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Evidence that the *SKI* Antiviral System of *Saccharomyces cerevisiae* Acts by Blocking Expression of Viral mRNA

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The *SKI2* gene is part of a host system that represses the copy number of the L-A double-stranded RNA (dsRNA) virus and its satellites M and X dsRNA, of the L-BC dsRNA virus, and of the single-stranded replicon 20S RNA. We show that *SKI2* encodes a 145-kDa protein with motifs characteristic of helicases and nucleolar proteins and is essential only in cells carrying M dsRNA. Unexpectedly, Ski2p does not repress M_1 dsRNA copy number when M_1 is supported by an L-A cDNA clone; nonetheless, it did lower the levels of M_1 dsRNA-encoded toxin produced. Since toxin secretion from cDNA clones of M_1 is unaffected by Ski2p, these data suggest that Ski2p acts by specifically blocking translation of viral mRNAs, perhaps recognizing the absence of cap or poly(A). In support of this idea, we find that Ski2p represses production of β-galactosidase from RNA polymerase I [no cap and no poly(A)] transcripts but not from RNA polymerase II (capped) transcripts.

Animals resist viral infection by antibody production, cell-mediated immunity, and interferon action. All of these mechanisms require intercellular communication in a multicellular organism. *Saccharomyces cerevisiae* has a purely intracellular antiviral system that protects it from viral cytopathology.

Most strains of the yeast *S. cerevisiae* carry several double-stranded RNA (dsRNA) viruses (L-A, M, and L-BC), single-stranded RNA (ssRNA) replicons (20S RNA and 23S RNA), and retroviruses (Ty1, Ty2, Ty3, and Ty4) (reviewed in references 7 and 72). The L-A virus encodes its major coat protein (*gag*) (32) and a *gag-pol* fusion protein formed by a −1 ribosomal frameshift event (14, 21, 34). The *pol* domain includes RNA-dependent RNA polymerase and ssRNA binding activities and has a role in packaging viral RNA as well as in its replication (20, 53). M dsRNAs (M_1 and M_2, etc.) each encode a secreted toxin (killer toxin) and immunity to that toxin (9, 13) and are satellites of L-A, being encapsidated in particles composed of the L-A-encoded major coat protein (10) and *gag-pol* fusion protein (53, 73). A deletion mutant of L-A, called X dsRNA, replicates in L-A-encoded viral particles and acts as a defective interfering mutant of L-A (17).

A system of six yeast chromosomal genes (*SKI2*, *-3*, *-4*, *-6*, *-7*, and *-8*) lowers the copy numbers of the L-A, M, X, and L-BC dsRNA viruses and of the ssRNA replicon 20S RNA (3, 17, 44, 54, 65). Mutants in these genes were first isolated on the basis of their superkiller phenotype, because of the increased copy number of M_1 dsRNA. By lowering the copy number of M dsRNA, the *SKI* products prevent the cell death at low temperatures that is otherwise associated with derepressed M dsRNA virus replication (16, 54). The cold sensitivity is not due simply to the load of virus particles, since loss of M from a *ski* mutant relieves the repression of L-A copy number by M, resulting in a large net increase of total viral particles, but the cells are nonetheless healthy (3, 54). Moreover, derepressed L-BC or 20S RNA has not been associated with any cytopathology. Nor is the cold sensitivity due to the overproduction of toxin, since *ski* mutants carrying deletion mutants of M_1 lacking essentially the entire preprotoxin region are nonetheless cold sensitive (54).

The *ski* mutations also suppress mutations in most of the *MAK* genes required for the replication of M dsRNA (reference 66; reviewed in reference 72). The *MKT1* and *MKT2* genes are necessary specifically for M_2 replication but not for that of M_1 (70), and the *ski* mutations also suppress *mkt* mutations (54).

The *SKI3* and *SKI8* genes have been cloned and characterized. The *SKI3* product is a 163-kDa nuclear protein (52) with several copies of an amino acid repeat pattern, the TPR repeat, of unknown function (62). The *SKI8* protein has two copies of a different repeat amino acid sequence pattern first identified in β-transducin (45). Deletion mutations of either *SKI3* or *SKI8* had no effect on cell growth unless an M replicon was present. In that case, the cells were cold sensitive for growth as discussed above. For this reason, we view the *SKI* system as a dedicated antiviral system. However, the mechanism by which the *SKI* proteins interfere with the propagation of the RNA replicons is completely unknown.

We report here that Ski2p resembles helicases and nucleolar proteins and present evidence that Ski2p acts by blocking the translation of viral mRNA.

MATERIALS AND METHODS

Strains and media. The yeast strains used in this study are shown in Table 1. *Escherichia coli* DH5α was used to propagate plasmid DNA, strain MV1190 and M13 helper phage K07 were used for the isolation of single-stranded plasmid DNA for DNA sequencing, and strain CJ236 was used for the production of uracil-containing single-stranded plasmid DNA for site-directed mutagenesis (40). Yeast strains were grown on YPAD, SD, H-His, H-Trp, H-Ura, or 4.7MB medium (70). *E. coli* strains were grown on LB, TB, or M9 medium (43).

The strains in which M or X is supported by an L-A cDNA clone were constructed as follows: a strain derived from strain 3221 harboring L-A and either M_1 or X (produced by cytoduction into strain 3221) was transformed with the L-A

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TABLE 1. Strains of *S. cerevisiae*

Strain	Genotype
2820	*MATα leu2 ura3 ski2-2* L-A-HN M_1
2603	*MATa trp1 his3* K^-
2604	*MATα trp1 leu2 his3 pho3 pho5* L-A-HN M_1
2603 × 2604	Diploid formed from strains 2603 and 2604
3221	*MATa ura3 his3 trp1 SKI*$^+$ L-BC L-A-o M-o Gal^+
4167-3B	*MATα his3 ura3 thr1 ade8 trp1 ski2-2* L-A-HN M_1
Cross 4169	2603 × 4167-3B
2629	*MATα leu1 kar1-1* L-A-HNB M_1

cDNA clone pTIL132 (73). Clones which acquire this plasmid are gradually cured of the L-A virus (67). The transformants were therefore grown to single colonies on H-Trp plates several times. dsRNA was isolated (19) and analyzed by Northern (RNA) blot hybridization to identify clones which no longer contained the L-A virus.

