A New Non-Mendelian Genetic Element of Yeast That Increases Cytopathology Produced by M_1 Double-Stranded RNA in *ski* Strains

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ABSTRACT

The *Saccharomyces cerevisiae SKI* (superkiller) genes are repressors of replication of M, L-A, and L-BC double-stranded (ds) RNAs; *ski* strains have an increased M dsRNA copy number and, as a result, are cold-sensitive for growth at 8°. Growth is normal, however, at higher temperatures. We have found a new cytoplasmic genetic element [D] (for *d*isease) that makes M_1 dsRNA-containing superkiller strains grow slowly at 30°, not at all at 37°, and only very poorly at 20°. These growth defects require three factors: a chromosomal *ski* mutation, the presence of M_1 dsRNA, and the presence of the new cytoplasmic factor, [D]. We have isolated mutants unable to *ma*intain [*D*] (*mad*), at least one of which is due to mutation of a single chromosomal locus. Further, [D] can be cured by growth at 37–39°. We present evidence that [D] is not M, L-A, L-BC or W dsRNAs or mitochondrial DNA, 2μ DNA, or [psi], but [D] depends on L-A for its maintenance. We also show that [D] is distinct from [B], a cytoplasmic element that allows M_1 dsRNA to be stably replicated and maintained in spite of defects in certain chromosomal *MAK* genes that would otherwise be necessary. [D] activity is blocked by the presence of another extrachromosomal element, called [DIN] (for [*D*] *in*terference). [D] and [DIN] may be different natural variants of the same molecule.

THERE are five families of dsRNA in strains of *Saccharomyces cerevisiae*: L-A, L-BC, M, T, and W (Table 1) (BEVAN, HERRING and MITCHELL 1973; VODKIN, KATTERMAN and FINK 1974; SOMMER and WICKNER 1982; WESOLOWSKI and WICKNER 1984). Of these, L-A, L-BC, and M are found in intracellular virus-like particles (VLPs) (HERRING and BEVAN 1974; SOMMER and WICKNER 1982). M encodes the precursor to a secreted protein toxin and immunity to that toxin, L-A encodes the major protein of the VLPs that separately encapsidate itself and M, while L-BC encodes its own VLP major coat protein (HOPPER *et al.* 1977; BOSTIAN *et al.* 1980; SOMMER and WICKNER 1982; EL-SHERBEINI *et al.* 1984; BOONE *et al.* 1986). Natural variants of M (M_1, M_2, etc.) determine distinct toxin-immunity specificities (*e.g.*, NAUMOV and NAUMOVA 1973; HARA, IIMURA OTSUKA 1983), while natural variants of L-A show varying interactions with M_1, M_2, and each other (WICKNER 1980; WICKNER and TOH-E 1982; SOMMER and WICKNER 1982; WICKNER 1983; HANNIG, LEIBOWITZ and WICKNER 1985). Structural variants of L-BC (L-B, L-C, etc.) are also known (SOMMER and WICKNER 1982). The dsRNAs and killer systems of *S. cerevisiae* have been reviewed recently (WICKNER 1986; WICKNER *et al.* 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 super*ki*ller phenotype of the mutants (TOH-E, GUERRY and WICKNER 1978; BALL, TIRTIAUX and WICKNER 1984). The high copy number of an M dsRNA replicon resulting from a *ski* mutation has been shown to result in cell death, specifically at 8° (RIDLEY, SOMMER and WICKNER 1984). This cold sensitivity is not a consequence of excess production of toxin or immunity protein since deletion derivatives of M_1 dsRNA (called S dsRNAs) lacking nearly the entire preprotoxin immunity protein coding region make *ski* mutants just as cold-sensitive as does the parent M_1 dsRNA. Nor is the cold sensitivity due to the total load of dsRNA or VLPs since elimination, by curing, 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, TIRTIAUX and WICKNER 1984). The resulting *ski* L-A M-o strain is, however, not detectably cold-sensitive (RIDLEY, SOMMER and WICKNER 1984).

We describe here a new non-Mendelian (cytoplasmic) genetic element ([D]) that slows or prevents cell growth of *ski* M_1 strains at any temperature, not only at 8°. [D] appears to be distinct from the previously defined nonchromosomal genetic entities, including those related and unrelated to the killer system.

MATERIALS AND METHODS

Strains and media: Some of the strains of *S. cerevisiae* used in this study are listed in Table 2. The nomenclature for the killer system is given in Table 1. Media were as previously described by WICKNER (1978).

