2 L-A-E M_1)	$4K^+:0$	12
2957 (*mak10-1* L-oM-o) × 4224-5A (pORF1+2 L-A-E M_1)	$4K^+:0$	12
2604 (L-A-HN M_1) × 2955(pORF1+2) (*mak10-1* L-o pORF1+2)	$4K^+:0$	7
2604 (L-A-HN M_1) × 2955(pTIL141) (*mak10-1* L-o pORF1)	$2K^+:2K^-$	8

[a] All crosses were carried out, diploids were sporulated, tetrads were dissected, and spores were germinated in the absence of tryptophan in order to insure the continued presence of pTIL131. The dependence of M_1 in the segregants on pTIL131 was demonstrated by streaking each spore clone of three tetrads of the cross 2957 × 4224-5A on either −Trp plates or on YPAD plates. All the single colonies on −Trp plates were killers, while all those on the YPAD plates were either nonkillers$^-$ or weak killers.

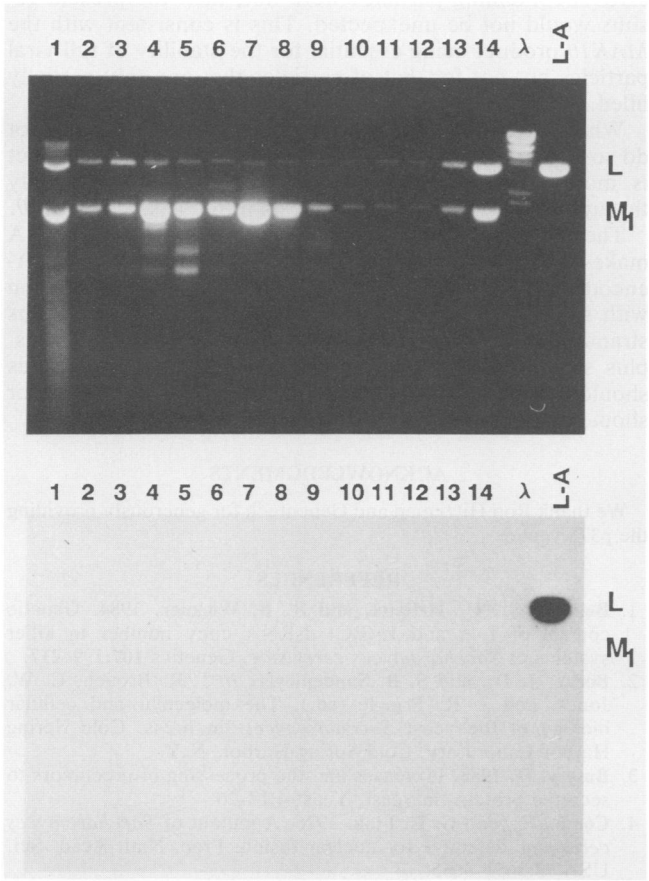

FIG. 3. Maintenance of M_1 but not L-A in a *mak10-1* strain by pORF1+2. Viral particles were prepared as previously described (6, 8, 13, 14) from stationary-phase cells of strain 2955(pORF1+2) into which L-A-HNB and M_1 from strain 2629 had been cytoduced. The final step in the purification was a CsCl gradient. RNAs were extracted from each fraction of the gradient and analyzed on a nondenaturing agarose gel. Ethidium bromide staining of the gel is shown in the upper panel. The the RNAs were denatured in the gel, blotted onto a nylon sheet, and hybridized with a ^{32}P-labeled L-A-specific probe. An autoradiogram of the sheet is shown in the lower panel. Lanes 1 to 14 are the CsCl gradient fractions, with lane 1 being the bottom fraction and lane 14 being the top fraction. Lanes λ and L-A show λ *Hin*dIII markers and purified L-A dsRNA, respectively. The positions of L-A and M_1 dsRNAs are shown as L and M_1, respectively, on the right. Note that there are two types of M_1 viral particles (6); one, which contains two M_1 dsRNA molecules per particle (M_1-H), forms a band at fraction 4, and the other, which contains one M_1 dsRNA molecule per particle (M_1-L), forms a band at fraction 7. The expected density of mature L-A particles is that of fraction 3. The dsRNA with the same mobility as that of L-A seen throughout the gradient is L-BC.