Genetic manipulations. Yeast strains were transformed by the lithium acetate method (35). Genetic crosses, sporulation, tetrad analyses, and cytoduction experiments were performed as described previously (54, 71). *E. coli* strains were transformed by the calcium chloride method (43). Killer toxin production was measured qualitatively by spotting or replica plating freshly grown cells of each strain on a 4.7MB plate (rich medium containing methylene blue to stain dead cells and buffered at pH 4.7) freshly seeded with a lawn of the sensitive strain 5X47. Killer zones were observed after 2 days of incubation at 20°C.

Cloning of the *SKI2* gene. A bank of yeast DNA in the vector YCp50 (56) (ATCC 37415) was propagated in *E. coli* (approximately 20,000 transformant colonies), and plasmid DNA was isolated and transformed into the *ski2-2* mutant strain 2820. Cells were spread onto 80 H-Ura plates at a density that would yield approximately 500 transformant colonies per plate and incubated at 8°C for 12 days and then transferred to 20°C to allow colonies to form. Approximately 20 colonies arose on each plate; 500 of these transformants were tested for killer activity and the ability to grow at 8°C. Of these, only 18 were able to grow at 8°C, and only one of these was a killer. Plasmid DNA was isolated from this strain and used to transform *E. coli* DH5α. When this plasmid was reintroduced into yeast strain 2820, all transformants were able to grow at 8°C and their killing zones were reduced to wild-type levels on plate tests (54), implying that the YCp50-based recombinant plasmid (herein designated YCp50-SKI2) was indeed complementing the *ski2* defect.

Plasmids and constructs. The following cloning vectors were utilized in this study: Bluescript KS^+ (Stratagene), pGEM-7Zf(+) (Promega), pJH-H1 (from John E. Hill), pRS316 (62), pFLAG-1 (IBI), YEp357 (49), and YEpFLAG (IBI).

To construct a plasmid in which an RNA polymerase I (rDNA) promoter was driving transcription of the *lacZ* gene, we used prib1 (47), in which the enhancer region and promoter region for the synthesis of the 35S rRNA precursor (15) have been brought together by eliminating the intervening 5S RNA gene. From prib1, with the polymerase chain reaction primers

*Kpn*I *Eco*RI
P1 = 5′ ACC GGTACC GAATTC TATGATCCGGG 3′

(underlined sequence is from 5′ end of enhancer) and

*Bam*HI *Xba*I
P2 = 5′ CC GGATCC TCTAGA AGT *CAT* TTT CGA ACT TGT CTT CAA 3′

(underlined sequence is complementary to 3′ end of promoter region; the italicized CAT makes an artificial ATG in frame on the *lacZ* strand), we amplified a 583-base segment containing the 321-bp enhancer region linked to 234 bp of the promoter region. This fragment was digested with *Eco*RI (upstream end) and *Xba*I (downstream end), purified, and ligated to YEp357 digested with the same enzymes. This places *lacZ* in frame with both the AUG that forms the first three bases of the 35S rRNA precursor transcript (and thus is probably not recognized by the ribosomes) and the artificial ATG created by the polymerase chain reaction (Fig. 1A).

Localization of *SKI2* and DNA sequencing. The cloned *SKI2* gene was on a *Sau*3A fragment of approximately 15 kb in size (Fig. 1). Almost the entire fragment was removed from the YCp50 vector by digestion with *Sal*I and *Sac*I (*Sal*I was in the vector DNA very close to the *Bam*HI cloning site, and *Sac*I was within a few hundred base pairs from the other end of the insert [Fig. 1]) and cloned into the *Sma*I site of the vector pRS316. Deletions of the inserted fragment were generated with *Not*I, *Spe*I, *Sma*I, *Nru*I, *Eco*RI, and *Xba*I (Fig. 1). The *SKI2* gene was localized to the 5,200-bp *Xba*I-*Sac*I fragment, which was subsequently sequenced by making unidirectional deletions from both directions with exonuclease III and S1 nuclease (29).

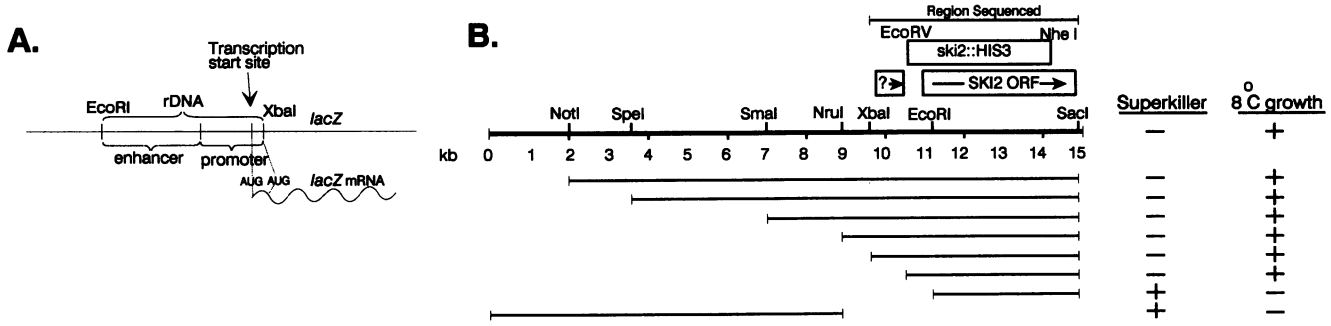

FIG. 1. (A) Structure of the rDNA-*lacZ* fusion plasmid. The region labeled "rDNA" was amplified from prib1 by polymerase chain reaction (47), and the remainder is from YEp357 (49). (B) Restriction map of the *SKI2* region and localization of the *SKI2* gene. Subclones were reintroduced into the *ski2-2* strain 2820, and the superkiller activity and growth at 8°C of transformants were tested. The bars (⊢⊣) show the region remaining in the subclones. Also shown are the region deleted in the disruption mutation, the region sequenced, and the extent of ORF1 (?) and of the *SKI2* ORF.