TABLE 1

Components of the *S. cerevisiae* killer systems

dsRNAs	
M_1	A 1.8-kb dsRNA that encodes the K_1 preprotoxin-immunity protein. Secretion of an active K_1 toxin is the K_1 phenotype
M_2	A 1.5-kb dsRNA that codes for a second toxin that kills M-o- and M_1-containing cells
L-A	A 4.5-kb dsRNA that is noninfectious and stably maintained like a plasmid, but encapsidated like a virus. It encodes its major capsid protein (81 kD). The genes [EXL], [NEX], and [HOK] (see below) are present in various combinations on various forms of L-A, denoted L-A-E ([EXL] alone), L-A-HN ([HOK] and [NEX]; found in all K_1 killers), L-A-HE ([HOK] and [EXL]), or L-A-H ([HOK] only). L-A depends on MAK3, MAK10, and PET18 for replication
L-B and L-C	These are 4.5-kb dsRNAs unrelated to L-A and present in VLPs with different major proteins. L-B and L-A or L-C and L-A are compatible. L-B and L-C show some sequence homology. L-BC means an L dsRNA related to L-B and L-C
T and W	These are 2.7-kb and 2.25-kb minor dsRNAs. They do not cross-hybridize with each other, other dsRNAs, or cell DNA. They are cytoplasmically inherited. The copy number of T and W is induced 10-fold by growth at 37° in some strains
Cytoplasmic genes	
[HOK] or H	*h*elper *of k*iller. This non-Mendelian gene supplies the helper function needed by M_1 or M_2 for replication in a wild type strain. It is located on certain forms of L-A dsRNA, namely, L-A-HN, L-A-HE, or L-A-H. To test for [HOK], the combination of L-A-E + M_1 is introduced from a *ski2* strain into the *SKI* M-o strain to be tested. L-A-E cannot support M_1 replication in a *SKI* host, but if [HOK] is present, M_1 will be maintained
[EXL] or E	*excl*uder of M (or M_1) dsRNA. This non-Mendelian trait prevents the replication of M_2 (or M_1) if [NEX] is absent, but not if [NEX] is present. [EXL] is located on certain forms of L-A, namely, L-A-E and L-A-HE. L-A-E acts by lowering the copy number of the L-A-H in the M_2 (or M_1) strain
[NEX] or N	M_2 (or M_1) *n*on*ex*cludable by [EXL], but does not prevent exclusion of M_2 by M_1. [NEX] is located on L-A-HN, the form of L-A found in wild type K_1 killer strains. L-A-HN also excludes M_2 in *mkt* strains, but not in *ski mkt* strains
[B]	A cytoplasmic gene that allows M_1 to dispense with certain of the *MAK* gene products
[D]	The introduction of [D]-carrying cytoplasm into *ski* mutants that contain M_1 dsRNA results in cells with a growth defect at 37° (temperature-sensitive) and at 20° (cold-sensitive). Cytoductants cannot grow at all at 37° and only poorly at 20°. These growth defects require three factors: (1) a chromosomal *ski* mutation, (2) the presence of M_1 dsRNA and (3) the presence of [D]
MAK	*ma*intenance of killer. *MAK* genes comprise at least 32 chromosomal genes necessary to maintain M_1 dsRNA. Mutants carrying the recessive alleles, *mak*, are K^-R^- M-o. At least some of the genes are also required for the maintenance of M_2. *MAK3*, *MAK10*, and *PET18* are needed by all forms of L-A
clo	A complex chromosomal defect resulting in loss of L-B or L-C
SKI	*super*killer. Mutants carrying the recessive alleles of *ski2*, *ski3*, *ski4*, *ski6*, *ski7*, or *ski8* produce more killer toxin and have increased copy number of M_1, M_2, L-A, and L-(BC). *ski* mutations eliminate the need for some of the *MAK* genes and prevent the exclusion of M_2 by L-A-HN in *mkt* strains. The *ski5* mutant has none of these traits and lacks a cell surface protease that normally degrades the toxin
MKT	*m*aintenance of [KIL-k_2] in the presence of [NEX]. Strains having a recessive allele, *mkt* (about 80% of laboratory strains), cannot maintain [KIL-k_2] at 30° if [NEX] (L-A-HN) is present.

Genetic analysis: Matings were carried out on unbuffered YPAD which, because its pH is about 6, inactivates the killer toxin (WOODS and BEVAN 1968; YOUNG and YAGIU 1978). Diploids were isolated by using the complementary nutritional requirements of their parents. Sporulation and dissection were by the usual methods [see MORTIMER and HAWTHORNE (1975) for references].

Cytoduction: A cytoplasmic genome can be transferred from one haploid strain to another without diploidization or other change in nuclear genotype by heterokaryon formation, using a *kar1* mutant that is defective in nuclear fusion (CONDE and FINK 1976). Usually recipient cells were ρ^0 (mitochondrial DNA eliminated by growth in the presence of ethidium bromide), and donor cells were ρ^+. In other cases, K^- strains were used as recipients and K^+ strains as donors. The procedure was the same as described by RIDLEY, SOMMER and WICKNER (1984). "A → B" refers to either the experiment of transferring cytoplasm from strain A to strain B by a cross using the *kar1* mutant (a cytoduction experiment) or to the cytoductants—strains with the same nuclear genotype as strain B but having received cytoplasm from strain A, as shown by acquisition of ρ^+ or K_1^+.

Assay of killing activity: Colonies to be tested for killing activity were replica-plated to MB medium that had recently been seeded with a lawn of the sensitive strain 5X47 (WICKNER 1978) (0.5 ml of a suspension with an optical density at 650 nm of 1), spread on each plate, and allowed to dry. The MB plates were incubated at 20° for 2–3 days. Killing was indicated by a clear zone surrounding the killer strain and surrounded in turn by growth of the lawn of sensitive cells.

[HOK] test: The presence or absence of L-A is conveniently tested by mating K^- clones to be tested with strains 1407 or 1408 (*ski2-2* L-A-E M_1). If the diploids formed are stable killers, then [HOK] is present and thus L-A is present (WICKNER and TOH-E 1982; SOMMER and WICKNER 1982; RIDLEY, SOMMER and WICKNER 1984).

dsRNA preparation: Different dsRNAs (L-A, L-BC, W, and M_1) were purified by cellulose chromatography as previously described (SOMMER and WICKNER 1982). To analyze dsRNA in cells, the rapid method for extraction described by FRIED and FINK (1978) was used, and the extracted dsRNAs were analyzed on 1.5% agarose gels. Northern blot hybridization was carried out as described by THOMAS (1980).

RESULTS

The disease phenomenon: Strain 1368 carries a cytoplasmically determined superkiller phenotype and