tions but not *mak18-1* or *mak27-1*. The expression of [B] from our cDNA clone requires only ORF1. Furthermore, the same pORF1 suppresses at least two *mak* mutations not normally suppressed by [B] (Table 7). That pORF1 suppresses mutations in many *MAK* genes is consistent with the notion that these genes are concerned with the efficiency of production of the major coat protein from L-A transcripts. Alternatively, since these *mak* mutations are also those which are suppressed by *ski* mutations, they could be involved in M_1's defense against the *SKI* antiviral system or be one of the actual targets of the *SKI* system.

A unifying hypothesis to explain the interactions of [HOK], [B], pORF1, *MAK* genes, and *SKI* genes. The *SKI* products reduce the copy numbers of M_1, M_2, L-A, and L-BC dsRNAs, and this function is essential or at least important for cell growth, depending on the temperature and other cytoplasmic elements in the strain (7, 24, 27). However, the precise target of *SKI* gene action is not yet known. L-A-E's defect in maintaining M_1 is suppressed by *ski* mutations or by supplying major coat protein from a normal L-A ([HOK] function) or from pORF1. This is consistent with the major coat protein being the target of the *SKI* antiviral system or with the coat protein protecting the actual target (genomic RNA?) from the *SKI* system. L-As carrying [B] make many *MAK* genes dispensable for M_1, and the same L-A maintains M_1 at a higher-than-normal copy number (30). Here we show that pORF1 is sufficient for [B] and for the suppression of two *mak* mutations not suppressed by [B]. However, all the *mak* mutations suppressed by pORF1 are suppressed by *ski* mutations. If the target of the *SKI* system were the major coat protein, then its overproduction by pORF1 might swamp the *SKI* system, making the cell a *ski* phenocopy.

TABLE 6. L-A expression vectors allow M_1 replication in a *mak10-1* strain

Characteristics of donor strain 2629 (*kar1-1*)	Characteristics of recipient strain 2955rho^0	Killer phenotype of cytoductants[a]
L-A-HNB M_1	*mak10-1*	All K^-
L-A-HNB M_1	*mak10-1* p375	All K^-
L-A-HNB M_1	*mak10-1* pORF1+2	All K^+
L-A-HNB M_1	*mak10-1* pORF1	All K-
L-A-HNB M_1	*mak10-1* pORF1fus2	All K-
L-A-HNB M_1	*mak10-1* pORF1oc+2	All K-
L-A-HNB M_1	*mak10-1* pORF1+2oc	All K-

[a] Cytoduction mixtures were plated on media selecting against the cytoduction donor and for retention of the L-A expression plasmid. Clones were tested for growth on minimal medium (to screen out diploids), for growth on glycerol plates (to identify clones that had received donor cytoplasm), and for killing of a sensitive strain. At least 10 cytoductants from each cytoduction were scored.

TABLE 7. Comparison of effects of pORF1, pORF1+2, and a *ski* mutation

Trait	Definition	Suppression by:		
		ski⁻	pORF1	pORF1+2
[B]	Ability of an L-A to make *MAK11* dispensable for M_1 replication	+	+	+
[HOK]	Ability of an L-A to complement defective L-A-E in supporting M_1 in a *SKI*⁺ host	+	+	+
mak18, *mak27*	Lose M_1 but not L-A or L-BC	+	+	+
mak10	Lose M_1 and L-A but not L-BC	−	−	+
mak3	Lose M_1 and L-A but not L-BC	−	−	−

This would have the effect of suppressing many *mak* mutations and allowing L-A-E to support M_1 replication.