***SKI2* disruption.** The 5,200-bp *Xba*I-*Sac*I fragment of YCp50-SKI2 (Fig. 1) was cloned into pGEM-7Zf(+) cut with the same enzymes. Most of the *SKI2* gene was deleted by digestion with *Eco*RV and *Nhe*I (residues 1204 to 4857 of Fig. 1 and 2) and replaced by the *HIS3* gene of pJH-H1 on an *Xba*I-*Sma*I fragment. The resulting *ski2*::*HIS3* deletion plasmid (p500) was digested with *Xba*I and *Sac*I and used to transform the diploid yeast strain 2603 × 2604 and the haploid strain 3221. The cells were spread onto H-His plates to select for recombinants which acquired a copy of the *HIS3* gene with concomitant loss of *SKI2*. Disruptions were verified by Southern blot analysis (data not shown).

Generation of antibody to Ski2p. Several in-frame fusions of the *SKI2* gene were constructed with the overexpression plasmid pFlag-1; however, most were toxic when introduced into *E. coli*. One fusion from nucleotides 3059 (*Xho*I) to 4268 (*Xmn*I) (Fig. 2) of *SKI2* did produce large quantities of a 45-kDa polypeptide in the form of insoluble inclusion bodies. This fusion was constructed as follows: the *Eco*RI-*Xmn*I internal fragment (bases 1832 to 4268) of the *SKI2* gene was cloned between the *Eco*RI and *Sal*I sites of pFlag-1. The *Xho*I site of the vector was then destroyed by digestion of the plasmid with *Bgl*II followed by S1 nuclease digestion and ligation. Finally, this plasmid was digested with *Eco*RI and *Xho*I, and the ends were filled in with Klenow fragment of DNA polymerase I and ligated; this yielded an in-frame fusion to the Flag peptide under the control of the inducible *tac* promoter. This plasmid was introduced into *E. coli* DH5α and induced with IPTG (isopropyl-β-D-thiogalactopyranoside) according to the manufacturer's instructions. Cells were pelleted at 5,000 × *g* for 10 min and washed with sterile distilled water. Finally, the cells were resuspended in sterile distilled water and sonicated (four 30-s pulses on ice, 1 min on ice between each) and centrifuged to recover insoluble material. The inclusion preparation obtained was >90% pure as judged by Coomassie blue staining of a sodium dodecyl sulfate–10% polyacrylamide gel. One milligram of this material was sent to the Berkeley Antibody Company, and rabbit antibody specific for Ski2p was produced. Immunoblot analyses were performed with alkaline phosphatase-conjugated Gamma-bind G (Genex) used to detect immune complexes on nitrocellulose filters.

Overexpression of *SKI2* in *S. cerevisiae*. The *Rsa*I-*Eco*RI fragment (nucleotides 1333 to 1832, Fig. 2) of pRS316-SKI2 was cloned into the KS+ vector cut with *Eco*RI and *Sma*I. A 3,390-bp *Eco*RI fragment from pRS316-SKI2 (the remainder of the *SKI2* gene) was cloned into the *Eco*RI site to regenerate the entire *SKI2* gene. This plasmid was digested with *Not*I, the ends were filled in with Klenow DNA polymerase, a second cut was made with *Sal*I, and the resulting 3.9-kb *SKI2*-bearing fragment was cloned into the YEpFLAG vector (a generous gift from IBI) cut with *Eco*RI and *Sal*I. This vector has a 2μm circle-based replicon, a *TRP1* selectable marker, an origin of replication derived from pBR322, an f1 origin, and an *ADH1* promoter for overexpressing cloned genes. A mutagenic oligonucleotide was constructed to make the methionine start codon of the *SKI2* gene be in the correct position to be the first methionine codon of the *ADH1*-promoted transcript. This construct was functional since it was able to complement a *ski2* mutation. To overexpress Ski2p, cells were grown at 30°C in H-Trp medium to late log phase, diluted 1/20 into YPAD broth, and again grown to late log phase. Extracts for immunoblotting were prepared essentially as described by Sambrook et al. (60).

Nucleotide sequence accession number. The GenBank accession number for the *SKI2* region is L13469.

RESULTS

Cloning and sequencing of *SKI2*. Killer strains of *S. cerevisiae* which have a *ski2* mutation are cold sensitive for growth. We utilized this characteristic as a positive selection for cloning the *SKI2* gene. One transformant of strain 2820 that could grow at 8°C and was still a killer and thus a candidate for having acquired the *SKI2* gene was obtained. Plasmid DNA from this strain was reintroduced into strain 2820 and retested. Cells which acquired the plasmid (YCp50-SKI2) were able to grow at 8°C and had a reduced zone of killing comparable to that of a wild-type strain. These results show that this recombinant plasmid suppresses the *ski2-2* mutation.

Genetic mapping of *SKI2* places it on chromosome 12 between *ura4* and *ilv5* (Table 2). That YCp50-SKI2 actually carries the *SKI2* gene was confirmed by using the cloned gene to probe a Southern blot of chromosomal DNA separated by pulsed-field electrophoresis and also an ordered lambda bank of the yeast genome (51). The former showed hybridization specifically with chromosome 12 (data not shown), and the latter showed that the clone was located between *ura4* and *ilv5* (54a).

TABLE 2. Genetic mapping of *SKI2*[a]

Gene	Parameter	*ura4*	*ilv5*
ilv5	PD	30	
	NPD	6	
	T	79	
	cM[b]	50	
ski2	PD	65	32
	NPD	2	3
	T	52	61
	cM	26	41

[a] Meiotic mapping was carried out as described previously (48). PD, parental ditype; NPD, nonparental ditype; T, tetratype.
[b] cM, centimorgans.

