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A translational fidelity mutation in the universally conserved sarcin/ricin domain of 25S yeast ribosomal RNA

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ABSTRACT

Recent evidence suggests that ribosomal RNAs have functional roles in translation. We describe here a new ribosomal RNA mutation that causes translational suppression and antibiotic resistance in eukaryotic cells. Using random mutagenesis of the cloned ribosomal RNA gene and in vivo selection, we isolated a C → U mutation in the universally conserved sarcin/ricin domain in *Saccharomyces cerevisiae* 25S ribosomal RNA. This mutation changes the putative CG pair, which closes the GAGA tetraloop in the sarcin/ricin domain, into a weaker UG pair without eliminating ribosomal sensitivity to ricin. We show that suppression of several UGA, UAG, and frameshift mutations is evident when a portion of the cellular ribosomal RNA contains the C → U mutation. Cells that contain essentially all mutant ribosomal RNA grow only 10% slower than the wild-type, but show increased suppression as well as resistance to paromomycin, G418, and hygromycin, and sensitivity to cycloheximide. Our results provide genetic evidence for the participation of the sarcin/ricin loop in maintaining translational accuracy and are discussed in terms of a hypothesis that this ribosomal RNA region normally undergoes a conformational change during translation.

Keywords: elongation; omnipotent suppressor; *Saccharomyces cerevisiae*; translational accuracy

INTRODUCTION

Accurate and efficient translation of mRNA is a critical step in the process of protein synthesis and is essential for cell viability. One approach used to investigate translational fidelity has been to isolate suppressor and antisuppressor mutations that decrease or increase translational accuracy, respectively. Many of these mutations also alter resistance to antibiotics. Such studies in both eukaryotes and prokaryotes have implicated a variety of translational apparatus components, such as tRNAs (Ozeki et al., 1980; Hinnebusch & Liebman, 1991), ribosomal proteins (Kurland et al., 1990; Hinnebusch & Liebman, 1991), and elongation (Vijenboom et al., 1985; Tapio & Kurland, 1986; Sandbaken & Culbertson, 1988) and termination factors (Weiss et al., 1984; Kawakami & Nakamura, 1990; Frolova et al., 1994; Stansfield & Tuite, 1994; Zhouravleva et al., 1995), in translational fidelity.

Ribosomal RNAs (rRNAs) have been shown recently to be the primary target for translational antibiotics (Cundliffe, 1990) and to retain peptidyl-transferase activity in the absence of most ribosomal proteins (Noller et al., 1992), suggesting that rRNAs have catalytic activity during translation. Indeed, a number of suppressor and antisuppressor mutations have been isolated in *Escherichia coli* and organelle rRNA (for reviews see Noller, 1991; Triman, 1994; see also O'Connor & Dahlberg, 1993). The eukaryotic analogues of several of these mutations were recently constructed by site-directed mutagenesis and shown to also affect translational accuracy in yeast (Chernoff et al., 1994, 1996; Liebman et al., 1995).

We now describe an in vitro random mutagenesis and in vivo selection scheme designed to isolate new suppressor mutations in yeast rRNA. Such analyses have not been undertaken previously in eukaryotes because of the highly repeated nature of ribosomal rRNA genes (rDNA) in most eukaryotes. In yeast, the 9-kb rDNA units, including regulatory sequences and cod-

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ing regions for 18S, 25S, 5.8S, and 5S rRNAs, are in a tandem array of 100–200 copies (*RDN* array) on chromosome XII. We use a novel plasmid system designed by E. Morgan (Chernoff et al., 1994) to isolate functional mutations in rRNA despite the large number of rDNA repeats. In this system, rRNA is expressed from plasmids containing a functional 9-kb rDNA repeat (pRDN), and deletions of part or all of the chromosomal *RDN* array are obtained using a recessive drug-resistant rDNA mutation (*hyg1*). We have used this system to isolate a new omnipotent (codon-nonspecific) suppressor, *rdn-5*, which acts on several UGA, UAG, and frameshift alleles. The *rdn-5* mutation is in the universally conserved sarcin/ricin domain of yeast 25S rRNA.

This sarcin/ricin domain is composed of a stem and loop with a GAGA tetraloop (Szewczak et al., 1993; Glück et al., 1994), and has been suggested to have important functions in translation and to interact with *E. coli* elongation factors (Moazed et al., 1988). Site-directed mutations generated previously in the GAGA tetraloop at the underlined position (G2661C,T) in *E. coli* 23S rRNA (Tapprich & Dahlberg, 1990; Melancon et al., 1992) decreased nonsense suppression and +1 frameshifting slightly, and G2661C was a synthetic lethal in combination with an antisuppressor mutation in ribosomal protein S12 (Tapprich & Dahlberg, 1990). Conformational changes involving the GAGA tetraloop and its putative closing CG pair have been proposed to occur during each elongation cycle (Wool et al., 1992). The new *rdn-5* suppressor mutation changes the wild-type CG closing pair of the tetraloop to a UG pair. We propose that the C → U change in this mutation may shift the equilibrium of the transitions in this domain toward the conformation associated with a higher mistranslation frequency.