In fact, introduction of pORF1 into a wild-type killer strain makes it a superkiller, as predicted by this model. This finding is most easily explained if the major coat protein is the target or the first molecule recognized by the *SKI* products. Supplying an excess of this target would titrate out the *SKI* products, allowing unrestrained replication of M_1, and all of the genetic consequences of the cell's being *ski*⁻ would follow. L-A-B could be interpreted, in accord with this model, as a variant of L-A that produces more major coat protein and as a result has a partial Ski⁻ phenotype. L-A-B both suppresses some (but not all) of the *mak* mutations suppressed by *ski* mutations and produces a partial superkiller phenotype (with larger killing zones and higher M_1 copy number than wild-type strains). An alternative model would have the excess of major coat protein providing better protection of M_1 from the *SKI* products.

pORF1+2 support of M_1 in a *mak10-1* host provides an in vivo assay for ORF2 functions. pORF1+2 supported M_1 replication in a *mak10-1* host. Both genetic and biochemical tests showed that L-A was lost from the strain. Subsequently making the strain *MAK*⁺ by mating it with a *mak3* strain produced wild-type cells in which pORF1+2 was maintaining M_1 in the absence of L-A. This indicates that M_1 depends on the products of L-A for its replication but not on the presence of a replicating L-A genome. Moreover, M_1 does not directly require the *MAK10* product for its replication; *MAK10* is needed only because L-A products are needed and L-A needs *MAK10*. Since pORF1 did not support M_1 in a *mak10-1* host, M_1 needs both ORF1 and ORF2. An ochre mutation early in ORF1 and an ochre mutation in ORF2 just after the end of ORF1 both eliminate the ability of pORF1+2 to maintain M_1. This provides a simple in vivo assay for the activity of ORF2 which can be used to study the role of the RNA polymerase consensus sequence in ORF2 (19) and the ssRNA-binding activity of ORF2 (13).

That pORF1+2 allows M_1, but not L-A, to replicate in a *mak10* host points again to the distinction between the mechanisms of replication of the L-A replicon and those of its satellites, M_1, M_2, and X (a deletion mutant of L-A). Biochemical studies indicate that both follow the same replication cycle, except for the head-full replication mechanism shown by the smaller replicons, the fact that M_1 and X require many *MAK* genes not needed by L-A, and the fact that M_1 and X must borrow coat proteins synthesized by L-A. pORF1+2 also supports M_1 in a wild-type host (in the absence of L-A), showing that this is not a phenomenon peculiar to *mak10* strains. Previous studies of L-A replication in a *mak10*^{ts} host showed that L-A dsRNA-containing particles were unstable, although L-A ssRNA particles were apparently unaffected (11). If that difference were due to the L-A dsRNA-containing particles being full while the L-A ssRNA-containing particles were not, then the present results would not be unexpected. This is consistent with the *MAK10* product being essential for the stability of full viral particles but not for that of particles that are only partially filled.

While pORF1+2 supported M_1 in a *mak10* host, it did not do so in a *mak3* host. This suggests that the *MAK3* product is more directly needed for M_1 replication, not simply through its being needed for L-A as in the case of *MAK10*.

The availability of a complete, expressed clone of L-A makes possible the detailed dissection of the role of L-A-encoded proteins in the replication process and in interaction with satellite genomes and with the host. Since viral plus strands are the species packaged to make new viral particles, plus strands made from the vector with the correct ends should replicate. This "launching" of L-A from the vector should allow its use as an RNA virus expression vector.

ACKNOWLEDGMENTS

We thank Ron Hitzeman and Genentech for generously providing the p375 vector.

REFERENCES

1. **Ball, S. G., C. Tirtiaux, and R. B. Wickner.** 1984. Genetic control of L-A and L-(BC) dsRNA copy number in killer systems of *Saccharomyces cerevisiae*. Genetics **107**:199–217.
2. **Boeke, J. D., and S. B. Sandemeyer.** *In* J. R. Broach, E. W. Jones, and J. R. Pringle (ed.), The molecular and cellular biology of the yeast *Saccharomyces*, in press. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Bussey, H.** 1988. Proteases and the processing of precursors to secreted proteins in yeast. Yeast **4**:17–26.
4. **Conde, H., and G. R. Fink.** 1976. A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. Proc. Natl. Acad. Sci. USA **73**:3651–3655.
5. **Dihanich, M., E. Van Tuinen, J. D. Lambris, and B. Marshallsay.** 1989. Accumulation of viruslike particles in a yeast mutant lacking a mitochondrial pore protein. Mol. Cell. Biol. **9**:1100–1108.