TABLE 2

Strains of *S. cerevisiae* used

Designation	Genotype	Source or reference
1368*	α *his4 kar1-1* L-BC L-A-HN M_1 [D]	P24-28C → 1020 (TOH-E and WICKNER 1980)
2404* [a]	α *his4 kar1-1* L-BC L-A-HN M-o [D]	1368 Δ K^-
2405*	α *his4 kar1-1* L-BC L-A-o M-o [D-o]	1368 Δ K^- H^-
1101*	α *his4 kar1-1* L-BC L-A-HN M_1 [D-o]	A364A → 1020
2507*	a *arg1-1 ski2-2* L-BC L-A-o M-o [D-o]	
RE23	a *arg1-1 ski2-2* L-BC L-A-HN M_1 [D-o]	1101 → 2507
RE24	a *arg1-1 ski2-2* L-BC L-A-HN M_1 [D]	1368 → 2507
RE123	α *his4 leu1 kar1-1 clo* L-BC-o (at 25°) L-A-o M_1-o [D-o]	This work
RE327	α *his4 kar1-1 leu1 clo* L-BC-o (at 25°) L-A-HN M_1	RE297 → RE123
RE329	α *his4 kar1-1* L-BC L-A-HN M_1^+ [D]	RE297 → RE121
RE297	a *arg1-1* L-BC L-A-HN M_1 [D]	This work
295	a *his4* L-BC L-A-HN M_1 [D]	This work
1747	a *arg9 mak10-1* L-o M-o [D-o]	MITCHELL, HERRING and BEVAN (1976)
RE57	α *arg1-1 kar1-1 ski2-2 mkt1-1* M-o [D-o]	Segregant from cross 1368 × 2507
1716	a *aro7 tyr1 leu2 can1 mak3-1* L-BC L-A-o M-o	This work
TF78-4A	a *ura1 leu1 can1 mak10*^{us}	T. FUJIMURA
1868	a *leu2 cdc16 mak11-1* [B-o] [D-o] [DIN] M-o	TOH-E and WICKNER (1980)
RE59	a *arg1-1 ski2-2* L-BC L-A-HN M-o [D]	2404 → 2507 ρ^o
2375	a *ura3 mkt1 ski4-1* Δ K^- H^-	
2-13	a *arg1-1 ski2-2 mad* [D-o] L-A-HN M_1	Mutants obtained from strain RE24 by EMS treatment
2-15	a *arg1-1 ski2-2 mad* [D-o] L-A-HN M_1	
2-16	a *arg1-1 ski2-2 mad* [D-o] L-A-HN M_1	
2-21	a *arg1-1 ski2-2 mad* [D-o] L-A-HN M_1	
2-24	a *arg1-1 ski2-2 mad* [D-o] L-A-HN M_1	
1405*	a *ade1 ski2-2* L-A-E M_1	
2530*	a *ade1 pep4 ski2-2* L-A-HN M_1	
2523*	a *ade1 pep4 ski2-2* L-A-HN M_1	
1407	α *thr1 ski2-2* L-A-E M_1	
1408	a *thr1 ski2-2* L-A-E M_1	
2265	α *ura3 leu2 mkt1 ski2-2* L-A-HN M_2 (at 30°)	
2267	a *ura3 leu2 mkt1 ski2-2* L-A-HN M_2 (at 30°)	
2601	a *ura3 his5* L-A-o M-o	

* Asterisks indicate that not only that strain, but also ρ^o derivatives obtained by growing the cells on ethidium bromide-containing plates, were used.

[a] The absence of M_1 dsRNA or derivatives thereof from strain 2404 was checked by agarose gel electrophoresis of dsRNA purified from strain 2404 by CF11 column chromatography as well as of dsRNA purified from VLPs from strain 2404.

a cytoplasmically determined ability to suppress M_1's requirement for several *MAK* gene products (TOH-E and WICKNER 1980). Since this strain's M_1 copy number is also 4-fold higher than other killer strains (BALL, TIRTIAUX and WICKNER 1984), its cytoplasm was introduced into strain 2507 (*ski2-2* L-BC L-A-o M-o) in order to produce a strain with extra-high M_1 copy number for biochemical studies (ESTEBAN and WICKNER 1986). All of the K_1^+ cytoductants grew very slowly at 30°, requiring 3–4 days to make tiny colonies. Subcloning of these cytoductants on YPAD at 30° produced large and small subclones from each cytoductant (Figure 1). The large subclones remained large on subcloning, while the small subclones continued to segregate large subclones for one or two further subclonings, but eventually stabilized to give very slow-growing tiny subclones. Both large and small subclones of 1368 → 2507 were unable to grow at 37° and grew only poorly at 20°. Even the large subclones did not grow at normal rates at 30° and formed noticeably smaller colonies than wild-type strains. The severity of this phenomenon clearly depends on some mitotically segregating factor.

Disease is due to a non-Mendelian (cytoplasmic) element: 1368 → 2507 gave diseased cytoductants, but using the donor 1101, isogenic for nuclear markers with 1368, but with a killer cytoplasm derived from a different source, the cytoductants 1101 → 2507 were healthy (Table 3). To distinguish whether the difference between the cytoplasms of 1368 and 1101 was a difference in their M_1 dsRNAs or in some other element, we heat-cured M_1 from 1368 (= strain 2404) and introduced this cytoplasm into either the *ski2-2* L-A-o M-o strain 2507 or into the healthy cytoductants 1101 → 2507. Only the second cytoduction produced diseased cytoductants. Thus the combined cytoplasms of 1101 and 2404, when introduced together into the 2507 nuclear background, produced disease. Neither one alone suffices. As shown by cytoduction 5 in Table 3, the order of introduction of these two elements does not matter.

