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SPL1-1, a *Saccharomyces cerevisiae* Mutation Affecting tRNA Splicing

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A genetic approach was used to isolate and characterize *Saccharomyces cerevisiae* genes affecting tRNA processing. Three mutants were isolated which were able to process and utilize splicing-deficient transcripts from inactivated *Schizosaccharomyces pombe* suppressor tRNA genes. Extragenic recovery of suppressibility was verified by the suppression of nonsense mutations in *LEU2*, *HIS4*, and *ADE1*. One mutant, *SPL1-1*, was chosen for detailed analysis on the basis of its increased synthesis of mature suppressor tRNA over wild-type cell levels as determined by Northern (RNA) analysis. This mutant exhibited strong suppression exclusively with the defective tRNA gene used in the mutant selection. Genetic analysis revealed that a single, dominant, haplo-lethal mutation was responsible for the suppression phenotype. The mutation mapped on chromosome III to an essential 1.5-kb open reading frame (L. S. Symington and T. D. Petes, Mol. Cell. Biol. 8:595–604, 1988), recently named *NFS1* (S. G. Oliver et al., Nature [London] 357:38–46, 1992), located adjacent (centromere proximal) to *LEU2*.

tRNA biosynthesis is a complex multistep process. In eukaryotes, tRNA genes are transcribed into tRNA precursors which contain 5′ and 3′ flanking sequences, lack the 3′-terminal CCA sequence, and in some cases also contain an intron (7, 38). After maturation of the 5′ and 3′ termini of an intron-containing precursor tRNA, the intervening sequence is excised and the two tRNA half-molecules are ligated. Splicing of the intervening sequence is a multistep process that occurs in an enzyme complex on the inner surface of the nuclear membrane (4). In *Saccharomyces cerevisiae*, at least five enzymatic activities are required for tRNA splicing. In the first step, an endonucleolytic cleavage liberates the intron and generates two tRNA half-molecules. Before ligation can occur, the 2′,3′-cyclic phosphate of the 5′-half-molecule is cleaved to a 2′-phosphate and the 3′-half-molecule is phosphorylated. After adenylation of the ligase enzyme, the two half-molecules are joined in an ATP-dependent ligation step. A specific phosphatase releases the splice junction 2′-phosphate from the mature-size tRNA molecule to complete the reaction. Knowledge of the gene organization, enzymology, and localization of the tRNA splicing pathway in *S. cerevisiae* is quite detailed. The endonuclease activity has been partially purified and associates with a membrane-bound complex containing three polypeptides of 31, 42, and 51 kDa (35). The cyclic phosphodiesterase, kinase, and ligase activities have been shown to copurify with a single 95-kDa polypeptide (10, 34) located on the inner surface of the yeast nuclear membrane (4). The three activities are arranged sequentially along the length of the ligase protein, and the encoding gene has been shown to be essential for cellular viability (33). The 2′-phosphatase activity requires NAD^+ as a cofactor and mediates the cleavage and transfer of the splice junction 2′-phosphate to an unidentified acceptor molecule, possibly the NAD^+ cofactor (22, 23, 49).

Genetic selections in *S. cerevisiae* have yielded several mutants that indirectly influence tRNA biosynthesis or affect a small subset of intron-containing tRNAs, e.g., *rna1-1*, *los1-1*, *STP1*, and *tpd1*. The mutants *rna1-1* and *los1-1* show in vivo accumulation of end-matured, intron-containing precursor tRNAs and unprocessed rRNAs, but exhibit wild-type tRNA splicing activity in cell extracts and are thought to be involved in transport of RNA species across the nuclear membrane and/or maintenance of the nuclear membrane structure (1, 14). A gene disruption of *STP1* is not lethal, but leads to accumulation of a specific group of intron-containing precursor tRNAs (characterized by an extra stem-loop structure in the anticodon loop), suggesting that *STP1* may encode a "helper" function that provides proper conformation of the pre-tRNA or directs the tRNA to the splicing complex or facilitates binding of enzyme and substrate (46). The *tpd1* mutant shows in vivo accumulation of intron-containing precursor $tRNA^{Ser}$ at the nonpermissive temperature, but has not been tested for in vitro splicing activity (45).

Biochemical approaches have also been used to isolate genes affecting tRNA biosynthesis. The *sen1-1* mutant confers temperature-sensitive growth and results in pre-tRNA accumulation in vivo and in vitro (48). Cloning and characterization of *SEN1* show it to be essential for cellular viability and required for processing of all 10 families of intron-containing tRNAs (5). Mutant *sen2-3* accumulates tRNA two-thirds molecules (5′ half-molecule plus intron), which suggests that it may be defective in cleavage at the 5′ splice site of the intervening sequence (12). Antibodies raised against the *SEN2* gene product react with the 42-kDa subunit associated with endonuclease activity, suggesting that *SEN2* may encode a subunit of the endonuclease complex (35). The *pta1-1* allele causes conditional lethality and in vivo accumulation of pre-tRNAs at the restrictive temperature, although cell extracts show normal splicing activity (29, 30).