RESULTS

Isolation of a mutation in rRNA that causes nonsense suppression

The pRDN plasmid, pRDN-hyg1ani1-UL (Table 1), was mutagenized with hydroxylamine in vitro (Fig. 1). The rDNA repeat on this plasmid contains *hyg1*, which causes recessive resistance to hygromycin, and *ani1*, which causes semi-dominant resistance to anisomycin. The mutagenized plasmid mixture was transformed into yeast strain L63-31V-D543 (Table 2), which contains several nonsense suppressible markers. About 4,000 Ura$^+$ transformants were inoculated as patches on −Ura master plates and velveteen replica-plated first to −Leu plates and from there to medium containing hygromycin (+Hyg). Growth on −Leu plates caused the plasmid to amplify to 100–200 copies per cell, which is approximately equal to the normal number of chromosomal rDNA repeats (see the Materials and methods; Chernoff et al., 1994). Growth on +Hyg selected for hygromycin-resistant (Hyg^R) cells, which contain deletions of different sizes in the wild-type chromosomal *RDN* array (Chernoff et al., 1994; Fig. 2). Transformants grown on the +Hyg master plates were velveteen replica-plated to media where suppression is required for growth (−Ade, −Lys, −Trp, and −His, see the Materials and methods). The replicas of the +Hyg plates, containing cells with *RDN* deletions, revealed one transformant that showed suppression of *ade1-14* (UGA), *lys2-L63* (UGA), and *trp1-289* (UAG). Suppression of *his7-1* (UAA) was not detected. Plasmid isolated from this transformant was amplified in *E. coli* and retransformed into L63-31V-D543. The suppression phenotype described above reappeared in Hyg^R rDNA deletion derivatives of these transformants (Fig. 1). This plasmid has been named pRDN-5-hyg1ani1-UL because it contains the suppressor mutation *rdn-5* in addition to *hyg1* and *ani1*.

The pRDN-5-hyg1ani1-UL plasmid was also transformed into L-1489 (Table 2) to test if pRDN-5-hyg1ani1-UL could cause suppression in a strain other than the one (L63-31V-D543) in which it was isolated originally. Indeed, as in L63-31V-D543, *ade1-14* (UGA) was suppressed and *his7-1* (UAA) was not suppressed in Hyg^R pRDN-5-hyg1ani1-UL transformants of L-1489. Suppression of another nonsense allele present in L-1489 but not in L63-31V-D543, *lys2-L864* (UAG), was not detected.

TABLE 1. pRDN Plasmids.

Name	Reference	rDNA	Plasmid markers
pRDN-wt-U	Chernoff et al. (1994)	Wild type	*URA3*
pRDN-wt-TL	Chernoff et al. (1994)	Wild type	*TRP1, LEU2-d*
pRDN-hyg1ani1-UL	Chernoff et al. (1994)	*hyg1, ani1*	*URA3, LEU2-d*
pRDN-hyg1-U	This study	*hyg1*	*URA3*
pRDN-hyg1-TL	This study	*hyg1*	*TRP1, LEU2-d*
pRDN-5-hyg1ani1-UL	This study	*rdn-5, hyg1, ani1*	*URA3, LEU2-d*
pRDN-5-hyg1-U	This study	*rdn-5, hyg1*	*URA3*
pRDN-5-TL	This study	*rdn-5*	*TRP1, LEU2-d*
pRDN-5-hyg1-TL	This study	*rdn-5, hyg1*	*TRP1, LEU2-d*

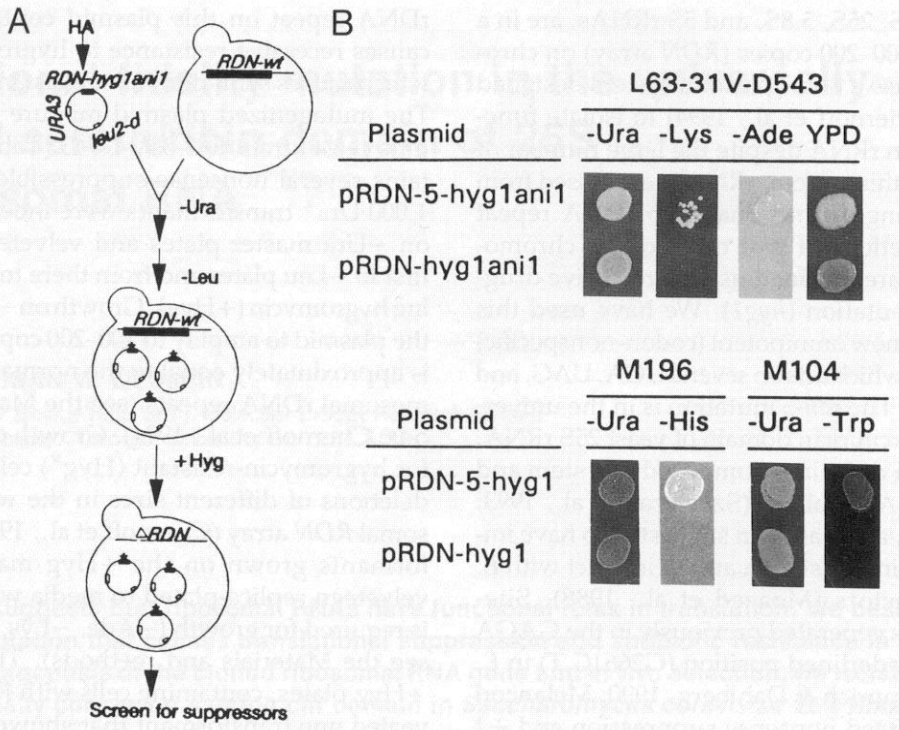


FIGURE 1. Isolation of the *rdn-5* suppressor mutation. **A:** Screen for rDNA plasmid-borne suppressor mutants. Plasmid pRDN-hyg1ani1-UL (Table 1) was mutagenized with hydroxylamine (HA) and transformed into yeast strain L63-31V-D543 (Table 2). About 4,000 Ura^+ transformants were grown on −Ura, transferred to −Leu, and from there to +Hyg plates, and were then screened for suppressor phenotypes by replica-plating to media where suppression is required for growth (−Ade, −Lys, −Trp, −His). **B:** Suppression is shown in different strains carrying the indicated *rdn-5* (pRDN-5-hyg1ani1-UL or pRDN-5-hyg1-U) and control plasmids (pRDN-hyg1ani1-UL or pRDN-hyg1-U). Transformants were replica-plated to +Hyg and suspensions of these Hyg^R rDNA deletion cells were spotted on appropriate media to detect suppression of *ade1-14* (UGA), *lys2-L63* (UGA), *his4-713* (CCUCCCU), and *trp1-1* (UAG), and on −Ura control plates. Suppression is measured by growth on plates. For each strain, vertical spots were on the same plate and horizontal spots were made from the same cell suspension. Plates were incubated for 2 days (M196 and M104, −Ura); 3 days (L63-31V-D543, −Ura, −Lys, and YPD); 7 days (L63-31V-D543, −Ade; M196, −His), and 11 days (M104, −Trp). Note the red color (dark) caused by *ade1-14* (UGA) on YPD in L63-31V-D543 is suppressed by *rdn-5*.