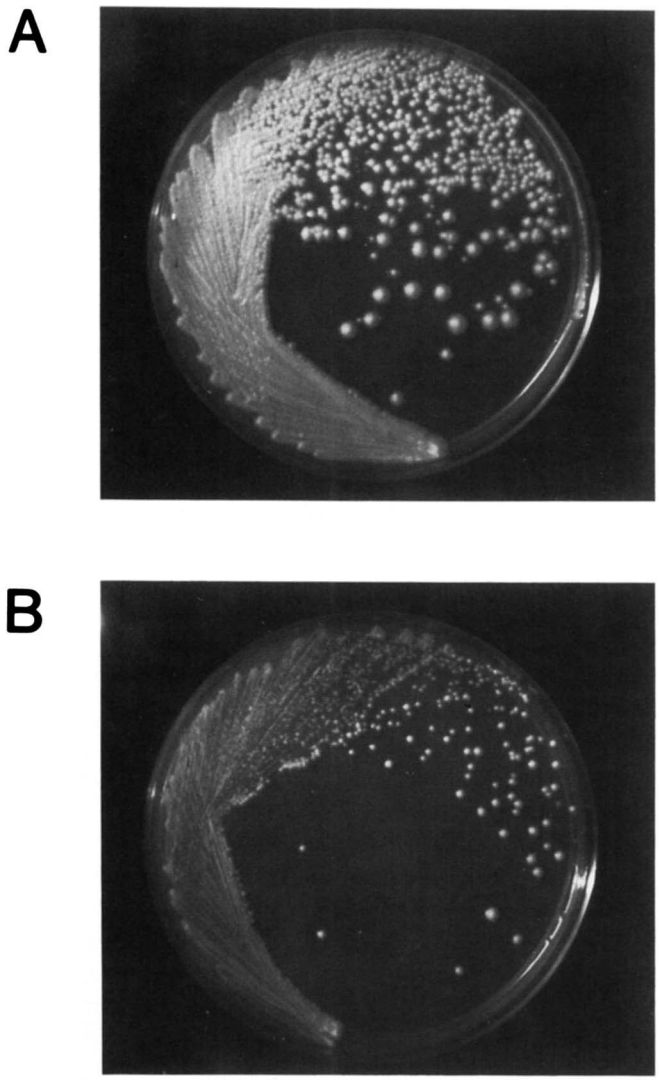

FIGURE 1.—Colony size of cytoductants 1368 → 2507 after subcloning and growth at 30° for 5 days on YPAD medium plates. (*A*) Mitotic segregation of tiny and almost normal growth rate colonies after subcloning each original cytoductant. (*B*) Pattern of growth of some of the diseased colonies that gave stable slow growing tiny subclones.

Strain 1368 has all the elements necessary to produce disease, and strain 2404 was derived from strain 1368 by heat-curing M_1 dsRNA (but not L-A dsRNA). Loss of M_1 was confirmed by Northern blot hybridization. That M_1 is in fact necessary for disease production was critically confirmed by selecting, from a diseased 1368 → 2507 cytoductant, 23 clones that had spontaneously acquired ability to grow at 37° and 23 clones that formed normal size colonies at 20°. All of these clones had become K^-. Thus selecting for loss of disease selects for loss of M_1, indicating that M_1 is necessary for the disease.

We will present evidence below that there are two cytoplasmic elements present in strain 2404 (*SKI* L-A-HN M-o) necessary (in addition to M_1) to produce disease; one, which we call [D] for *d*isease, is not located on L-A or M, while the other is L-A, which is necessary to maintain [D].

Disease expression requires a ski⁻ mutation: Tetrad analysis of the cross 1368 × 2507 showed that in each of 24 tetrads, two spore clones were healthy and two were diseased or dead (Figure 2 and Table 3). Of 31 healthy spore clones checked, all were *SKI*, while the six diseased spore clones tested were *ski2*. Thus disease requires a single nuclear mutation present in 2507 and closely linked to *ski2-2*, presumably *ski2-2* itself. Cytoduction and meiotic crosses using 1368 with several other *ski2-2* strains or a *ski4-1* strain gave similar results (Table 3). However, *ski2-11* and *ski3-11* did not become diseased (data not shown). The presence of [DIN] (see below) in the *ski2-11* and *ski3-11* strains has not, however, been ruled out.

The L-A in 2404 is necessary for the disease phenomenon: Strain 2404 (*α kar1* L-A-HN M-o [D]) was streaked for single colonies at 40°. Single colonies were tested for [HOK], an L-A marker, and [HOK^+] and [HOK-o] clones were tested for their ability to produce disease by cytoduction into (1101 → 2507) and by crosses with the same strain (Table 4). Each of the [HOK^+] clones had the ability to produce disease as judged by cytoduction or by meiotic crosses. In contrast, the [HOK-o] (*i.e.*, L-A-o) clones had lost this ability. Thus the L-A in strain 2404 is necessary to produce disease.

That the L-A of strain 2404 is not *sufficient* to produce disease when introduced into 2507 will be dealt with further below.

[DIN] is an extrachromosomal element that interferes with disease production: [DIN] is another extrachromosomal element present in some strains, for example, strain 1868. It is defined by its ability to eliminate disease-producing activity from a strain when it is introduced by cytoduction. The presence of [DIN] can be tested by two sequential cytoductions. (1868 → 1368) → 2507 results in only healthy cytoductants and (1868 → 1368) × 2507 gives 4 healthy:0 meiotic products (14 tetrads examined), whereas 1368 → 2507 produces diseased cytoductants and 2 healthy:2 sick meiotic products (see Table 3). Thus the cytoplasm of 1868 contains a *d*isease-*in*terference or [DIN] activity. [DIN] can be cured by growth of cells for 4–5 days at 40°C. [DIN] is present in many laboratory strains, but experiments in Table 3 show that [DIN] is not present in strains 1101, 1368, 2507, or 2404.

[D] requires chromosomal MAD gene(s) for its maintenance: We used the growth defects at 20° and 37° of *ski2-2* M_1 [D] strains to select mutants unable to maintain [D]. Strain (1368 → 2507) was mutagenized with 1% ethyl methane sulfonate in 0.1 M potassium phosphate, pH 7.6, and plated at 20° on YPAD. Fast-growing clones were picked, purified, and then

TABLE 3

A cytoplasmic gene controls "disease"

Cyto-duction	Donor	Recipient	Cytoductants		Meiotic analysis: No. of tetrads		Segregation[a]	
1	1368	→ 2507	All grow slowly at 30°		24[b]	D	3 tetrads	2 large:2 tiny
	SKI L-A-HN M_1	*ski2-2* L-A-o M-o	No growth at 37°, poor growth at 20°	= D			5 tetrads	2 large:1 tiny
							14 tetrads	2 large:0
							2 tetrads	1 large:1 tiny
2	1101	→ 2507	Healthy = H		16	H	14 tetrads	4 large:0
	SKI L-A-HN M_1	*ski2-2* L-A-o M-o					1 tetrad	3 large:0
							1 tetrad	2 large:0
3	2404 = 1368 Δ K^-	→ (1101 → 2507)	D		16	D	15 tetrads	2 large:0
	SKI L-A-HN M-o	ρ^o					1 tetrad	1 large:0
4	2404	→ 2507 ρ^o	H		15	H	12 tetrads	4 large:0
							3 tetrads	3 large:0
5	1101	→ (2404 → 2507)	D		14	D	13 tetrads	2 large:0
							1 tetrad	2 large:1 tiny
6	1368	→ 1405 (*ski2-2*)	D		15	D		
7	1368	→ 2530 (*ski2-2*)	D		16	D		
8	1368	→ 2523 (*ski2-2*)	D		17	D		
9	1368	→ 2375 (*ski4-1*)	D		15	D		

[a] The D pattern of meiotic segregation is 2 healthy:2 diseased or dead spore clones. The H pattern of meiotic segregation is 4 healthy spore clones:0 diseased or dead.