It is known that *Schizosaccharomyces pombe* UGA (opal) suppressor tRNA genes function in *S. cerevisiae* as active suppressors in vivo (13). The resulting *S. pombe* tRNAs can

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TABLE 1. *S. cerevisiae* strains constructed in this study

Strain	Relevant genotype	Description
M1-rs29	*MATa leu2-2*$_{UGA}$ *his4-260*$_{UGA}$ *ura3-52 trp1-1*	Haploid strain to generate splicing mutants
M8	*MATa leu2-2*$_{UGA}$ *his4-260*$_{UGA}$ *ura3-52 trp1-1* *MATα leu2-2*$_{UGA}$ *his4-260*$_{UGA}$ *ura3-52 TRP1*	Diploid strain to generate splicing mutants
CK105	*MATa SPL1-1 leu2-2*$_{UGA}$ *his4-260*$_{UGA}$ *ura3-52 trp1-1* *MATα spl1 leu2-2*$_{UGA}$ *HIS4 ura3-52 TRP1*	*SPL1-1* splicing mutant
CK119	*MATα SPL1-1 leu2-2*$_{UGA}$ *HIS4 ura3-52 trp1-1* *MATa spl1 leu2-2*$_{UGA}$ *his4-260*$_{UGA}$ *ura3-52 TRP1*	Outcrossed CK105 mutant
CK115	*MATa SPL1-1 leu2-2*$_{UGA}$ *his4-260*$_{UGA}$ *ura3-52 trp1-1 ade1-UGA* *MATα* spl1 *leu2-2*$_{UGA}$ *his4-260*$_{UGA}$ *ura3-52 TRP1 ade1-UGA*	Outcrossed CK105 mutant

easily be distinguished from the homologous *S. cerevisiae* tRNAs since they show minimal cross-hybridization by Northern (RNA) analysis because of their wide sequence divergence from *S. cerevisiae* (47). Therefore, we used a number of nonfunctional *S. pombe* UGA suppressor tRNAs inactivated by single-base mutations (32, 47) to select for *S. cerevisiae* mutants capable of splicing the intervening sequence of the defective tRNA transcripts to yield active opal suppressor tRNAs. Restored activity of the tRNAs was assayed by suppression of nonsense alleles *LEU2*, *HIS4*, and *ADE1*.

MATERIALS AND METHODS

General. The *S. cerevisiae* strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Complete and minimal media and plates were prepared as described previously (39). Selective minimal media and plates were supplemented with the amino acids and nucleotides needed to cover the auxotrophies that were not being assayed. 5-Fluoroorotic acid (FOA) plates were synthetic complete plates which contained 6.7 g of yeast nitrogen base (without amino acids with ammonium sulfate), 20 g of dextrose, and 1 g of amino acid mix supplemented with 875 mg of FOA (SCM Specialty Chemicals, Gainesville, Fla.) per liter and 30 mg of uracil. All components for FOA plates, except the agar, were dissolved by using minimal heat and were filter sterilized.

Sporulation, ascus dissection, and tetrad analysis were performed as described before (39). For analysis of marker segregation, random spore analysis was also performed (36).

Mutagenesis and mutant selection. Functional and defective *sup3-e* and *sup9-e* alleles and *sup8-e* were cloned on YCp50 and YRp17 (28, 32, 47). These constructs were transformed into the wild-type strain M8 by the lithium acetate method (15). *S. cerevisiae* transformants were grown to saturation in minimal medium selective for plasmid maintenance. A total of 10^9 cells was resuspended in 0.2 volume of 50 mM sodium phosphate buffer, pH 7.0. Incubation at 30°C for 30 min with 0.15 ml of ethyl methanesulfonate (Eastman Organic Chemicals, Rochester, N.Y.) resulted in 20 to 30% lethality. The mutagenesis was stopped with 6% sodium thiosulfate, which was followed by washes with double-distilled water. The mutagenized cells were divided into 10 flasks each containing 10 ml of minimal medium lacking uracil and grown overnight at 30°C. Cells were then plated on minimal plates lacking uracil and incubated at 30°C for 3 to 4 days. Colonies were replicated to minimal plates lacking leucine and histidine to assay for UGA suppressor activity. Growth on minimal plates after approximately 4 days indicated suppression of the nonsense codons in the *leu2-2*$_{UGA}$ and *his4-260*$_{UGA}$ alleles.