Plasmid YCp50-SKI2 contained an insert of approximately 15 kb (Fig. 1). To localize the *SKI2* gene, several subclones were tested for *SKI2* complementing activity. This localized the gene to a 5.2-kb *Xba*I-*Sac*I fragment, which was then sequenced (Fig. 2). Two substantial open reading frames (ORFs) were identified. ORF1 encodes a polypeptide of 216 amino acids with a predicted molecular mass of 23 kDa. This peptide has significant similarity (1) to the *fsh* (female sterile homeotic) protein of *Drosophila* species (28) and to the human Ring3 protein (4) in a region where these two proteins are nearly identical:

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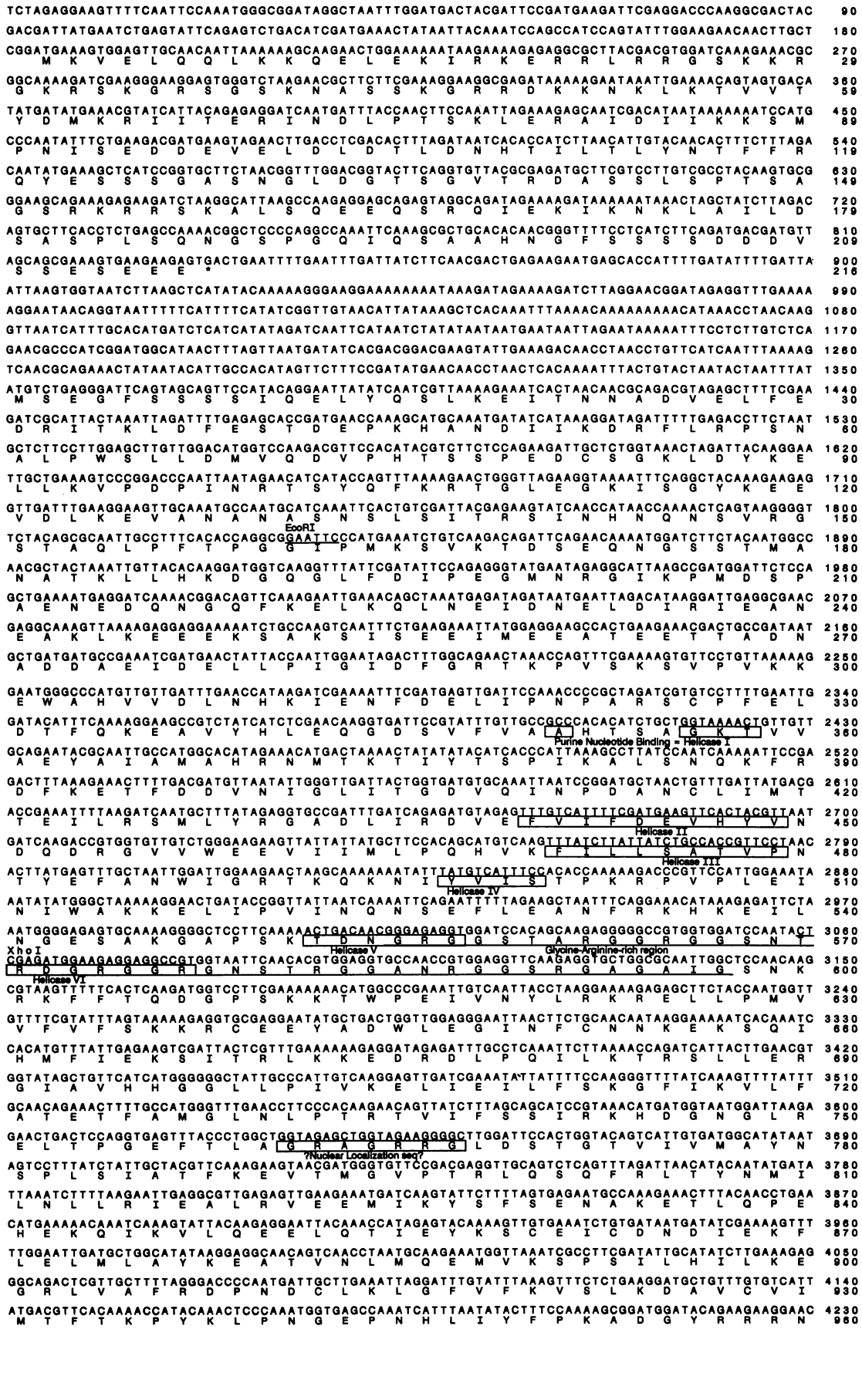
ORF1   58 VTYDMKRIITERINDLPTSKLERAIDIIKKSMPNISEDDEVELDLD 103
          ++YD KR ++ IN LP  KL R +II +  P+++ + E+++D
fsh   952 MSYDEKRQLSLDINKLPGDKLGRVVHIIQNREPSLRDSNPDEIEID 997
Ring3 595 MSYDEKRQLSLDINKLPGEKLGRVVHIIQAREPSLRDSNPEEIEID 640
```

ORF2 encodes a polypeptide of 1,286 amino acids with a predicted molecular mass of 145 kDa. ORF1 was deleted with exonuclease III and S1 nuclease, leaving ORF2 still intact, and the resulting construct was still able to complement a *ski2* mutation, suggesting that ORF2 encodes the *SKI2* product. In an attempt to mark Ski2p with an antigenic


EcoRI
Purine Nucleotide Binding = Helicase I
Helicase II
Helicase III
Helicase IV
Glycine-Arginine-rich region
XhoI
Helicase V
Helicase VI
?Nuclear Localization seq?

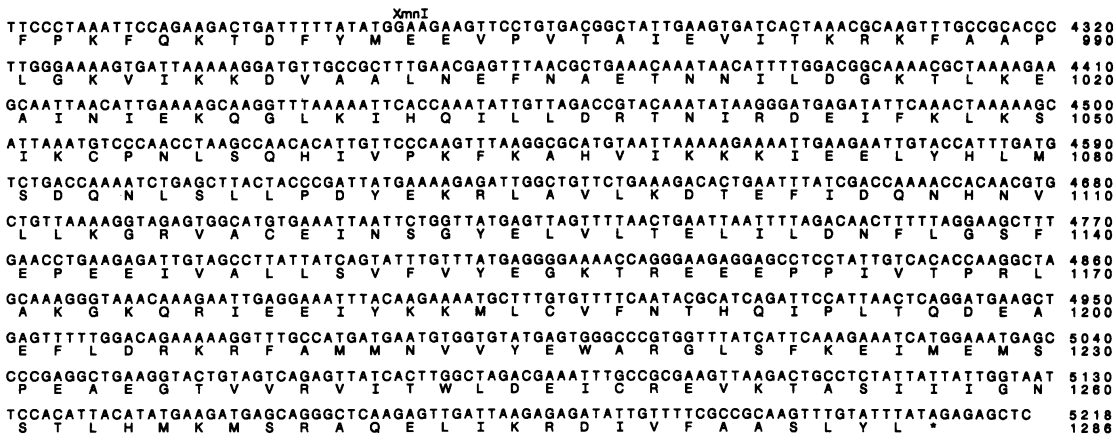


FIG. 2. The *SKI2* region DNA and protein sequence.

tag for the purpose of determining its intracellular location, we constructed variants in which the amino acid sequence DYKDDDDK was appended to either the NH_2 or the COOH terminus of ORF2. Unfortunately, neither of these constructions complemented the *ski2-2* mutation. However, this result does confirm that ORF2 is *SKI2*.

The *SKI2* ORF was expressed, without any amino acids appended to either end, from YEpFLAG. This construct complemented the *ski2-2* mutation and yielded a detectable protein of the expected size on Western blots (immunoblots) with antibodies produced against a 45-kDa fragment of *SKI2* made in *E. coli* (see Materials and Methods). However, we were unable to reliably detect the *SKI2* protein in whole extracts or in various subcellular fractions of wild-type cells which were not overexpressing Ski2p, indicating that it is produced in low amounts.

***SKI2* is essential for cell viability only in the presence of M.** We replaced most of the chromosomal copy of *SKI2* with the *HIS3* gene, forming a deletion-insertion mutation (Fig. 1) in both a diploid strain (2603 × 2604) originally homozygous for *SKI2* and *his3* and carrying L-A and M_1 and a *SKI*$^+$ *his3* L-A-o M-o haploid strain (strain 3221). The heterozygous *SKI2/ski2*::*HIS3* diploid was allowed to sporulate, and tetrads were dissected and tested for viability (the ability to germinate and grow at 30°C) and killer phenotype (super- or normal killers and the ability to grow at 8°C). All *HIS3* segregants (*ski2*::*HIS3* disruptants) were viable at 30°C, were superkillers, and were cold sensitive; *his3* segregants (*SKI2*) were viable at 30°C and 8°C and were normal killers. The identical disruption mutant made in strain 3221 (L-A-o M-o) showed no cold sensitivity or other growth phenotype, consistent with earlier data on chemically induced *ski2* mutants (54) and deletions of *SKI8* and *SKI3* (52, 63). Thus, the *SKI2* gene is essential for cell growth at 8°C only in the presence of the M_1 replicon.

Ski2p is homologous to helicases and nucleolar proteins. The deduced amino acid sequence of Ski2p was compared with the data base with the BLAST3 program (1). Ski2p has a glycine-arginine-rich region with homology to several nucleolar proteins (Fig. 3A) such as nucleolin (*NSR1* product in *S. cerevisiae* [11, 39, 41]), fibrillarin (*NOP1* product in *S. cerevisiae* [30, 61]), and the products of *GAR1* (23), *SSB1* (36), and *NOP3* (58). This type of sequence has also been identified as an RNA-binding motif (37). Further analyses revealed that Ski2p possesses all six amino acid motifs which are shared by the superfamily of DNA and RNA helicases (Fig. 3B) (24, 25, 31). All of the described motifs are present in the correct order and with the proper spacing.