[b] Among the surviving spore clones of these 24 tetrads, 37 were checked for their *ski2* marker as follows. Spore clones were cured of M dsRNA by growth at 37–39°. The M-o clones were then mated with the *ski2-2 mkt1* M_2 L-A-HN strains 2265 and 2267. Diploids were selected, subcloned at 30°, and tested for the K_2 killer phenotype. Since both 1368 and 2507 are *mkt1*, all segregants are *mkt1*. Only *ski2* segregants will form diploids where M_2 is stable at 30° due to the *ski2* mutation's suppression of L-A-HN exclusion of M_2 in *mkt1* strains. All 31 healthy spore clones tested were found by this test to be *SKI*, while the 6 diseased spore clones tested were all *ski2*.

tested for K_1, growth at 20°, growth at 37°, and the acquisition of [DIN] activity (Table 4). All of these mutants were K_1^+, and all but one could grow at 37°. One showed slight [DIN] activity. That none of the mutants had lost L-A was shown by mating each mutant with a *SKI* L-A-o M-o tester strain. Since the diploids were stable killers in each case, the presence of L-A was confirmed (RIDLEY, SOMMER and WICKNER 1984). That each of these mutants had lost [D] was shown by cytoductions of the form (mutant → 1101) → 2507 and by crosses of the type (mutant → 1101) × 2507 (Table 5). The mutant's inability to maintain [D] when it was reintroduced from strain 1368 was tested by cytoductions of the type ((1368 → mutant) → 1101) → 2507. In each case, the final cytoductants were healthy, indicating that [D] was lost in the mutants. The control cytoduction ((1368 → parent) → 1101) → 2507 gave only diseased cytoductants, as expected. Thus we call these mutants *mad* (for *ma*intenance of [*D*]). This confirms that loss of a cytoplasmic element other than L-A results in loss of disease-producing activity.

The absence of [DIN] activity and the inability to maintain [D] when it is re-introduced suggest that the *mad* mutant defect is either chromosomal or at least nuclear-limited. We wanted to test the segregation of diploids of the type *ski2-2/ski2-2 mad/+* M_1 [D], expecting that the spore clones would directly show the effect of *mad* as 2 healthy:2 sick segregation. Unfortunately, all such diploids were so sick that they would not sporulate, and healthy K^- derivatives were rapidly selected. This result shows that *mad* is recessive to *MAD*. We therefore first analyzed *ski2-2/+ mad/+* M_1 [D] diploids (Table 6). These diploids all showed more healthy spores than the *ski2-2/+ +/+* M_1 [D] diploid formed by the mating 1368 × 2507, which gave 2 healthy:2 sick in almost all tetrads. With mutants 2-16 and 2-21, the most frequent tetrad class was the tetratype class expected if *ski2-2* and *mad* were unlinked, namely, 3 healthy:1 sick. For mutants 2-13, 2-15, and 2-24, the parental ditype class was the most frequent, suggesting linkage of these *mad* mutations with *ski2-2* or, conceivably, a non-Mendelian mutation with a nuclear-limited effect.

We further attempted to examine *mad* segregation using diploids of the type *ski2-2/ski2-2 mad/+* M-o [D-o]. Sporulation or germination was inadequate in the case of mutants 2-16 and 2-21. The results for mutant 2-15 are shown in Table 7. M_1 and D were introduced into each spore clone by cytoduction from strain 1368 or RE295 or by meiotic crosses with the same strains. If the cytoductants were healthy and/or the segregation showed more healthy spore clones than 2 healthy:2 sick, the original spore clone was judged to be *mad*. In each of the five tetrads examined, segregation seemed to be 2 *MAD*:2 *mad*, indicating that mutant 2-15 has a *mad* mutation in a single chromosomal gene.

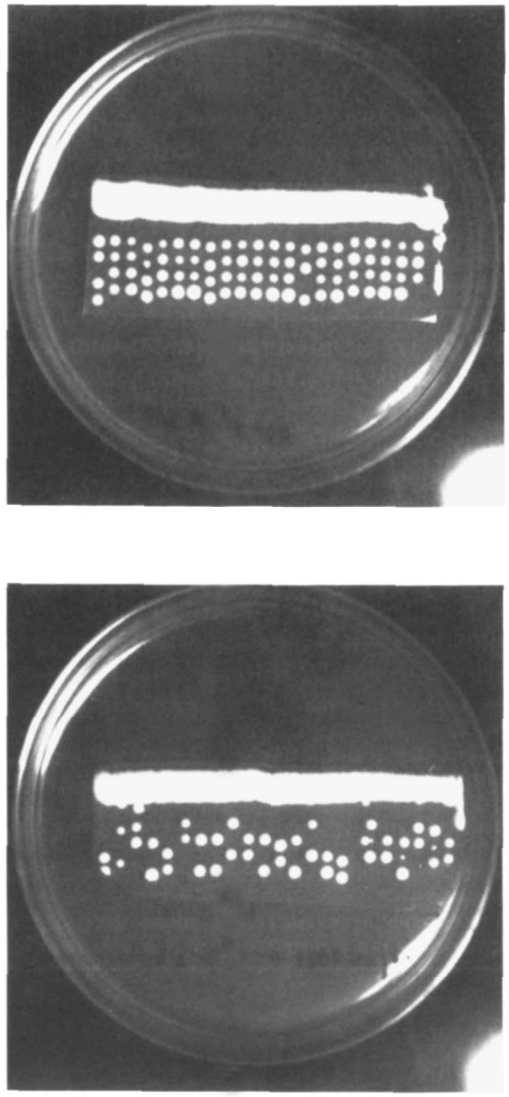

FIGURE 2.—Effect of [D] presence on the growth pattern of the 4 spore clones of each tetrad obtained by meiosis and sporulation of M_1 dsRNA-containing diploids heterozygous for the *ski2-2* mutation. (*A*) [D] is absent and all spore clones are healthy. (*B*) [D] is present. Only *SKI* segregants show normal growth rate. *ski2-2* segregants are unable to grow or they gave very slow growing tiny colonies. Parental strains used in both crosses were isogenic for nuclear markers.