TABLE 2. Yeast strains.

Name	Reference	Genotype[a]
L63-31V-D543	Chernoff et al. (1994)	*MATa ade1-14*(UGA) *his7-1*(UAA) *lys2-L63*(UGA) *trp1-289*(UAG) *ura3-52 leu2-3,112* [*psi*$^-$]
L-1489	Chernoff et al. (1994)	*MATa ade1-14*(UGA) *his7-1*(UAA) *lys2-L864*(UAG) *trp1-Δ1 ura3-52 leu2-3,112* [*psi*$^-$]
L-1494	Chernoff et al. (1994)	Isogenic to L-1489 except that it contains the complete deletion in *RDN* and pRDN-wt-TL.
L-1521	Chernoff et al. (1994)	Isogenic to L-1489 except that it contains the complete deletion in *RDN* and pRDN-wt-U.
L-1548	This study	Isogenic to L-1489 except that it contains the complete deletion in *RDN* and pRDN-5-TL.
74-D694	Chernoff et al. (1995)	*MATa ade1-14*(UGA) *his3-Δ200 ura3-52 leu2-3,112 trp1-289*(UAG) [*psi*$^-$]
L-1581	This study	Isogenic to 74-D694 except that it contains pJG2RA integrated into the *LEU2* locus
M196	Sandbaken and Culbertson (1988)	*MATa leu2-3,112 his4-713*(fs) *met2-1*(fs) *ura3-52 trp1-Δ1*
M104	Sandbaken and Culbertson (1988)	*MATα leu2-3*(fs) *his4-519*(fs) *trp1-1*(UAG) *ura3-52*
1865	Sandbaken and Culbertson (1988)	*MATa leu2-3*(fs) *his4-520*(fs) *ura3-52*
1867	Sandbaken and Culbertson (1988)	*MATa leu2-3*(fs) *his4-507*(fs) *ura3-52*
1870	Sandbaken and Culbertson (1988)	*MATa leu2-3*(fs) *his4-504*(fs) *ura3-52*
1645	Sandbaken and Culbertson (1988)	*MATa leu2-3*(fs) *his4-38*(fs) *ura3-52*
1589	Sandbaken and Culbertson (1988)	*MATa his4-506*(fs) *ura3-52 trp1*

[a] (fs), +1 frameshift mutation.

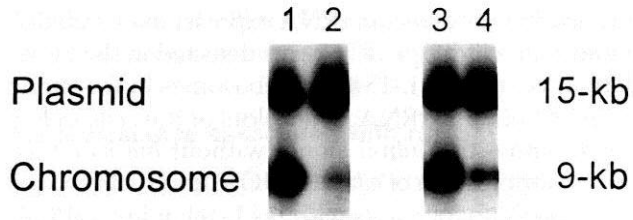


FIGURE 2. Chromosomal rDNA repeats are removed partially in Hyg^R deletion cells. Total yeast DNA was isolated (Rose et al., 1990) from L63-31V-D543 transformants containing pRDN-hyg1ani1-UL (lanes 1, 2) or pRDN-5-hyg1ani1-UL (lanes 3, 4) grown in −Leu (lanes 1, 3) or +Hyg (lanes 2, 4) liquid media. DNA digested with *Mlu* I, which recognizes a unique site in the 9-kb chromosomal rDNA repeats and in the 15-kb pRDN plasmids, was separated on 0.7% agarose gels and blotted as described (Maniatis et al., 1982). Blots were hybridized with ^{32}P-labeled 9-kb rDNA.

The *rdn-5* mutation is a C → T change in the universally conserved sarcin/ricin domain in 25S rRNA

To localize the *rdn-5* mutation, rDNA restriction fragments from pRDN-5-hyg1ani1-UL were used to replace the corresponding fragments in pRDN plasmids that lack *rdn-5* (Fig. 3). The resulting plasmids, each containing an rDNA fragment from pRDN-5-hyg1ani1-UL cloned in an intact *hyg1* rDNA unit, were transformed into L63-31V-D543.

Tests for suppressor activity in Hyg^R rDNA deletion derivatives of these transformants localized the *rdn-5* mutation to a region close to the 3′ end of 25S rRNA (Fig. 3). Plasmids containing either the *Mlu* I–*Pfl*M I or *Tth111* I–*Sal* I fragment from pRDN-5-hyg1ani1-UL caused the same suppressor phenotype as pRDN-5-hyg1ani1-UL. Sequence analysis of the overlapping region, *Tth111* I–*Pfl*M I, from *rdn-5* and non-*rdn-5* plasmids, revealed a single nucleotide alteration. The *rdn-5* mutation is a C → T change located 3,022 nt from the 5′ end of mature yeast 25S rRNA (Miller & Bodley, 1991) within 206 bases of the *ani1* mutation. The location of the *rdn-5* mutation is analogous to *E. coli* C2658 in 23S rRNA, and is in the universally conserved sarcin/ricin domain of 25S rRNA (Fig. 3).