***SKI2* acts on L-A, L-BC, and 20S RNA but not on Ty1.** Since the *SKI* system limits the copy number of L-A and its satellites, as well as of the unrelated replicons L-BC and 20S RNA (3, 17, 44, 54, 65), we tested its action on Ty1 retrovirus transposition with plasmids pBDG207 and pBDG208 (22), in which transcription of a *HIS3*-marked Ty1 element is controlled by the *GAL1* promoter. His$^+$ clones that have lost the plasmid (marked with *URA3*) are those in which a transposition has occurred. There was no effect of a *ski2-2* mutation on the frequency of this event (Table 3).

TABLE 3. *ski2-2* has no effect on Ty1 transposition[a]

Genotype	Host strain	Ty1 plasmid[b]	No. of colonies that are His$^+$ Ura$^-$/total no. of Ura$^-$ colonies on:	
			Galactose	Glucose
ski2-2	4169-4B	BDG207	20/60	0/50
		BDG208	12/55	0/59
SKI	4169-4C	BDG207	15/51	
		BDG208	10/49	
ski2-2	4169-7B	BDG207	17/66	
		BDG208	3/31	
SKI	4169-11A	BDG207	15/43	
		BDG208	6/38	
ski2-2	4169-11C	BDG207	11/50	0/40
		BDG208	6/42	0/40

[a] Plasmids BDG207 and BDG208 (22) each carry a Ty1 containing the *HIS3* gene. The Ty1 *HIS3* is under the control of the *GAL1* promoter. The plasmids also carry the *URA3* gene. Transposition was monitored as described previously (22); briefly, after a period of growth on galactose to induce transcription of Ty1, cells were plated on glucose media and colonies that had lost the plasmid (Ura$^-$) and remained His$^+$ were scored as transpositions.

[b] BDG207 = pGTyH3 (*HIS3* gene in same orientation as the Ty transcript); BDG208 = pGTyH3 (*HIS3* gene in orientation opposite to that of the Ty transcript).

***SKI2* regulation of M_1 and X copy number depends on their being supported by the L-A virus.** When M_1 or X dsRNAs are maintained by the L-A helper virus, their copy number is derepressed 5- to 10-fold by a *ski* mutation (17, 54, 65). In

A.

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                    ****   ******      *** *         ******
SKI2        SAKGAPSKTDNGRGGSTARGGRGGSNTRDGRGGRGNSTRGGAN RGGSRGAGAIGSN   599
SSB1        TPGQMQRGGFRGRGGFRGRGGFRGGFRGGYRGGFRGRGNFRGRAAPEVVSMDKKGK   174
NOP1        RPGSRGGSRGGSRGGFGGRGGSRGGARGGSRGGFGGRGGSRGGARGGSRGGFGGRG    59
GAR1        GRGGASMGRGGSRGGFRGGRGGSSFRGGRGGSSFRGGSRGGSF RGGSRGGSRGGFR   201
Nucleolin   KGEGGFGGRGGGRGGFGGRGGGRGGRGGFGGRGRGGFGGRGGF RGGRGGGGDFKPQ   700
FibrillarinRGGFGDRGGRGGRGGFGGGRGRGGGFRGRGRGGGGGGGGGGGGGRGGGGFHSGGNR    70
NSR1        GGSRGFGGRGGGRGGNRGFGGRGGARGGRGGFRPSGSGANTAPLGRSRNTASFAGS   409
HSV         SPSQGTDGPARGGGSGGGRGPGGGRGGPRGSRGRGGRGRGGRGGGRRGRQGGPGP   332
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B.

	domain I	domain II	domain III
Consensus for "Helicases"	..A..G.GKT.. G S	..hhhhDE.H.. AD	..hhhhSAThP.. T
SKI2	349VAAHTSAGKTVV	440 FVIFDEVHYV	471 FILLSATVP

	domain IV	domain V	domain VI
Consensus for "Helicases"	..F..S.. Y T	...TDh..RG..	..HR.GR.GR Q
SKI2	497 YVIS	552TDNG-RG	570 TRDGRGGR
		560TARGGRG	

FIG. 3. Homology of Ski2p to nucleolar proteins and helicases. (A) Homology of the glycine-arginine-rich region of the *SKI2*-encoded protein with those of nucleolar proteins. Nucleolar proteins are characterized by a glycine-arginine-rich region (23). *SSB1*, *NOP1*, *GAR1*, and *NSR1* are *S. cerevisiae* genes. HSV is the herpes simplex virus nuclear latency-related protein. (B) Helicase consensus sequence patterns in *SKI2*. A superfamily of proteins, including several well-characterized DNA and RNA helicases, has been described previously (24, 25, 31). There are six characteristic amino acid sequence patterns, designated I to VI from NH_2 to the COOH end of the proteins. These patterns are found in *SKI2*, in the correct order, between residues 350 and 580. h, hydrophobic residue; a period indicates any amino acid.

order to narrow the possible sites of action of the *SKI* system, we tested the effect of a *ski2*::*HIS3* disruption on the copy number of X (Fig. 4B, lanes 3 and 4) or M_1 (Fig. 4A [all lanes] and B [lanes 1 and 2]) when these satellites were maintained by the L-A cDNA clone, pI2L2 (73), in the absence of the L-A virus. We found (Fig. 4B) that the copy number of X dsRNA is unchanged by the *ski2* mutation (compare lanes 3 and 4) and that that of M_1 dsRNA is actually decreased (Fig. 4A and B). Note that, as an internal control, in Fig. 4B, L-BC copy number is elevated in the *ski2* host (lanes 2 and 4) compared with the *SKI*$^+$ host (lanes 1 and 3) while X copy number is unchanged and M_1 copy number falls. Similarly, in Fig. 4A, in the absence of L-A (lanes 3 to 8), L-BC copy number is elevated in the *ski2* host (lanes 5 to 8) compared with the *SKI*$^+$ host (lanes 3 and 4).