The results of similar analysis of mutants 2-13 and 2-24 was less clearcut, and further studies will be necessary to define the nature of the mutation in those cases.

Combining the results of all crosses involving the *mad* mutation in strain 2-15 of the type *ski2-2 mad* × *SKI MAD* K_1^+ [D], from Tables 5 and 6, the segregation is PD = 66, T = 55, NPD = 13 where tetrads with 4 healthy spore clones are PD, those with 3 healthy clones are T, and those with 2 healthy clones are NPD tetrads. This suggests that the *mad* mutation in strain 2-15 is loosely linked to *ski2-2*. The presence of extraneous lethality from the mutagenesis means that this is an underestimate of the linkage of *ski2-2* and the *mad* mutation in strain 2-15.

TABLE 4

Relation of L-A to pathogenesis

	Subclone of 2404	[HOK]	Cytoductants into (1101 → 2507)	Spore clone germination of diploids with (1101 → 2507) No. of tetrads	4	3	2	1	0
A.	364-4	H^+	Tiny	12	0	2	9	1	0
	360-8	H^+	Tiny	14	0	2	12	0	0
	359-2	H^+	Tiny	15	0	1	13	1	0
	359-6	H^+	Tiny	13	0	1	12	0	0
	359-1	H^-	Large	15	13	2	0	0	0
	359-3	H^-	Large	18	17	1	0	0	0
	360-1	H^-	Large	18	15	2	1	0	0
	359-4	H^-	Large	13	11	1	1	0	0
			Cytoductants into RE218[a]						
B.	359-1	H^-	Large	19	15	2	2	0	0
	359-2	H^+	Tiny	18	1	6	11	0	0
	359-3	H^-	Large	18	12	5	1	0	0

[a] RE218 = ((2-15 (*mad*) → 1101) → 2507 = **a** *arg1 ski2-2* L-A-HN M_1 [D-o].

Relationship of [D] and other non-Mendelian elements

Mitochondrial DNA: Elimination of ρ from strain 1368 by growth in the presence of 100 μg/ml of ethidium bromide did not eliminate [D] as judged by the ability of 1368 ρ^o to produce tiny cytoductants in the cytoduction 1368 ρ^o → 2507. Likewise, none of the *mad* mutants are ρ° although all have lost [D]. Thus [D] is not located on ρ. Cytoductants produced by 1368 ρ^o → 2507 ρ^o were unable to grow at 37°C, but were stable killers and grow normally at 20° or 30°.

M_1 dsRNA: The *mad* mutants lose [D] but retain M_1, and strain 2404, for example, has been cured of M_1, but retains [D]. Thus [D] is not a feature of M_1 dsRNA.

L-A dsRNA: The *mad* mutants that lose [D] all retain L-A as shown by crossing them with the *SKI* [HOK-o] (and thus L-A-o) strain 2405. All the meiotic segregants were killers. Had the *mad* mutants lost L-A, their M_1 would only be maintained in the *ski2* segregants (the *ski*-dependent phenomenon) (TOH-E and WICKNER 1979; RIDLEY, SOMMER and WICKNER 1984). Thus [D] is not located on L-A dsRNA.

Transfection experiments with L-A and M_1 from a [D] strain: To confirm that [D] and [DIN] were not simply natural variants of L-A, we examined transfectants of an L-A-o strain (2601) with purified L-A- and M_1-containing VLPs from the [B] strain 1368 prepared by H. Uemura (H. UEMURA and R. B. WICKNER, unpublished data) by the method of M. EL-SHERBEINI and L. A. BOSTIAN (unpublished data). The L-A and M_1 VLPs were cytoduced from the transfectants into the *ski2-2* L-A-o M-o strain RE57, and the cytoductants were tested for growth at various temperatures.

TABLE 5

Properties of *mad* mutants

Mutant	K_1	[DIN][a]	Growth 20°	Growth 37°	[D] cytoduction test[b]	Total tetrads	[D] Meiotic test[c] Healthy:Sick 4:0	3:1	2:2	1:3	0:4	Ability to maintain [D][d] *mad*
2-13	K_1^+	–	++	++	[D-o]	12	6	5	1	0	0	–
2-15	K_1^+	–	++	++	[D-o]	14	6	5	3	0	0	–
2-16	K_1^+	–	++	++	[D-o]							–
2-21	K_1^+	slight	++	++	[D-o]	14	9	4	1	0	0	–
2-24	K_1^+	–	++	–	[D-o]	13	12	1	0	0	0	–
Parent[e]	K_1^+	–	±	–	[D]	16	0	1	12	1	2	+

[a] Tested by the cytoductions, (mutant → 1368 ρ^o) → 2507. If the final cytoductants were diseased, then [DIN] activity was not present in the mutants. If the final cytoductants were healthy, [DIN] activity was present in the mutant. In the case of mutant 2-21, the cytoductants were detectably larger than the parent or other mutants, but much smaller than normal.

[b] The presence or absence of [D] in the mutants was tested by the two-step cytoduction (mutant → 1101) → 2507. In each case, the final cytoductants were healthy, indicating that none of the mutants had [D].

[c] The cross (mutant → 1101) × 2507 was dissected, and spores were germinated at 30°. The scarcity of 2 healthy:2 sick segregation in each case shows that [D] was absent from the mutants. The parent strain had [D] and so segregated mostly 2 healthy:2 sick.

