KOR. JOUR. MICROBIOL. May, 1991 p. 75-79 Vol. 29, No. 2

A Yeast Chromosomal Gene that Induces Defective Interfering Particles of L-A dsRNA Virus in *ski*$^-$ Host Cells.

Hyun-Sook Lee
Department of Microbiology, College of Natural Science, Gyeongsang National University, Chinju 660-701, Korea

ski$^-$ 기주 세포에서 L-A dsRNA 바이러스의 Defective Interfering Particle을 유도하는 효모 유전자

이현숙
경상대학교 자연과학대학 미생물학과

ABSTRACT: The yeast L-A virus (4.6 kb dsRNA genome) encodes the major coat protein and a "gag-pol" fusion minor coat protein that separately encapsidate itself and M_1, a 1.8 kb dsRNA satellite virus encoding a secreted protein toxin (the killer toxin). The yeast chromosomal *SKI* genes prevent viral cytopathology by lowering the virus copy number. Thus, *ski*$^-$ mutants are ts and cs for growth. We transformed a *ski*2-2 virus-infested mutant with a yeast bank in a high copy cloning vector and selected the rare healthy transformants for analysis. One type of transformant segregated M-O L-A-O cells with high frequency. Elimination of the DNA clone from the *ski*2-2 strain eliminated this phenotype and introduction of the DNA clone recovered from such transformants into the parent *ski*2-2 strain, or into *ski*3 or *ski*6 mutants gave the same phenotype. This killer-curing phenotype was due to the curing of the helper L-A dsRNA virus. The 6.5 kb insert only had this activity when carried on a high copy vector and in *ski*$^-$ cells (not in *SKI*$^+$ cells). This 6.5 kb insert acts as a mutagen on L-A dsRNA producing a high rate of deletion mutations.

KEY WORDS □ Yeast killer, Defective interfering particles of L-A, *ski*$^-$ mutation.

In *Saccharomyces cerevisiae,* the relative ease of genetic and biochemical manipulation allows the study of host-virus interactions during a persistent infection to be dissected in way that are very difficult to accomplish in mammalian cells.

There are five families of dsRNA in *S. cerevisiae* (L-A, L-BC, M, T, and W). Three of which were found encapsidated in intracellular noninfectious virus particles. L-A encodes the major coat protein of the particles in which L-A and M are separately encapsidated, while L-BC encodes its own coat protein. Strains carrying M dsRNA secrete a protein toxin, and are immune to that toxin. M encodes this toxin as a larger precursor protein, and the precursor protein apparently serves as the immune protein. Natural varieties of M (M_1, M_2, etc) determine distinct toxin-immunity specificities, while natural varients of L-A show varying interactions with M_1, M_2 and each other (Wickner, 1986).

The replication of L-A, L-BC, and M is repressed by a set of chromosomal genes, called SKI genes, so named for the superkiller phenotype of the mutants (Ball *et al.,* 1984). The high copy number of an M dsRNA replicon resulting from a ski mutation has been shown to result in cell death at 8℃ (Ridley *et al.,* 1984). This cold sensitivity is not a consequence of excess production of toxin or immunity protein since delection derivatives of M_1 dsRNA (called S dsRNA) lacking nearly the entire preprotoxin immunity protein coding region make *ski*$^-$ mutants just as does the parent M_1 dsRNA.

Nor is the cold sensitivity dur to the total load of or virus particles since elimination, by heat curing at 39℃, of M from a *ski*$^-$ L-A M strain results in a strain with much more total dsRNA-mostly L-A because of relief of the M repression of L-A copy number (Ball *et al.,* 1984). The resulting *ski*$^-$ L-A M-O strain is, however, not

Table 1. *Strains*

Designatin		Nuclear genotype	dsRNA
2820	α	*leu2 ura3 ski2-2*	L-(BC), L-A-HN, M_1
2917	α	*leu2 ura3 ski2-2 mkt1*	L-(BC), L-A-HN, M_2
2259	α	*leu2 ura3-52 ski3 mkt1*	L-(BC), L-A-HN, M_2
2260	α	*leu2 ura3-52 ski3 mkt1*	L-A-HN, M_2
2634	α	*leu2 ura3 his5 ski3 mkt1*	L-(BC), L-A-HN, M_2
2911	*a*	*leu2 ura3-52 ade3 his6 ski6-2*	L-(BC), L-A-HN, M_1
2913	α	*leu2 ura3-52 his6 ski7-1*	L-(BC), L-A-HN, M_1
4282	α	*leu2 ura3 his3* SKI8::URA3 (*ski8*)	L-(BC), L-A-HN, M_1
TF229	*a*	*leu2 his*(3,4), *ski2-2*	L-(BC), L-A-HN
2604	α	*leu2 his3 trp1*	L-(BC), L-A-HN, M_1
1947	α	*leu2 ura3 his4*	L-(BC), L-A-HN, M_2
1877	*a*	*ade1 kar1-1 mak10 can1*	L-(BC)