The suppressor phenotype of *rdn-5* is independent of the *hyg1* and *ani1* mutations

To rule out possible effects of the *hyg1* and *ani1* mutations on the phenotypes caused by *rdn-5*, the *rdn-5* mutation was regenerated using PCR site-directed mutagenesis on a 15-kb otherwise wild-type pRDN plasmid (see the Materials and methods). The resulting plasmid, pRDN-5-TL, on which *rdn-5* is the only rDNA mutation, was transformed into strain L-1521 (Chernoff et al., 1994), which contains an essentially complete deletion of the chromosomal *RDN* array and is kept alive by a 14-kb wild-type pRDN plasmid, pRDN-wt-U

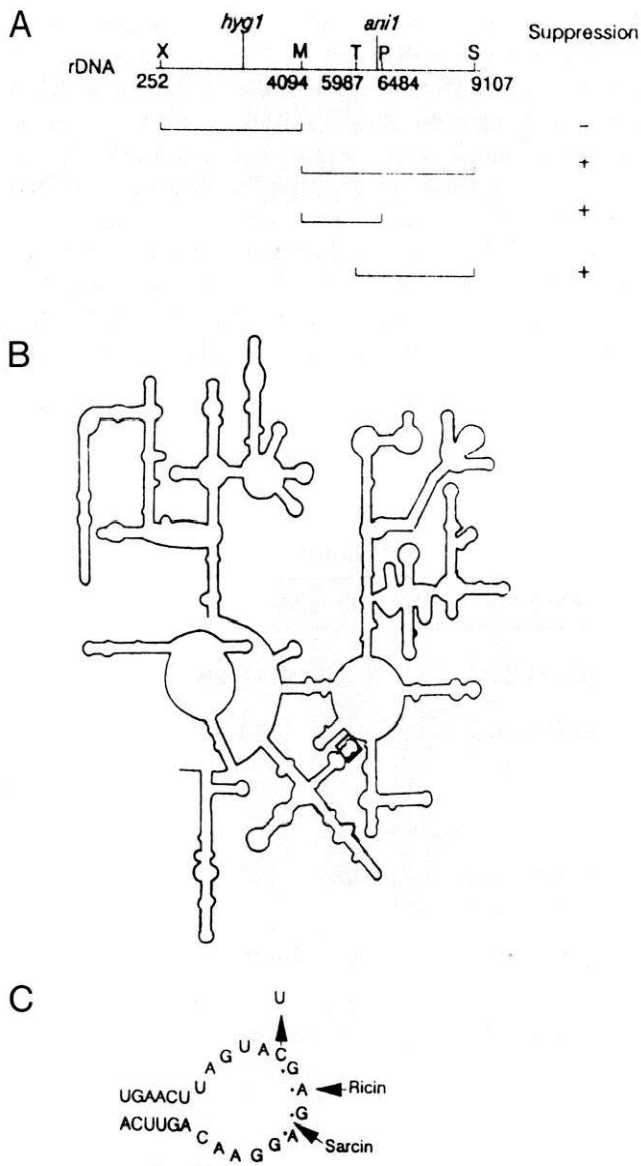
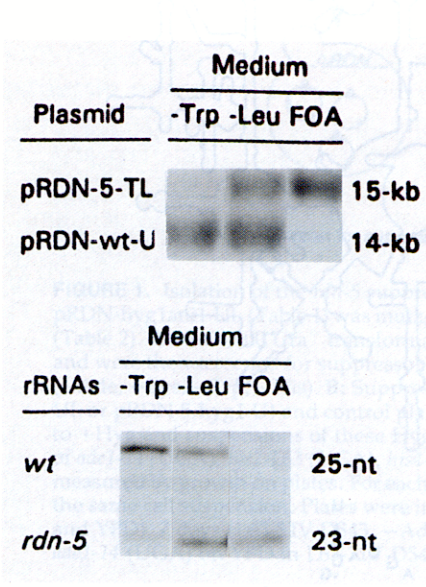


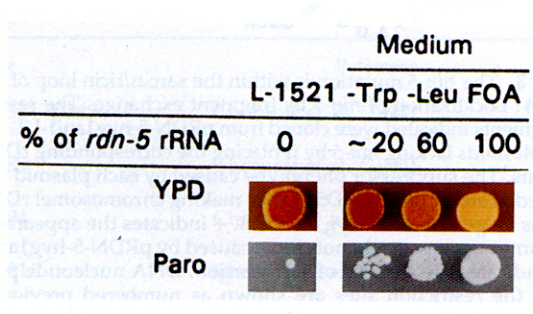
FIGURE 3. The *rdn-5* mutation is within the sarcin/ricin loop of 25S rRNA. **A:** Localization of *rdn-5* by fragment exchange. The restriction fragments indicated were cloned from pRDN-5-hyg1ani1-UL into pRDN plasmids lacking *rdn-5* by replacing the corresponding rDNA fragments. The suppressor phenotype caused by each plasmid was examined in strain L63-31V-D543 after making chromosomal rDNA deletions by growth on +Hyg medium. + indicates the appearance of the same suppressor phenotype as caused by pRDN-5-hyg1ani1-UL; − indicates the absence of suppression. DNA nucleotide positions of the restriction sites are shown as numbered previously (Gutell et al., 1993) X, *Xho* I; M, *Mlu* I; T, *Tth111* I; P, *Pfl*M I; S, *Sal* I. **B:** Secondary structure of the 3′ half of *S. cerevisiae* 25S rRNA adopted from Gutell et al. (1993). The region corresponding to the sarcin/ricin domain is boxed and shown in detail in C. **C:** Diagram of the sarcin/ricin loop. The tetraloop recognized by ricin is marked with dots. The *rdn-5* mutation is marked as a C → U change. Ricin specifically depurinates the adenosine indicated and sarcin specifically cleaves the phosphodiester bond indicated (Wool et al., 1990).