[d] [D] was reintroduced into the mutants from 1368, then the cytoplasm was transferred into 2507 as in footnote *b*. The sequence of cytoductions was ((1368 → mutant) → 1101) → 2507. If the final cytoductants were sick, then the mutant could maintain [D]. If they were healthy, then the mutant was *mad*.

[e] The parent strain of the mutants is (1368 → 2507) = RE24.

TABLE 6

Meiotic segregation of *mad* mutations

Healthy spores	×1101 (*SKI* M_1 [D-o]) 4	3	2	1	0	×2404 (*SKI* M-o [D]) 4	3	2	1	0	×1368 (*SKI* M_1 [D]) 4	3	2	1	0
2-13 *mad*	5	5	1	0	0	12	12	2	1	5	11	3	1	0	1
2-15 *mad*	6	3	3	0	0	11	8	8	0	0	11	4	0	0	1
2-16 *mad*	7	4	4	0	0	9	17	2	2	1	2	14	5	1	0
2-21 *mad*	12	3	1	0	0	6	14	5	3	4	0	9	8	3	0
2-24 *mad*	10	4	2	0	0	12	14	6	0	0	10	4	1	0	2
2507	14	1	1	0	0	12	3	0	0	0	0	0	22	2	0
(1101 → 2507)						0	0	15	1	0					

All of the crosses shown here are isogenic with respect to nuclear genotype except for mutations introduced into the *mad* mutants in their mutagenesis. The crosses of *mad* mutants with 1368 show less lethality (or disease) than the control cross of 2507 with 1368. This is in spite of the presence of nonspecific lethality produced by the mutagenesis as shown in the crosses of mutants with 1101 (compare with 2507 × 1101). The segregation pattern for *mad* mutants 2-16 and 2-21 seems to be simple two-gene segregation. In these *mad ski2* × *MAD SKI* [D] M_1 crosses, only the *mad ski2* segregants are sick, and *mad* and *ski2* appear to be unlinked. In the crosses of strains 2-13, 2-15, and 2-24 with strain 1368, most of the tetrads are 4 healthy:0. This suggests linkage of these mutations to *ski2*.

All grew well at 20°, 30° and 37°, indicating that L-A alone is not responsible for the "disease" phenomenon and confirming the conclusion of the *mad* mutant experiments.

[D] depends on L-A for its maintenance or replication: If L-A and [D] are completely independent, then the derivatives of 1368 heat-cured of L-A should still have [D], while the *mad* mutants that lose [D] are known to still have the L-A from strain 1368. These two cytoplasms were combined in a *ski2* host by the cytoductions and crosses described in Table 4B; however, the disease phenomenon did not reappear, except in the control crosses and cytoductions. Since the *mad* mutant carries the L-A of strain 1368 and lacks [D], the heat-cured L-A-o derivative of strain 1368 must also lack [D]. We suggest that [D] is absent from the heat-cured L-A-o strains because its replication depends on L-A.

L-BC dsRNA: To test whether loss of L-BC results in loss of [D], we used the chromosomal *clo* mutation that results in loss of L-BC (WESOLOWSKI and WICKNER 1984). Cytoplasm of strain RE297 carrying L-BC and [D] was transferred to the *clo* strain RE123. These cytoductants were grown at 25° (the nonpermissive temperature for *clo*) to eliminate L-BC, and then cytoduction into the *ski2-2* tester strain was carried out. These cytoductants were sick, showing that [D] was not lost by the *clo* cytoductants. That L-BC was lost from the *clo* cytoductants was checked by cytoducing into the *mak10* L-o strain 1747. The *mak10* mutation only affects L-A and M, not L-BC (WICKNER and TOH-E 1982; SOMMER and WICKNER 1982). The resulting *mak10* cytoductants were also L-o as shown by minipreparations of dsRNA. Thus the *clo* strain lost L-BC, as expected, but did not lose [D]. We conclude that [D] is not a feature of L-BC.

W dsRNA: The *mad* mutants all had W dsRNA although they had lost [D].

2μ DNA: [D] is transmitted with 100% efficiency in cytoduction while 2μ DNA is only transmitted at 50% efficiency (LIVINGSTON and KLEIN 1977).

[Psi]: [Psi] is a non-Mendelian genetic element that increases the efficiency of ochre suppressors and is

TABLE 7

Segregation of *mad1-1*

Cross 74:
mutant 2-15 (**a** *arg1 ski2-2 mad1-1* Δ K^- [D-o]) × RE57 (*α arg1 ski2-2 MAD* K^- [D-o])

		Healthy spore clones						
	Total tetrads	PD 4	T 3	NPD 2	1	0	Cytoductants[a]	*MAD* genotype of cross 74 segregants
74-1A × 295	16	0	0	10	1	5	Tiny	*MAD*
74-1B × 295	17	0	0	17	0	0	Tiny	*MAD*
74-1C × 1368	17	5	10	3	0	0		*mad*
74-1D × 1368	16	10	4	1	1	0	Large	*mad*
74-2A × 295	17	0	1	13	2	1	Tiny	*MAD*
74-2B × 1368	16	4	4	4	2	2	Large	*mad*
74-2C × 295	16	1	5	9	1	0	Tiny	*MAD*
74-2D × 1368	16	8	4	1	2	1	Large	*mad*
74-4A × 1368	19	1	9	7	2	0	Tiny	*MAD*
74-4B × 295	20	4	14	1	1	0		*mad*
74-4C × 1368	20	14	5	1	0	0	Large	*mad*
74-4D × 295	19	0	8	11	0	0	Tiny	*MAD*
74-5A × 295	21	0	1	17	3	0	Tiny	*MAD*
74-5B × 295	21	1	7	11	2	0	Tiny	*MAD*
74-5C × 1368	21	6	12	3	0	0	Large	*mad*
74-5D × 1368	20	13	6	1	0	0	Large	*mad*
74-6A × 295	18	0	0	15	3	0	Tiny	*MAD*
74-6B × 1368	19	12	6	1	0	0	Large	*mad*
74-6C × 295	20	6	8	6	0	0		*mad*
74-6D × 1368	20	0	7	7	5	3	Tiny	*MAD*

[a] Cytoduction from strain 295 or strain 1368 (as indicated) into the spore clones of cross 74 was carried out. The size of the cytoductant clones on YPAD at 30° after 3 days is indicated.

uniformly cured by growth on rich medium containing 1 mM guanidine-HCl, but is not cured by growth at elevated temperatures (TUITE, MUNDY and COX 1981). We find that [D] is cured at elevated temperatures (see section above on L-A) and is not cured by growth on 5 mM guanidine-HCl, indicating that [D] and [Psi] are different elements.