detectably cold sensitive (Ridley *et al.*, 1984). All *ski*$^-$ mutations could suppress mutations in certain chromosomal genes (*mak*$^-$ mutations) normally needed by M dsRNA for its replication (Sommer *et al.*, 1987; Toh-E *et al.*, 1980).

Thus, while *mak*$^-$ mutants lose M dsRNA, *ski*$^-$ *mak*$^-$ double mutants, in many cases, maintain M dsRNA at normal or elevated copy number. The *MKT*1 gene is needed specifically by M_2 dsRNA if L-A-HN variant is present (Sommer *et al.*, 1982). This requirement is temperature dependent, with M_2 loss observed at 30℃, but not at 20℃. The *ski*$^-$ mutations also suppress this effect of mkt 1$^-$ mutations so that a ski$^-$ mkt 1$^-$ L-A-HN M_2 strain can stably maintain M_2 even at 30℃ (Ridley *et al.*, 1984).

In the absence of M dsRNA, an effect of *SKI* products on L-A and L-BC dsRNA copy number was detectable, with *ski*$^-$ mutants showing three to fivefold higher copy number than wild type strains (Ball *et al.*, 1984). This effect on L-A is not directly seen in strains carrying M dsRNA because M itself represses L-A and the increased M_1 due to the *ski*$^-$ mutation, repressed L-A more than the *ski* mutation derepresses L-A. The paradoxical results that a *SKI*$^+$ L-A$^+$ M$^+$ strain has more L-A than a *ski*$^-$ L-A$^+$ M$^+$ strain.

We transformed a *ski*2-2 virus infested mutant with a yeast bank in a high copy cloning vector (YEp13) and selected the rare healthy transformants for analysis. Here we report that a particular 6.5 kb yeast chromosomal DNA fragment, when carried on a high copy vector, cures *ski*$^-$ strains of the L-A and M viral genomes. This phenotype was due to the mutagenic effect on L-A dsRNA producing a high rate of deletion mutations.

MATERIALS AND METHODS

The genotypes of each mutant and wild type strains of *S. cerevisiae* are described in Table 1.

Table 2. *Plasmids*

Plasmids	Essential features
YEp13	LEU2, ARS1, pBR322, Amp^r
YEp^{LAM}-2	6.5 kb of Sau3A fragment of *S. cerevisiae* (LAM) inserted at BamHI site of YEp13; a functional L-A mutator gene is present
pRS316	URA3, ARS1, CEN4, pBR322, Amp^r
pRS316-LAM	6.5 Kb LAM DNA from YEp^{LAM}-2 inserted at ClaI/SalI sites of pRS316
pTIL05	L-A cDNA was inserted at the SmaI site of a Bluescript vector SK$^+$

Transformation of yeast was performed with LiCl and polyethylene glycol (Ito *et al.*, 1983). dsRNA from the colonies recovered from cold sensitivity due to the loss of M replicon were extracted to screen a yeast bank for a gene which cures L-A and/or L-BC virus.

General yeast methods (Mortmier *et al.*, 1975), media (Wickner, 1978) and assays of killing have been described previously (Wickner, 1987). Complete medium minus leucine or uracil are reffered to as −LEU and −URA medium, respectively. Cold sensitivity for growth was tested on −LEU or −URA medium plates at 8℃ and 20 ℃ (Ridley *et al.*, 1984).