5a. **Dinman, J., T. Icho, and R. B. Wickner.** Proc. Natl. Acad. Sci. USA, in press.

6. **Esteban, R. E., and R. B. Wickner.** 1986. Three different M_1 RNA-containing viruslike particle types in *Saccharomyces cerevisiae*: in vitro M_1 double-stranded RNA synthesis. Mol. Cell. Biol. **6**:1552–1561.
7. **Esteban, R. E., and R. B. Wickner.** 1987. A new non-Mendelian genetic element of yeast that increases cytopathology produced by M_1 double-stranded RNA in *ski* strains. Genetics **117**:399–408.
8. **Esteban, R. E., and R. B. Wickner.** 1988. A deletion mutant of L-A double-stranded RNA replicates like M_1 double-stranded RNA. J. Virol. **62**:1278–1285.
9. **Fried, H. M., and G. R. Fink.** 1978. Electron microscopic heteroduplex analysis of "killer" double-stranded RNA species from yeast. Proc. Natl. Acad. Sci. USA **75**:4224–4228.
10. **Fujimura, T., and R. B. Wickner.** 1986. Thermolabile L-A

virus-like particles from *pet18* mutants of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6**:404–410.

11. **Fujimura, T., and R. B. Wickner.** 1987. L-A double-stranded RNA viruslike particle replication cycle in *Saccharomyces cerevisiae*: particle maturation in vitro and effects of *mak10* and *pet18* mutations. Mol. Cell. Biol. **7**:420–426.
12. **Fujimura, T., and R. B. Wickner.** 1988. Replicase of L-A virus-like particles of Saccharomyces cerevisiae: in vitro conversion of exogenous L-A and M_1 single-stranded RNAs to double-stranded form. J. Biol. Chem. **263**:454–460.
13. **Fujimura, T., and R. B. Wickner.** 1988. Gene overlap results in a viral protein having an RNA binding domain and a major coat protein domain. Cell **55**:663–671.
14. **Fujimura, T., and R. B. Wickner.** 1989. Reconstitution of template-dependent in vitro transcriptase activity of a yeast double-stranded RNA virus. J. Biol. Chem. **264**:10872–10877.
15. **Hannig, E. M., M. J. Leibowitz, and R. B. Wickner.** 1985. On the mechanism of exclusion of M_2 double-stranded RNA by L-A-E double-stranded RNA in *Saccharomyces cerevisiae*. Yeast **1**:57–65.

15a. **Hitzeman, R.** Personal communication.

16. **Hitzeman, R. A., D. W. Leung, L. J. Perry, W. J. Kohr, H. L. Levine, and D. V. Goeddel.** 1983. Secretion of human interferons by yeast. Science **219**:620–625.
17. **Hizi, A., L. E. Henderson, T. D. Copeland, R. C. Sowder, C. V. Hixson, and S. Oroszlan.** 1987. Characterization of mouse mammary tumor *gag-pol* gene products and the ribosomal frameshift site by protein sequencing. Proc. Natl. Acad. Sci. USA **84**: 7041–7045.
18. **Hopper, J. E., K. A. Bostian, L. B. Rowe, and D. J. Tipper.** 1977. Translation of the L-species dsRNA genome of the killer-associated virus-like particles of *Saccharomyces cerevisiae*. J. Biol. Chem. **252**:9010–9017.
19. **Icho, T., and R. B. Wickner.** 1989. The double-stranded RNA genome of yeast virus L-A encodes its own putative RNA polymerase by fusing two open reading frames. J. Biol. Chem. **264**:6716–6723.
20. **Jacks, T., H. D. Madhani, F. R. Masiarz, and H. E. Varmus.** 1988. Signals for ribosomal frameshifting in the Rous sarcoma virus *gag-pol* region. Cell **55**:447–458.
21. **Jacks, T., and H. E. Varmus.** 1985. Expression of the Rous sarcoma virus *pol* gene by ribosomal frameshifting. Science **230**:1237–1242.