If Ski2p were acting to block viral transcription, replication, or packaging or were directly attacking the viral particles or the free M_1 or X (+) ssRNA, it should have lowered M_1 and X copy number even when they were supported by the L-A cDNA clone. This is because M_1 (+) ssRNA is an intermediate in the replication cycle. Its destruction should lower virus copy number. This suggests that the effects of the *SKI* system on M_1 or X copy number are via their effects on expression of L-A-encoded proteins, but only when these are expressed from the virus and not when they are expressed from the cDNA clone.

Western blot analysis of L-A coat proteins showed that there was no effect of the *ski2* mutation on the amount of coat proteins produced from the L-A cDNA clone (data not shown), but there was an approximately fivefold increase in *ski2* mutants when the L-A virus was the source of the mRNA (67a).

Toxin production from M_1 dsRNA is inhibited by *SKI2*, but toxin production from M_1 cDNA clones is not. We compared the K_1 killer toxin production of isogenic *SKI*$^+$ and *ski2*::*HIS3* strains (Table 4). When M_1 was maintained by the L-A virus, the toxin produced was increased in the *ski2*::*HIS3* strain, as expected (65). When M_1 was supported by the L-A cDNA clone, even though the copy number of

M_1 was decreased in the *ski2*::*HIS3* strain, the toxin produced was increased.

To test whether this result could be an effect of the *SKI* system on the processing or secretion of the K_1 killer toxin, we tested the effect of the *ski2*::*HIS3* mutation on toxin production from either of two cDNA clones of M_1: pP-T_{316}, in which the preprotoxin gene is controlled by the *PGK1* promoter (74), or pVT100-U/KT, in which its transcription is driven by the *ADH1* promoter (68). In each case, there was no detectable effect of the *ski2* mutation (Table 4), confirming the earlier results of Hougan et al. (33) with a *ski3* mutation.

Thus, Ski2p inhibits expression of toxin from the M_1 virus but not from the M_1 cDNA clone and does not appear to act on the processing or secretion steps. If Ski2p were acting on M_1 transcription, replication, or packaging or directly attacking the M_1 (+) ssRNA, then it would be expected to lower M_1 dsRNA copy number, since each of these is a step in the viral replication cycle. This suggests that Ski2p blocks M_1 (+) ssRNA translation.

***SKI2* inhibits expression of β-galactosidase from a Pol I promoter but not from Pol II promoters.** The above results suggested that the *SKI* system specifically detects viral transcripts and blocks their expression. Since L-A and M transcripts are uncapped (12) and L-BC and 20S RNA replicate in the cytoplasm and are probably likewise uncapped, we tested whether the expression of the uncapped transcripts made by RNA polymerase I (38, 50) from an rDNA promoter was affected by a *ski2*::*HIS3* mutation (Table 5). We examined six independent *ski2*::*HIS3* disruptants, testing four transformants of each with the rDNA-*lacZ* plasmid. We found a 2.5- to 4.3-fold increase in expression of β-galactosidase in the *ski2*::*HIS3* mutant hosts for the rDNA promoter-*lacZ* construct, but no effect for a *CYC1* promoter-*lacZ* construct (1.07-fold) or for an *RPL16A* promoter-*lacZ* construct (0.8-fold) (Table 5). We confirmed that the effect on expression was due to the *ski2* mutation by showing that they cosegregate in five meiotic tetrads (data not shown). The effect was seen whether cells were harvested early or late in log phase. This effect on expression of Pol I transcripts is similar in magnitude to the two- to fivefold effect on L-A, L-BC, or 20S RNA copy number seen in *ski* mutants (3, 44).

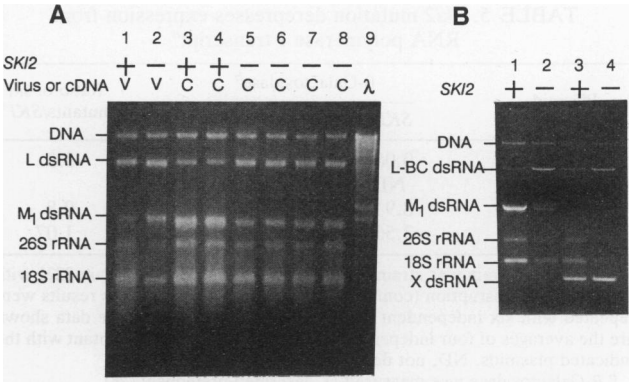