Relationship of [B] and [D]: [B] is a nonchromosomal genetic element, also present in strain 1368, that *b*ypasses M_1 dsRNA's usual requirement for *MAK11* and several other *MAK* genes for its replication. The molecular identity of [B] is not known. The *mad* mutants, which had lost [D], were tested for [B] by transferring their cytoplasm to strain 1101 (*α kar1* M_1^+ B-o D-o) and then crossing the cytoductants with the *mak11-1* strain 1868 (Table 8). The control crosses with and without [B] gave the expected uniform 4 K^+:0 and 2 K^+:2 K^- segregation, respectively. The *mad* [D-o] mutants 2-24 and 2-15 retained [B] while mutant 2-21 apparently lost [B]. The result with mutant 2-13 was equivocal. We conclude that [B] and [D] appear to be distinct elements.

A nuclear-limited dominant factor that eliminates [D]: When strains 1716 (*mak3-1*) and TF78-4A Δ (*mak10^{ts}*) were crossed with strain 1368 ([D] M_1), all of the 14 spore clones tested (by cytoduction into 2507) showed no [D] activity. Neither 1716 nor TF78-4A Δ had [DIN] activity as shown by (1716 or TF78-4A Δ → 1368 ρ^0) → 2507 or (1716 or TF78-4A Δ → 1368 ρ^0) × 2507. This shows that a nuclear-limited (noncytoducible) factor is capable of eliminating [D] or preventing its expression. This could be either a dominant chromosomal allele in strains 1716 and TF78-4A Δ or a non-Mendelian gene that does not leave the nucleus. Further studies will be needed to clearly define this factor.

DISCUSSION

We have found a new genetic element affecting the *S. cerevisiae* killer system. [D], together with a variant of L-A, increases the severity of the cytopathology resulting from M dsRNA's derepressed replication in a *ski2* host. Our studies to date have shown [D] to be distinct from most of the known cytoplasmic entities in *S. cerevisiae*.

[D] might produce its effect by further increasing the copy number of M_1 dsRNA beyond that resulting from the *ski2* mutation. Alternatively, [D] might be another entity whose replication is normally controlled by *SKI2* and whose derepressed replication, when combined with that of M_1 dsRNA, causes more severe effects than either M_1 or [D] alone. It is clear that [D] is not necessary for M_1 replication since *mad* mutants lose [D] but not M_1. Also, [D] alone does not

TABLE 8

Relation of [B] and [D]

Parents	Total tetrads	Meiotic segregation		
		4 K^+:0	3 K^+:1 K^-	2 K^+:2 K^-
Controls				
1868 *mak11-1* × 1101 [D-o] [B-o]	12	0	1	11
1868 *mak11-1* × (RE59 → 1101) [D] [B]	12	12	0	0
Experimental				
1868 *mak11-1* × (2-13 *mad* → 1101) [D-o] [?B?]	12	4	4	4
1868 *mak11-1* × (2-15 *mad* → 1101) [D-o] [?B?]	12	8	4	0
1868 *mak11-1* × (2-21 → 1101) [D-o] [?B?]	12	0	4	8
1868 *mak11-1* × (2-24 → 1101) [D-o] [?B?]	12	11	1	0

cause significant ill effects in a *ski2* host in the absence of M_1.

[DIN] is a cytoplasmic element found in many strains that interferes with [D] expression and is cured by growth at high temperature. Perhaps [DIN] and [D] are different forms of the same replicon. [DIN] might be analogous to the suppressive (S) mutants of M_1 dsRNA that are deletion mutants of the internal part of M_1. These S mutants interfere with replication of the parent M_1 molecule (FRIED and FINK 1978). Likewise [DIN] may interfere with [D] replication, while [DIN] itself, for some reason, does not make the cells sick.

The role of the L-A in strain 1368 in the disease phenomenon is not yet completely clear. We have shown that curing of L-A is accompanied by the loss of [D], so apparently [D] depends on L-A for its maintenance. Whether an L-A from another strain, such as 1101, could substitute for the L-A in 1368 has not yet been rigorously tested.

Two lines of evidence—the *mad* mutants and the transfection experiments—both show that [D] is *not* simply another L-A trait like [HOK], [NEX], and [EXL].

[D] and [DIN] are elements found in apparently wild type strains. This study has utilized these natural variants to find new elements affecting the killer system. The same approach previously led to the detection of the elements [EXL], [HOK], and [NEX], located on L-A, whose study revealed the existence of L-BC, T, and W, the dependence of L-A on *MAK3*, *MAK10*, and *PET18*, and the relations between L-A, M_1, and M_2 dsRNAs and *SKI* and *MKT* genes, which are seen as the [EXL], [HOK], and [NEX] phenomena (WICKNER 1980, 1983; WICKNER and TOH-E 1982; SOMMER and WICKNER 1982). We take this to confirm the value of the natural variant approach as opposed to restricting studies to a set of isogenic strains.

The fact that L-A, [D], and M_1 are all cytoplasmic elements found in wild type strains of *S. cerevisiae* emphasizes that the *SKI* genes are essential to a wild type host even if only to prevent the severe cytopathology described here. Also, the variety of genetic effects now attributable to variants of L-A ([EXL] = E, [NEX] = N, [HOK] = H) points to the need to more clearly define the nature of these interactions. It is unclear whether these effects are all due to coat protein variants or whether L-A might encode some other products or have *cis*-sites responsible for some of these effects.

Further efforts will be directed at identifying the molecular identity of [D] and [DIN] and to further define the *MAD* genes. It will also be of interest to study the cytopathology of *ski2-2* M_1 [D] strains.

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