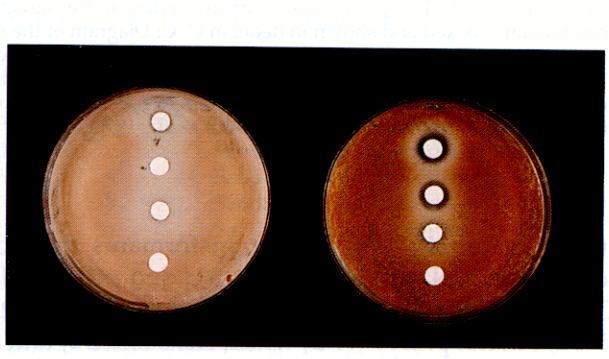
(Chernoff et al., 1994). The transformants therefore contain two plasmids, pRDN-wt-U and pRDN-5-TL. Transformants were grown on −Leu to amplify pRDN-5-TL, but not pRDN-wt-U, which lacks *LEU2-d*; and on

FOA, to select for loss of all copies of the *URA3*-containing plasmid, pRDN-wt-U. Primer extension analysis was used to measure the relative levels of rRNA expressed from the *rdn-5* and wild-type pRDN plasmids in cells grown on −Trp, −Leu, and FOA (Fig. 4). This analysis also confirms that the rRNA transcribed from the *rdn-5* plasmid contains the C → U change that corresponds to the C → T change in the *rdn-5* DNA (see the Materials and methods). Because the amount of wild-type and *rdn-5* rRNA is proportional to the amount of the corresponding pRDN plasmid (Fig. 4A), *rdn-5* has no obvious defect in rRNA expression or stability. Because no wild-type rRNA was detected in the FOA$^+$ derivative (named L-1548), all ribosomes in this strain are made of *rdn-5* rRNA. The colors of the cells in Figure 4B show that *rdn-5* alone, without *hyg1* or *ani1*, causes suppression of *ade1-14* (UGA) and that the level of suppression increases with the level of *rdn-5* rRNA. Suppression of *ade1-14* by the *rdn-5* single mutation was also demonstrated by growth of L-1548 on −Ade (data not shown).

We have compared the growth rate of cells that contain a pure population of either *rdn-5* (L-1548) or wild-type (L-1521) ribosomes. In complete (YPD) medium, *rdn-5* and wild-type cells have similar generation times, 131 min and 118 min, respectively.







The *rdn-5* mutation causes suppression of a +1 frameshift mutation

To test the ability of *rdn-5* to suppress other mutant alleles, plasmid pRDN-5-hyg1-TL (containing the site-directed *rdn-5* mutation in addition to *hyg1*) was transformed into a variety of yeast strains (M196, M104, 1865, 1867, 1870, 1645, and 1589, kindly provided by M. Culbertson) containing frameshift and nonsense mutations (Table 2). An examination of Hyg^R rDNA deletions of these transformants (Fig. 1) revealed that pRDN-5-hyg1-TL mediates suppression of *trp1-1* (UAG) and a +1 frameshift mutation *his4-713* (ACC CCU GAA to ACC CCCU GAA) (Donahue et al., 1981), which is also suppressed by mutations in elongation factor EF-1α (Sandbaken & Culbertson, 1988) and termination factor *SUP35* (Wilson & Culbertson, 1988;

FIGURE 4. The *rdn-5* mutation alone, without *hyg1* or *ani1*, is viable and causes suppression. A transformant of L-1521, which contains a complete deletion of the chromosomal *RDN* array and two pRDN plasmids, pRDN-5-TL and pRDN-wt-U, was grown on −Trp to select for pRDN-5-TL, on −Leu to amplify pRDN-5-TL, and on FOA to lose pRDN-wt-U. **A:** Top, Southern analyses showing the relative plasmid copy numbers. Total DNA extracted (Rose et al., 1990) from yeast cells grown on the indicated media was digested with *Mlu* I, which recognizes a unique site in the 14-kb pRDN-wt-U and 15-kb pRDN-5-TL plasmids. The Southern blot was hybridized with the 9-kb rDNA probe. Bottom, Primer extension analyses of total yeast RNAs isolated from the same cells. Percentage of *rdn-5* rRNA in the total rRNA, determined by scanning the autoradiogram, are listed in B. **B:** Levels of suppression and paromomycin resistance increase as the level of *rdn-5* rRNA increases. The red color of cells on YPD indicates lack of suppression of the *ade1-14* (UGA) marker; white indicates strong suppression; pink indicates intermediate suppression. Paro plates are YPD medium containing 1 mg/mL paromomycin. **C:** Effects of paromomycin, G418, and hygromycin on *rdn-5* cells. Cells (10^8) of L-1548 (100% *rdn-5*, on the left) and L-1521 (100% wild type, on the right) were spread on YPD plates. Ten microliters of each drug were pipetted onto filter paper discs from top to bottom: 29 mg/mL paromomycin, 3 mg/mL G418, 3 mg/mL hygromycin, and zero control. Lighter background color shows suppression by *rdn-5*. Clear and white circles around the discs indicate, respectively, killing and phenotypic suppression caused by the antibiotics.

Zhouravleva et al., 1995). The other frameshift and nonsense mutations in these strains were not suppressed.

Drug-resistance associated with *rdn-5*

By comparing antibiotic-induced zones of growth inhibition, we show that L-1548 cells containing essentially all *rdn-5* rRNA are more resistant to paromomycin, G418, and hygromycin than L-1494 cells containing only wild-type rRNA (Fig. 4C). The paromomycin resistance of *rdn-5* is further demonstrated in Figure 4B, where *RDN* deletion cells containing both wild-type (pRDN-wt-U) and *rdn-5* (pRDN-5-TL) plasmids were spotted on Paro plates. Paromomycin-resistant derivatives were found to be Ura$^-$, indicating loss of the wild-type plasmid, pRDN-wt-U, and that the *rdn-5* plasmid conferred resistance.

Although the *rdn-5* mutation caused resistance to the antibiotics that induce misreading, it did not eliminate antibiotic-induced (phenotypic) suppression of the red color associated with the *ade1-14* mutation in L-1548 (Fig. 4C). Phenotypic suppression of *ade1-14* in L-1548 was also demonstrated by paromomycin induced growth on −Ade (data not shown).

In contrast to the antibiotic resistance described above, *rdn-5* cells are more sensitive to cycloheximide. This antibiotic inhibits elongation and acts on large ribosomal subunits (reviewed in Gale et al., 1981). The *rdn-5* cells (L-1548) had a zone of growth inhibition 50% bigger than isogenic wild-type cells (L-1494) around a disc with 5 μL of 1 mg/mL cycloheximide (data not shown).