Escherichia coli strain DH5α was used for propagation of plasmids. The essential feature of the plasmids used are descirbed in Table 2. The yeast genomic library DNA in the multicopy plasmids YEp13 was purchased from American Type Culture Collection.

dsRNA was purified on a CF11 cellulose chromatography and analyzed on agarose gels as previously described (Toh-E *et al.*, 1980) or by the rapid method described by Fried and Fink (1978). Briefly, spheroplasts produced by mureinase were lysed by sodium dodecyl sulfate (SDS), and extract

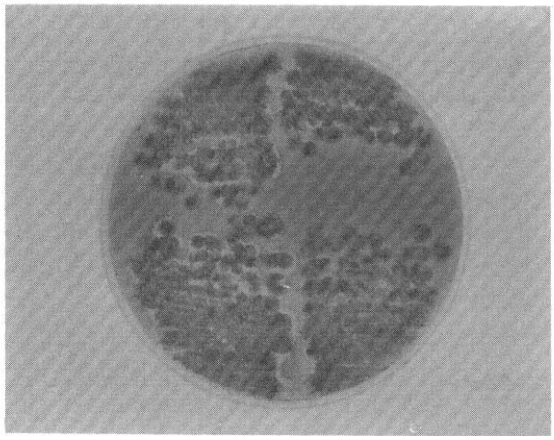

Fig. 1. *Killer-losing phenotype of ski⁻ cells after plasmid transformation (YEp^{LAM}-2).*

was digested with proteinase K, extracted with phenol, and precipitated with ethanol; dsRNA was purified by CF11 cellulose chromatography and analyzed on 1% agarose gels. In the rapid method, cells treated with 2-mercaptoethanol were extracted with SDS-phenol mixture, and the extract was precipitated with ethanol and analyzed on 1% agarose gels.

Northern blot hybridization was carried out as described by NYTRAN. Prepared viral dsRNA was separated by electrophoresis on formaldehyde containing 1.4% agaros gel prepared in MOPS buffer pH 7.0. After transfer to Nylon-66 membrane (NYTRAN), the blots were hybridized to labeled L-A transcription product of full-length L-A cDNA inserted in plasmid pTIL05 (Icho *et al.*, 1989). The hybridization were carried out in 50% (vol/vol) formamide at 60℃.

RESULTS AND DISCUSSION

Yeast strains 2820 and 2917 are *ski*2, *leu*⁻, *ura*⁻ mutants with high transformation frequency. They carry a cytoplasmically determined superkiller phenotype and grow slowly at 20℃ and do not grow at 8℃. The yeast gene bank ligated to the BamHI site of YEp13 shuttle vector (Amp^r, Leu^{2+}) was used to transform these two *ski*2 strains. After 10 days on −LEU plates at 20℃, the faster growing cells (big colonies) were picked restreaked on −LEU plates for single colony isolations. After 10 days of growth at 20℃ replicas were made on −LEU plates to test cold sensitivity at 8℃ and on 4.7 MB plates for killer activity. Among more than 500 fast growing clones 27 gradually lost killer phenotype (Fig. 1) at both 20℃ and 30℃, and grew at 8℃. These clones are not SKI$^+$ because they lost killer phenotype even at 20℃. Plasmids from the 27 clones were extracted, amplified in *E. coli*, and were used to tranform again the yeast strains 2820 and 2917. dsRNA of the tranformed 2820 and 2917 cells were extracted. As we expected there was no M dsRNA, but the amount of L-A and L-BC was not increased. Amount of L-A dsRNA was more than 10 times higher in M dsRNA missing cells when M dsRNA was cured spontaneously or by heat (Ball *et al.*, 1984).

Northern hybridization with transcript of L-A cDNA showed there was no L-A dsRNA in these clones. Plasmid YEp^{LAM}−2 carring the yeast DNA fragment of 6.5 kb from the killer-losing cells was used for further analyis. Restriction map of the 6.5 kb yeast DNA fragment (LAM:L-A Mutator) is shown In Fig. 2.

The other *ski*⁻ mutant cells, 2259 (*ski*3), 2260 (*ski* 3), 2634 (*ski*3), 2911 (*ski*6), 2913 (*ski*7), 4282 (*ski*8), and *SKI*$^+$ cells, 2604 and 1947, were transformed with YEp^{LAM}−2. Transformants of the *ski*3 (strain 2259, 2260 and 2634) and *ski*6 strains clearly showed killer curing phenotype as in the *ski*2 strains but SKI$^+$ cells did not. Northern hybridization confirmed that there were no L-A dsRNA in the *ski*⁻ transformant cells (Fig. 3). The 6.5 kb fragment of yeast DNA in YEp^{LAM}−2 was ligated into the ClaI and SalI sites of centromeric vector (single copy shuttle vector) pRS316. This plasmid (designated pRS316-LAM) was used to transform *ski*2 (strain 2917 and 2820), *ski*3 (strain 2259 and 2260), and *ski*6 cells. None of the pRS316-LAM transformed cells expressed killer curing phenotype.