21a. **Matsumoto, Y., R. Fishel, and R. B. Wickner.** 1990. Circular single-stranded RNA replicon in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **87**:7628–7632.

22. **Mead, D. A., E. S. Skorupa, and B. Kemper.** 1985. Single stranded DNA SP6 promoter plasmids for engineering mutant RNAs and proteins: synthesis of a 'stretched' preproparathyroid hormone. Nucleic Acids Res. **13**:1103–1118.
23. **Rhee, S.-K., T. Icho, and R. B. Wickner.** 1989. Structure and nuclear localization signal of the *SKI3* antiviral protein of *Saccharomyces cerevisiae*. Yeast **5**:149–158.
24. **Ridley, S. P., S. S. Sommer, and R. B. Wickner.** 1984. Superkiller mutations in *Saccharomyces cerevisiae* suppress exclusion of M_2 double-stranded RNA by L-A-HN and confer cold sensitivity in the presence of M and L-A-HN. Mol. Cell. Biol. **4**:761–770.
25. **Sommer, S. S., and R. B. Wickner.** 1982. Yeast L dsRNA consists of at least three distinct RNAs: evidence that the non-Mendelian genes [HOK], [NEX] and [EXL] are on one of these dsRNAs. Cell **31**:429–441.
26. **Sommer, S. S., and R. B. Wickner.** 1987. Gene disruption indicates that the only essential function of the *SKI8* chromosomal gene is to protect *Saccharomyces cerevisiae* from viral cytopathology. Virology **157**:252–256.
27. **Toh-e, A., P. Guerry, and R. B. Wickner.** 1978. Chromosomal superkiller mutants of *Saccharomyces cerevisiae*. J. Bacteriol. **136**:1002–1007.
28. **Toh-e, A., and Y. Sahashi.** 1985. The *PET18* locus of *Saccharomyces cerevisiae*: a complex locus containing multiple genes. Yeast **1**:159–172.
29. **Toh-e, A., and R. B. Wickner.** 1980. "Superkiller" mutations suppress chromosomal mutations affecting double-stranded RNA killer plasmid replication in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA **77**:527–530.
30. **Uemura, H., and R. B. Wickner.** 1988. Suppression of chromosomal mutations affecting M_1 virus replication in *Saccharomyces cerevisiae* by a variant of a viral RNA segment (L-A) that encodes coat protein. Mol. Cell. Biol. **8**:938–944.
31. **Wickner, R. B.** 1978. Twenty-six chromosomal genes needed to maintain the killer double-stranded RNA plasmid of *Saccharomyces cerevisiae*. Genetics **88**:419–425.
32. **Wickner, R. B.** 1979. Mapping of chromosomal genes of *Saccharomyces cerevisiae* using an improved genetic mapping method. Genetics **92**:803–821.
33. **Wickner, R. B.** 1980. Plasmids controlling exclusion of the K_2 killer double-stranded RNA plasmid of yeast. Cell **21**:217–226.
34. **Wickner, R. B.** 1983. Killer systems in *Saccharomyces cerevisiae*: three distinct modes of exclusion of M_2 double-stranded RNA by three species of double-stranded RNA, M_1, L-A-E, and L-A-HN. Mol. Cell Biol. **3**:654–661.
35. **Wickner, R. B.** 1989. Yeast virology. FASEB J. **3**:2257–2265.
36. **Wickner, R. B.** *In* J. R. Broach, E. W. Jones, and J. R. Pringle (ed.), The molecular and cellular biology of the yeast *Saccharomyces*, in press. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Wickner, R. B., and M. J. Leibowitz.** 1976. Chromosomal genes essential for replication of a double-stranded RNA plasmid of *Saccharomyces cerevisiae*: the killer character of yeast. J. Mol. Biol. **105**:427–443.
38. **Wickner, R. B., and M. J. Leibowitz.** 1979. *mak* mutants of yeast: mapping and characterization. J. Bacteriol. **140**:154–160.
39. **Wickner, R. B., and A. Toh-e.** 1982. [HOK], a new yeast non-Mendelian trait, enables a replication-defective killer plasmid to be maintained. Genetics **100**:159–174.