The rdn-5 mutation does not eliminate sensitivity to ricin

We used yeast strain L-1581, which was engineered to contain the ricin A chain gene (*RTA*) under the control of the *GAL1* promoter (Gould et al., 1991) to test for in vivo sensitivity to the ribotoxin ricin. L-1581, which contains only wild-type ribosomes, dies upon induction of ricin, but not when expression of ricin is repressed. This is also true of Hyg^R derivatives of pRDN-5-hyg1-TL transformants of L-1581, which contain enough *rdn-5* ribosomes to cause suppression of *ade1-14* (Fig. 5A).

Unlike *E. coli* systems (Poot et al., 1993), our system easily provides a pure population of mutant ribosomes, simplifying the interpretation of biochemical studies. We were thus able to test a preparation of pure *rdn-5* ribosomes isolated from L-1548 for ricin sensitivity in vitro (Morris & Wool, 1992). The 367-nt fragment (RAF) resulting from ricin modification is evident in *rdn-5* ribosomes, as well as in wild-type ribosomes (Fig. 5B). The appearance of RAF is both ricin and aniline dependent. These experiments show that mutation of the wild-type CG tetraloop closing pair to UG does not eliminate ricin recognition.

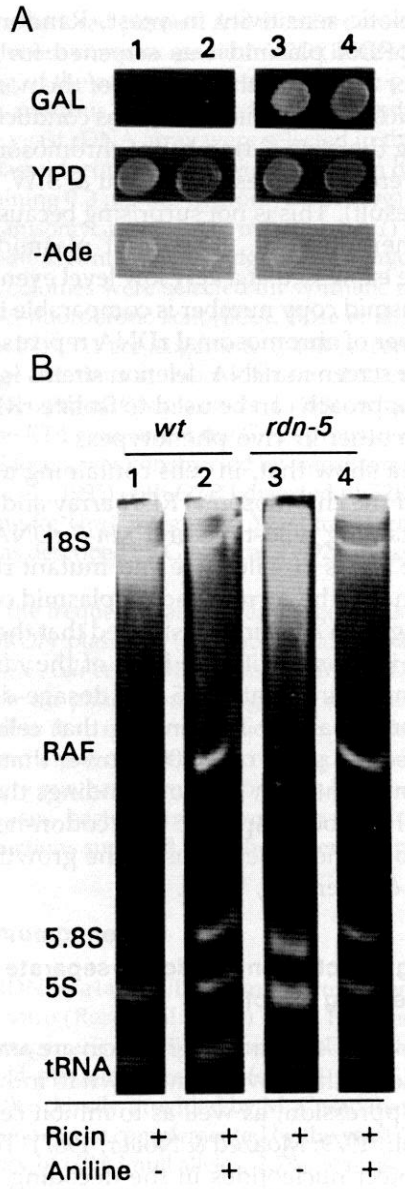


FIGURE 5. Ribosomes containing *rdn-5* rRNA remain sensitive to ricin. **A:** Cells containing *rdn-5* ribosomes are sensitive to ricin induction. Strain L-1581 (lanes 1, 2), which carries an integrated copy of the ricin A chain gene (*RTA*) under the *GAL1* promoter, and the isogenic control strain, 74-D694, lacking *RTA* (lanes 3, 4) were transformed with either pRDN-5-hyg1-TL (lanes 1, 3) or pRDN-hyg1-TL (lanes 2, 4). Hyg^R cells were obtained and tested for growth on glucose (YPD) and galactose (GAL) media. **B:** Pure *rdn-5* ribosomes are modified by ricin in vitro. Ribosomes isolated from isogenic complete chromosomal rDNA deletion strains containing pRDN-wt-TL, L-1494 (lanes 1, 2) or pRDN-5-TL, L-1548 (lanes 3, 4) were incubated with ricin and then treated with aniline (+) or left untreated (−). The RNA was electrophoresed in polyacrylamide gels and visualized by EtBr staining. RAF, fragment from the 3′ end of 25S rRNA formed by aniline scission after ricin depurination.

DISCUSSION

Genetic selection for rRNA mutations

This study describes the isolation and characterization of an rRNA mutation that affects translational fidelity

and antibiotic sensitivity in yeast. Randomly mutagenized pRDN plasmid was screened for nonsense suppressor activity in the absence of the normal chromosomal *RDN* array. Similar screens conducted in cells containing the normal number of chromosomal rDNA repeats were not successful (R. Liu & S.W. Liebman, unpubl. result). This is not surprising because we now know (Chernoff et al., 1994) that plasmid-encoded rRNAs are expressed at a very low level even when the pRDN plasmid copy number is comparable to the normal number of chromosomal rDNA repeats. The success of our screen in rDNA deletion strains implies that a similar approach can be used to isolate rRNA mutations with other in vivo phenotypes.

Our data show that, in cells containing a complete deletion of the chromosomal *RDN* array and two plasmids containing wild-type and *rdn-5* rDNA, respectively, the levels of wild-type and mutant rRNAs are proportional to the corresponding plasmid copy numbers. Using such a strain, we showed that the suppression level increases with the level of the *rdn-5* rRNA (Fig. 4), indicating that *rdn-5* is a dosage-dependent suppressor mutation. The finding that cells with all *rdn-5* ribosomes grow only 10% slower than the wild type is consistent with previous findings that a moderate level of codon-specific and codon-nonspecific suppression is not deleterious to the growth of yeast (Liebman & Sherman, 1976).

The killing effect of antibiotics is separate from their misreading effects

Paromomycin, G418, and hygromycin are aminoglycoside antibiotics that have been shown to induce translational suppression, as well as to inhibit cell growth (Singh et al., 1979; Moazed & Noller, 1987). These antibiotics protect nucleotides in the decoding region of 16S rRNA (Moazed & Noller, 1987) and have been hypothesized to cause phenotypic suppression by increasing the nonspecific affinity of the ribosomal A-site for tRNA (Gale et al., 1981). The killing effects of these antibiotics are not well understood, although it has been suggested that hygromycin may inhibit a step in translocation by sequestering peptidyl-tRNA at the ribosomal A site (Gale et al., 1981). Previously, resistance to paromomycin, G418, and hygromycin was only associated with alterations in the small ribosomal subunit (Moazed & Noller, 1987; Noller, 1991; Vincent & Liebman, 1992; Chernoff et al., 1994). The *rdn-5* mutation is the first report that an alteration in the ribosomal large subunit causes resistance to these antibiotics and increases mistranslation.