Thus, the killer curing phenotype caused by YEp^{LAM}−2 transformation into *ski*2, *ski*3, and *ski*6 cells was due to the loss of L-A particles which acts as helper virus for M virus. The 6.5 kb insert of YEp^{LAM}-2 plasmid (designated LAM: L-A Mutator)

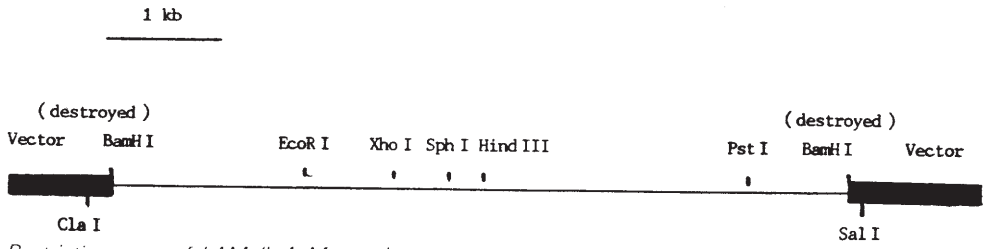

Fig. 2. *Restriction map of LAM (L-A Mutator).*

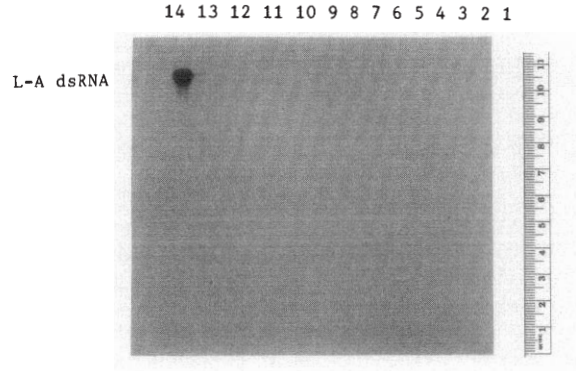

Fig. 3. *Northern blot hybridization confirms there were no L-A dsRNA in YEp^{LAM}-2 transformant ski$^-$ cells. The probe was T_7 RNA polymerase transcript of L-A cDNA. dsRNA was electrophoresed on 1.4% agarose gel containing formaldehyde, transfered to Nylon-66 membrane and hybridized for 14 hours with denatured ^{32}P-labeled probe.*
Lanes: 1-4, YEp^{LAM}-2 transformed *ski*2 (2820) strain; 5-8, YEp^{LAM}-2 transformed *ski*6 (2911) strain; 9-12, YEp^{LAM}-2 transformed *ski*3 (2260) strain; 13, original *ski*2 (2820) strain; 14, *ski*2 strain (TF229) cured from M dsRNA by heat treatment at 39℃.

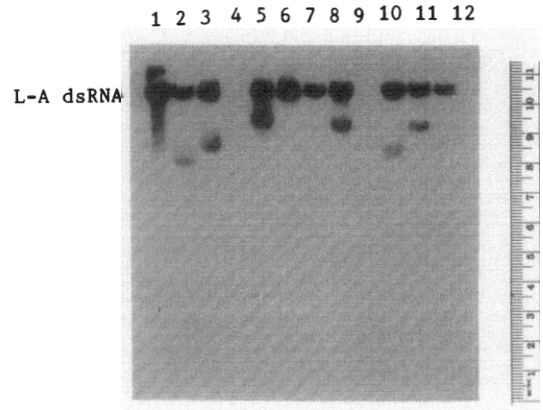

Fig. 4. *Identification of L-A Diffective Interfering Particles (DI) in YEp^{LAM}-2 transformed TF229 cells by Northern blot hybridization. The probe was T_7 RNA polymerase transcript of L-A cDNA. dsRNA was electrophoresed on 1.4% agarose gel containing formaldehyde, transformed to Nylon-66 membrane and hybridized for 14 hrs. With dentured ^{32}P-labeled probe.*
Lanes: 1 dsRNA from TF229 control cells; 2-12, dsRNA from YEp^{LAM}-2 transformed TF229 cells. Lane 2, 3, 5, 6, 8, 10, 11, and 12 show DI L-A dsRNA and Lane 4 and 9 show curing of L-A dsRNA.

had this activity only when carried on a high copy number plasmid and in *ski*$^-$ background.