FIG. 4. The *SKI* system does not repress M_1 or X dsRNA copy number when these replicons are supported by an L-A cDNA clone (C) but does repress them when they are maintained by the L-A virus (V). dsRNA was extracted (19), and equal amounts of nucleic acid were analyzed by electrophoresis on a 1.3% agarose gel in Tris-borate-EDTA buffer (43) and stained with ethidium bromide. Similar results were obtained when cells were lysed by being vortexed with glass beads in the presence of sodium dodecyl sulfate and phenol-chloroform (not shown). Each strain was isogenic with strain 3221. In panel B, all samples were from cells lacking the L-A virus. The cells for panel B, lanes 1 and 2, have M_1 dsRNA, and those for lanes 3 and 4 have X dsRNA. The 5- to 10-fold increase of M_1 and X dsRNAs in *ski2* mutants with L-A virus has been previously shown (3, 17, 54, 65). Only in panel A, lanes 1 and 2, is L-A dsRNA present. In all other samples, the only L-sized dsRNA is L-BC. Panel A, lane 9, has λ *Hin*dIII fragments.

DISCUSSION

Ski2p works on cytoplasmic RNA replicons, not on Ty1. The products of the *SKI* genes lower the copy numbers of several apparently unrelated RNA replicons: the L-A dsRNA virus and its satellites M and X (3, 17, 65), the L-BC dsRNA virus (3), and the 20S ssRNA replicon (44), whose dsRNA replicative intermediate is called W dsRNA (46, 55, 69). All of these RNA replicons are found in cytoplasmic fractions, where they would not have access to the cellular capping apparatus, and there is evidence that L-A and M are, in fact, uncapped (12). We show here that transposition of Ty1, mediated by capped RNA polymerase II transcripts, is not affected by a *ski2* mutation.

TABLE 4. *SKI2* effects on killer toxin production and satellite RNA copy number[a]

Source of *gag* and *gag-pol*	Satellite dsRNA replicon	Source of toxin	*SKI*$^+$ host[b]		*ski2*::*HIS3* host[b]	
			Copy no.[c]	Toxin[d]	Copy no.[c]	Toxin[d]
L-A virus	M_1	M_1 dsRNA	+	+	++	+++
L-A cDNA clone	M_1	M_1 dsRNA	+++	++	++	+++
L-A virus	X		+		++	
L-A cDNA clone	X		++		++	
None		M_1 cDNA clone (from D. Thomas) with *ADH1* promoter		++++		++++
None		M_1 cDNA clone (from D. Tipper) with *PGK1* promoter		+		+

[a] All comparisons were done with derivatives of strain 3221. M_1 dsRNA or X dsRNA was supported by either L-A-HNB virus or clone pI2L2, the cDNA clone of L-A-HNB.

[b] + to ++++ represents lowest to highest copy number or level of toxin.

[c] Copy number data are from Fig. 4.

[d] Killer toxin production was measured qualitatively by spotting freshly grown cells of each strain on a 4.7MB plate freshly seeded with a lawn of the sensitive strain 5 × 47. Killer zones were observed after 2 days of incubation at 20°C.

TABLE 5. *ski2* mutation derepresses expression from RNA polymerase I transcript[a]

Plasmid	β-Galactosidase[b]		Ratio of *ski* mutants/*SKI*$^+$
	SKI$^+$	*ski2::HIS3*	
rDNA-*lacZ*	0.064	0.21	3.3
YEp357[c]	ND	ND	
RPL16A-lacZ[d]	8.9	7.0	0.8
CYC1-lacZ[e]	3.56	3.82	1.07

[a] The *SKI*$^+$ strain was strain 3221, and the *ski2* strain was strain 3221 with the *ski2::HIS3* disruption (confirmed by Southern blotting). The results were repeated with six independent disruptants (not shown), and the data shown are the averages of four independent transformants of one disruptant with the indicated plasmids. ND, not detectable (<0.01).

[b] β-Galactosidase was measured as described previously (26).

[c] YEp357 is the *lacZ*-containing vector without any yeast promoter used to construct the rDNA-*lacZ* fusion plasmid (49).

[d] The CEN-ARS plasmid p1083 containing the promoter of the *RPL16A* gene for ribosomal protein L16 linked to *lacZ* (35a, 57).

[e] The plasmid pLG699Z in which the *CYC1* promoter is linked to *lacZ* (26).

Ski2p action localized to viral gene expression. While Ski2p represses M_1 and X copy number when they are supported by the L-A virus, we find that it does not do so when they are supported by an L-A cDNA clone. If the Ski2p were acting primarily on the viral replication or transcription steps, or by directly attacking the viral (+) ssRNA or viral particle itself, it would be expected to lower M_1 and X copy number even when these replicons are supported by the L-A cDNA clone. We therefore propose that the *SKI* system affects expression of L-A virus-encoded message, but not that of nuclear (L-A cDNA clone) message (Fig. 5).