Because omnipotent suppressor mutations described previously have generally been associated with hypersensitivity to these misreading-inducing antibiotics, antibiotic sensitivity has been suggested to result from an excess of errors during translation (Surguchov et al., 1984). However, a few omnipotent suppressors that are resistant to misreading-inducing antibiotics have been reported (Wakem & Sherman, 1990). The *rdn-5* suppressor mutation clearly separates the dual effects of the antibiotics on misreading and killing. In the *rdn-5* mutant cells, paromomycin, G418, and hygromycin continue to cause phenotypic suppression even though the *rdn-5* mutation causes resistance to these antibiotics (Fig. 4C). We propose that the decoding function of the *rdn-5* ribosome remains accessible to these antibiotics, allowing them to continue to cause phenotypic suppression. However, the *rdn-5* mutation may alter the normal interaction between the sarcin/ricin domain and the elongation factor EF-2, thereby antagonizing the killing effect of the antibiotics during translocation.

A hypothetical mechanism to explain the effect of *rdn-5* on mistranslation

The *rdn-5* mutation changes the putative CG pair that closes the GAGA tetraloop in the sarcin/ricin domain to UG and causes suppression and antibiotic resistance (Fig. 3). The conformation of an oligoribonucleotide that mimics the sarcin/ricin domain of rat 28S rRNA as determined by NMR spectroscopy shows the GAGA tetraloop and CG pair as part of a compact structure stabilized by unusual interactions (Szewczak et al., 1993). In vitro studies (Wool et al., 1992; Glück et al., 1994) showed that when the GAGA tetraloop is closed by CG, GC, AU, or UA Watson–Crick base pairs, it is recognized by both sarcin and ricin. However, when the closing pair is disrupted by being changed to CC, AA, or UU, ricin, but not sarcin, recognition is abolished. The UG mutation of the CG pair has not been studied in the in vitro system. Our data show that the *rdn-5* ribosomes continue to be recognized by ricin, suggesting that the mutation of the CG closing pair to UG does not destroy the conformation required for ricin recognition. On the basis of the different identities required for sarcin and ricin recognition in vitro, the sarcin/ricin loop was proposed to switch between two conformations during each elongation cycle—the alternate conformations being stimulated by the binding of different elongation factors (Nierhaus et al., 1992; Wool et al., 1992). Recent genetic data is consistent with an alternative conformation model. It has been shown in *E. coli* that when the CG pair is changed to CC in the presence of wild-type rDNA, the accumulation of the mutant 23S rRNA relative to wild type is greatly reduced in 70S ribosomes (Marchant & Hartley, 1994) and the analogous mutation appears to be a recessive lethal in yeast (R. Liu & S.W. Liebman, unpubl. result). One explanation of these results is that the mutant ribosomes can no longer oscillate between the alternate conformations. In the context of this model, *rdn-5*, which changes the CG pair to UG, may shift the normal equilibrium between the alternate conformations.

We propose that *rdn-5* ribosomes spend more time in the conformation associated with higher mistranslation frequencies.

MATERIALS AND METHODS

Plasmids

The pRDN plasmids used in this study are listed in Table 1. They are all ampicillin-resistant, contain both the yeast 2-μm and *E. coli* pBR322 replicators, and carry a single intact yeast 9-kb rDNA repeat. The plasmid rDNA repeats include the coding regions for 18S, 5.8S, 25S, and 5S rRNA, the polI promoter/enhancer, and the 5S polIII promoter. Some of the plasmids contain mutations in the rDNA. The recessive *hyg1* mutation in 18S rRNA causes resistance to hygromycin (Hyg^R) (E. Morgan, cited in Chernoff et al., 1994). Cells containing a pRDN plasmid with the *hyg1* mutation become resistant to hygromycin when at least some of the wild-type chromosomal rDNA repeats are missing. Deletions of different sizes in the chromosomal *RDN* array occur via homologous recombination. In this way, large deletions of essentially all the chromosomal rDNA repeats can be obtained that are stable under nonselective growth for at least 40 generations (Chernoff et al., 1994). The semi-dominant *ani1* mutation in 25S rRNA causes resistance to anisomycin (E. Morgan, cited in Chernoff et al., 1994). The *rdn-5* mutation in 25S rRNA isolated in this study causes translational suppression. The pRDN plasmids carry either the *URA3* or *TRP1* transformation selection markers and some contain a defective yeast *LEU2* gene, *LEU2-d*. This *LEU2-d* marker is used to select for high plasmid copy number, because only transformants containing high copy number of the *LEU2-d* gene can grow on medium lacking leucine (−Leu) (Rose & Broach, 1990).

The construction of the pRDN plasmids, pRDN-hyg1ani1-UL (previously called pRDN-hyg1), pRDN-wt-TL (previously called pRDN-wt), and pRDN-wt-U was described in Chernoff et al. (1994). Plasmid pRDN-5-hyg1ani1-UL was obtained by random mutagenesis of pRDN-hyg1ani1-UL and contains the *rdn-5* mutation. Construction of plasmids pRDN-5-hyg1-U and pRDN-5-TL is described under Site-directed mutagenesis. All remaining plasmids were constructed from these plasmids by the exchange of restriction fragments.

pJG2RA (kindly provided by M. Lord, Gould et al., 1991) is an integrating plasmid carrying *URA3* and *LEU2-d* and the ricin A chain gene (*RTA*) cloned under the *GAL1* promoter. This plasmid contains a unique *Eco*R I site within the *LEU2-d* gene.