To investigate the mode of L-A curing mechanism, we transformed YEp^{LAM}-2 plasmid to yeast *ski*$^-$ strain without M particles (TF229). Northern hybridization with transcript of full-length L-A cDNA showed that there were smaller sizes (1.9 kb, 1.5 kb, 1.3 kb) of L-A dsRNA in the transformant (Fig. 4). These smaller L-A dsRNAs were cytoduced to yest *SKI*$^+$ strains, 2604 and 1947. The cytoductants showed also L-A losing phenotype, though YEP^{LAM}-2 plasmid did not affect directly on L-A dsRNA in *SKI*$^+$ strains as shwon before. The L-A smaller dsRNA might act as defective interfering particles. Thus this cloned 6.5 kb yeast DNA acts as a mutagen on L-A dsRNA in *ski*$^-$ strain, producing a high rate of deletion mutations.

적 요

Yeast (*Saccharomyces cerevisiae*)의 L-A virus (약 4.6 kb dsRNA genome)는 major coat protein과 "gag-pol" fusion minor coat protein (RNA replicase 라고 사료됨)을 만들며 자신이 만든 capsid에 L-A dsRNA와 그 satellite인 killer toxin을 분비하는 M dsRNA를 encapsidation시킨다. Yeast chromosomal SKI gene들은 이 virus들의 cytopathology 및 virus copy 수를 억제한다. *ski*$^-$ 변이주들은 ts (temperature sensitive) 및 cs (cold sensitive)이다. 본 연구팀은 L-A 및 M virus에 감염된 *ski*2-2 변이주 세포내에 high copy cloning vector (YEp13)에 ligation으로 만든 yeast gene bank를 형질전환한 결과 건강한 형질전환주를 얻었다. 그 중 높은 빈도로 M-O L-A-O Segregation 되는 세포를 얻었으며 이 형질전환주로부터 plasmid를 제거했을 때는 이러한 표현형이 없어지는 것으로 미루어 보아 이는 plasmid의 형질전환에 의한결과로 사료되었다. Cloning된 plasmid를 *ski*2-2, *ski*3$^-$, *ski*6$^-$ 변이주에 형질전환 했을 때 동일한 M-O L-A-O 표현형이 나타났으며 이 plasmid에 cloning된 6.5 kb chromosomal DNA insert가 high copy vector에 존재할 때만 이러한 현상이 나타났다. 이 6.5 kb insert는 L-A dsRNA에 mutagen 역할을 하여 deletion mutant를 만들고, 이 deletion mutant는 defective interfering paticle 역할을 하여 helper virus인 L-A를 치유함으로 M-O L-A-O cell을 만든것으로 사료된다.

ACKNOWLEDGEMENT

This work was supported in part of by the 1990 reserch grant for genetic engineering from Minstry of Education.

REFERENCES

1. **Ball, S.G., Tirtiaux, C., and Wickner, R.B.,** (1984). Genetic control of L-A and L-(BC) dsRNA copy number in killer systems of *Saccharomyces ccerevisiae. Genetics,* **107**, 199-217.
2. **Fried, H.M. and G.R. Fink,** (1978). Electron microscopic heteroduplex analysis of "killer" double-stranded RNA species from yeast. *Proc. Natl. Acad. Sci. U.S.A.,* **75**, 4224-4228.
3. **Icho, T. and Wickner, R.B.,** (1989). The double-stranded RNA genome of yeas virus L-A encoded its own putative RNA polymerase by fusing two open reading frames. *J. Biol. Chem.,* **264**, 6716-6723.
4. **Ito, H., Fukoda, Y., Murata, K., and Kimura, A.,** (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.,* **153**, 163-168.
5. **Mortmier, R.K., Hawthorne, D.C.,** (1975). Genetic mapping in yeast. *Methods Cells Biol.,* **11**, 221-223.
6. **Ridley, S.P., Sommer, S.S., and Wickner, R.B.,** (1984). Superkillr 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.
7. **Sommer, S.S., Wickner, R.B.,** (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 The dsRNAs. *Cell,* **31**, 429-441.
8. **Sommer, S.S., Wickner, R.B.,** (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.
9. **Toh-E. A., Wickner, R.B.,** (1980). "Superkiller" mutations suppress chromosomal mutations affecting double-stranded RNA killer plasmid replication in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* 77, 527-530.
10. **Wickner, R.B.,** (1978). Twenty-six chromosomal genes needed to maintain the killer double-stranded RNA plasmid of *Sacchrromyces cerevisiae. Genetics,* **88**, 419-425.
11. **Wickner, R.B.,** (1986). Double-stranded RNA replication in yeast: the killer system. *Annu. Rev. Biochem.,* **55**, 373-395.

(Received February 18, 1991)
(Accepted March 28, 1991)