Although the M_1 copy number is not elevated by a *ski2* mutation when M_1 is supported by the L-A cDNA clone, the secreted killer toxin is increased. That this is not an effect of the *SKI* system on the processing or secretion of preprotoxin is shown by the fact that a *ski3* mutation (33) or a *ski2* mutation (this work) has no effect on toxin production from an M_1 cDNA clone. This result again points to expression from viral message as the site at which the *SKI* system acts. Supporting M_1 from an L-A cDNA clone also mimics the effects of a *ski* mutation (73), with cells showing a superkiller phenotype and suppression of the normal requirement for several *MAK* gene products for M_1 propagation. All of these observations point to the *SKI* system distinguishing between message produced by a cytoplasmic RNA replicon and that produced by a nuclear cDNA clone (Fig. 5).

Does the *SKI* system block translation of uncapped mRNA? The ability of the *SKI* system to repress copy number of several unrelated RNA replicons suggests that, rather than

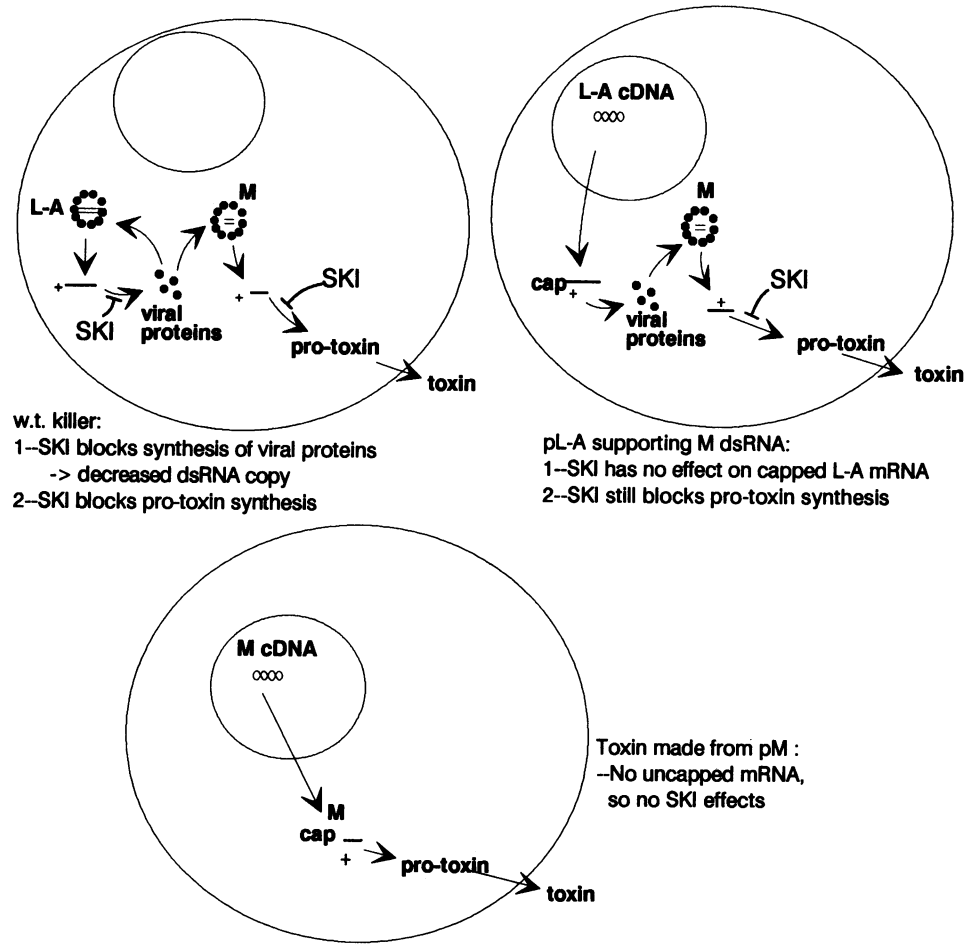

FIG. 5. Translational model of *SKI* action. The *SKI* system blocks translation of unmodified mRNA. The modification detected by the *SKI* system could be the 5′ cap, the 3′ poly(A), or some other difference between viral and cellular mRNAs.

its recognizing some sequence feature of all of these messages, a modification of cellular messages not found in viral messages is the explanation. L-A mRNA binds to poly(U) Sepharose (8), although genomic (+) strands are not polyadenylated (64), and M_1 (+) strands have an encoded poly(A) (8, 19, 27), so that this is less likely to be the differentiating signal. We suggest that the *SKI* system limits translation of uncapped mRNAs (Fig. 5).

In support of the suggestion of a translational site of action and RNA modification as the feature recognized, we show that the *SKI* system represses the expression of β-galactosidase from an RNA polymerase I promoter, the rDNA promoter, which directs the synthesis of uncapped, unpolyadenylated transcripts (38, 50). No such repression could be detected when β-galactosidase was made from either of two RNA polymerase II promoters. The 5′ cap is necessary for stability of RNA and its transport from the nucleus, as well as for its translation, and so the Ski2p effect on expression of β-galactosidase from an RNA polymerase I promoter could be at a step before translation, but that would not explain its effect on the various cytoplasmic RNA replicons. The magnitude of the effect of Ski2p on rDNA-promoted β-galactosidase production (two- to fivefold) is similar to the magnitude of its effect on L-A, L-BC, or 20S RNA copy number.

Can this hypothesis explain why the copy number of M_1, supported by the L-A cDNA clone, is lower in a *ski2* host? Possibly the increased translation of M_1 (+) ssRNA in the *ski2* cell interferes with its packaging, resulting in lower dsRNA copy number. Note that X dsRNA, which is probably not translated (17), is not so affected (Fig. 4B).

Poliovirus mRNA (free [plus] strand) is uncapped (2), and the poliovirus 2A protease leads to cleavage of one component (p220) of the cap-binding protein complex, thereby specifically turning off host translation (5, 42). Our hypothesis for the mechanism of action of the *SKI* products is the converse of this: the host defends itself from viruses by recognizing their transcripts as viral or nonself by the absence of the 5′ cap or 3′ poly(A) structure and limiting their translation.

Another finding relating RNA caps and L-A is that of Blanc et al. (6), who recently showed that cap structures become covalently linked to *gag* in vitro. The in vivo role of this phenomenon and its relation to the *SKI* system are as yet unclear.

How is the structure of Ski2p related to its function? We have shown that *SKI2* encodes a large protein, one section of which has all the motifs characteristic of helicases and of nucleolar ribonuclear proteins. Ski3p is a nuclear protein (52), but our efforts to localize Ski2p were frustrated by the low levels of protein in wild-type cells. The relation of the structural features of Ski2p to its function is not yet clear, but if it proves to be a nucleolar protein as suggested by the presence of a glycine-arginine-rich domain, it might affect translational specificity by altering ribosomal structure. Confirmation of our hypothesis will require using in vitro translation and methods for transient expression of RNA (18, 59).

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