Strains and cultivation conditions

Standard yeast cultivation conditions were used (Sherman et al., 1986). *Saccharomyces cerevisiae* strains used in this study are listed in Table 2. Suppression of nonsense (UGA, UAA, or UAG) and frameshift (fs) mutations in these strains was scored by velveteen replica-plating or by spotting cell suspensions (Sherman & Lawrence, 1974) onto synthetic glucose medium, SC, lacking a specific component (Rose et al., 1990), e.g., −Ade, which causes cell growth to be dependent upon suppression of the corresponding mutation, e.g., *ade1-14* (UGA). In addition, strains containing the *ade1-14* mutation accumulate a dark red pigment and therefore are dark red on complete glucose complex medium YPD (Rose et al., 1990). Suppression of the *ade1-14* allele abolishes the pigment accumulation and thus the cells are light pink on YPD. Deletions of the yeast rDNA array were selected in the presence of pRDN plasmids containing the *hyg1* mutation on YPD medium containing 0.3 mg/mL hygromycin (+Hyg). The presence of the anisomycin-resistance mutation (*ani1*) was scored on YPD medium containing 0.1 mg/mL anisomycin (+Ani). Ura^- yeast colonies were selected on synthetic media with 0.7 mg/mL 5-fluoroorotic acid (FOA, Rose et al., 1990).

L-1494 and L-1521 are isogenic to L-1489 except that they contain large deletions of the chromosomal *RDN* locus and are kept alive with pRDN plasmids. L-1581, which contains a copy of the *RTA* gene under the *GAL1* promoter integrated at the *LEU2* locus, was constructed by transforming 74-D694 (Chernoff et al., 1995) with *Eco*R I digested pJG2RA plasmid and selecting for Ura^+ integrants. Yeast transformation was performed as described by Gietz et al. (1992) unless otherwise specified.

To avoid the frequent plasmid rearrangements associated with the pRDN plasmids (Chernoff et al., 1994; Liebman et al., 1995), *E. coli* cells (DH5α) containing pRDN plasmid were grown on plates containing 0.05 mg/mL ampicillin (L-amp) rather than in L-amp liquid prior to DNA isolation. Bacteria were made competent according to Maniatis et al. (1982) or were purchased frozen from BRL Life Technologies, Inc. In all cases, bacterial transformation was done according to instructions supplied with the frozen competent cells.

Random mutagenesis

Plasmid pRDN-hyg1ani1-UL was mutagenized with hydroxylamine in vitro (Rose et al., 1990) for 41 h. To estimate the level of mutagenesis, small portions of the hydroxylamine mutagenized plasmid were transformed into *E. coli* $pyrF^-$ strain MC1066 (kindly provided by M. Casadaban). Because the $pyrF^-$ gene can be complemented by the yeast *URA3* gene, the frequency of *URA3* null alleles, 2.7%, was used as a measure of the level of mutagenesis of the plasmid. The remaining hydroxylamine-mutagenized plasmid was transformed (Ito et al., 1983) into yeast strain L63-31V-D543.

DNA sequencing

DNA sequencing was performed using the cycle-sequencing kit from USB. Sequencing gels of both strands of the *Tth111* I–*Pfl*M I region from plasmid containing the *rdn-5* mutation and plasmid lacking the *rdn-5* mutation were run side by side on a 6% polyacrylamide gel. The *rdn-5* mutation was identified as the only nucleotide change, C3022T, throughout the region.

Site-directed mutagenesis

The *rdn-5* mutation was remade with the two-step PCR procedure (Higuchi, 1990). The wild-type pRDN plasmid pRDN-wt-TL was used as template. Flanking primers, CGTTCTAGC ATTCAAGGT and GATTCTGACTTAGAGGCGTT, upstream

and downstream of the *Tth111* I and *Pfl*M I sites, respectively, and complementary mutagenic primers TTAGTATGAGAG GAACAGTT and AACTGTTCCTCTCATACTAA, each containing one mismatch at the underlined position, were used.

The *Tth111* I–*Pfl*M I piece of the amplified fragment was gel purified and cloned into plasmid pRDN-hyg1-U (see Table 1), generating pRDN-5-hyg1-U. DNA sequence analysis confirmed that the desired C → T change was the only alteration in this region. The *Mlu* I–*Sal* I fragment from pRDN-5-hyg1-U, containing *rdn-5* without *ani1*, was cloned into pRDN-wt-TL to make plasmid pRDN-5-TL (Table 1).

Primer extension analysis and sequencing of rRNA

Primer extension analysis of rRNA was performed as described (Chernoff et al., 1994) on total yeast RNAs (Schmitt et al., 1990). HPLC-purified oligonucleotide primer AUCC GAAUGAACUGUUCCUC was purchased from AMITOF and was 5′ end-labeled. If *rdn-5* (C3022U) rRNA serves as the template in the presence of dideoxy-ATP, DNA synthesis terminates at U3022, producing a fragment of 23 nt. If wild-type rRNA (C3022) is used as a template, DNA synthesis proceeds to the next U, which is two bases further away, producing a fragment of 25 nt and enabling a distinction between wild-type and mutant rRNA. This confirms that the *rdn-5* rRNA contains the C3022U change corresponding to the C3022T change in the DNA sequence. Products were separated on 20% polyacrylamide gels and visualized by autoradiography. Percentage of *rdn-5* rRNA in the total rRNA was determined by scanning the autoradiogram with a Digital Imaging System IS-1000 (Alpha Innotech Inc.).

In vitro assay for ribosome sensitivity to ricin

Ribosomes were assayed for ricin sensitivity as described previously (Morris & Wool, 1992). Briefly, isolated ribosomes (Grant et al., 1974) were treated with 0.01 μg/μL ricin A chain peptide, kindly supplied by I. Wool (University of Chicago), for 15 min at 37 °C. RNA was then phenol extracted and treated with aniline in acetic acid buffer (2.8 M acetic acid and 1 M aniline), or left untreated and separated on a 5% polyacrylamide gel containing 10% glycerol. The gel was visualized by EtBr staining.

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