Suppressing colonies were picked onto FOA plates to select for colonies lacking the plasmid. The resultant Ura$^-$ colonies were replica plated to minimal plates lacking leucine and histidine to ensure that loss of suppression segregated with plasmid loss. Ura$^-$ Leu$^+$ His$^+$ colonies were presumed to be *S. cerevisiae* chromosomal tRNA suppressor mutants and were discarded. Ura$^-$ Leu$^-$ His$^-$ colonies were transformed with the original tRNA construct to ensure that suppression was restored upon retransformation. Mutants which exhibited a Ura$^+$ Leu$^+$ His$^+$ phenotype upon retransformation were chosen for further analysis.

Suppression assays. Opal suppressor activity was assayed in the original transformed or retransformed mutant strains. Suppression of the *leu2-2*$_{UGA}$ and *his4-260*$_{UGA}$ markers was assayed by scoring growth on minimal plates lacking leucine or histidine or both. The strength of the suppression was quantitated by counting the number of days required for full growth of the colonies. Suppression of the *ade1-UGA* marker was assayed by scoring growth and color on minimal plates with limiting adenine (7 mg/liter).

TABLE 2. List of plasmids

Designation	Relevant characteristics	Source (reference)
YRp17/tRNA clones (e.g., YRp17/*sup3-e* A46)	3.15-kb *ClaI*/*SalI* inserts into YRp17	Willis et al. (47)
YCp50/tRNA clones (e.g., YCp50/*sup3-e* A46)	1.0-kb *Hin*dIII/*Bam*HI inserts into YCp50	Nichols et al. (28)
E5F, H9G, K3B, D10H, G285-2D, J11D, 31C-B, 52D-H9G, D8B, A2C, C2G, G4B	YIp5 subclones of chromosome III covering the 140 kb between *MAT* and *LEU2*	Newlon et al. (27)
pRS316	Yeast centromeric shuttle vector *CEN6/ARSH4, URA3*	Sikorsky et al. (40)
pSPL3.5	*nfs1* clone in pRS316	This study
*Bcl*I, *Bcl*I/*Xho*I, *Bcl*I/*Sma*I clones of pSPL3.5	Disruptions and deletions of the 3.5-kb insert of pSPL3.5	This study

Northern blot analysis of *S. pombe* tRNA gene expression. Low-molecular-weight RNA was prepared by phenol extraction at 37°C (37). The RNA was resolved by electrophoresis on a 10% polyacrylamide–8 M urea gel (0.7 mm thick). Each lane contained 30 μg of RNA, and electrophoresis was performed at 500 V. Following electrophoresis, the gel was equilibrated in TAE buffer (40 mM Trizma base, 20 mM sodium acetate, 1 mM EDTA, adjusted with acetic acid to pH 7.4) for 15 min. The RNA was electrophoretically transferred to a Zeta-Probe membrane (Bio-Rad Laboratories) at 60 V for 5 h, using a Bio-Rad Trans-Blot Cell.

Prehybridization, hybridization, and posthybridization wash conditions were as described by the manufacturer, with the final wash at 60°C for 15 min. A 1.0-kb DNA fragment containing the *sup3-e* A39 gene was used as a probe. This fragment was labeled with [α-^{32}P]dATP to a specific activity of 10^8 cpm/μg by priming denatured DNA fragments with random hexamers, using Klenow enzyme (Pharmacia). The probe was separated from unincorporated [α-^{32}P]ATP by elution over a G-50 spin column (20).

Sequencing. M13 subclones were constructed by using restriction endonuclease fragments from the 3.5-kb insert in pSPL3.5 (20). Single-stranded DNA was isolated (24) and sequenced by the dideoxy method on a Du Pont Genesis 2000 automated DNA sequencing apparatus.

RESULTS

Selection scheme for isolation of mutants affecting tRNA intron splicing. For mutant selection we used inactive alleles of two efficient *S. pombe* UGA suppressor serine tRNAs (*sup3-e* and *sup9-e*) which differ in sequence by a C→U change at the tip of the extra arm (Fig. 1). These tRNA genes are cotranscribed with a downstream initiator methionine tRNA gene by using the internal control regions of the serine tRNA gene (21) (Fig. 2, top). These suppressors are active in suppressing *S. cerevisiae* UGA mutations (13). Previously, many mutants were made from these suppressors which displayed loss of suppressor activity or reduced suppressor activity in vivo (32, 47). Several of these inactive tRNA genes were employed to select for mutant enzymes in *S. cerevisiae* capable of processing the inactive tRNA precursors to mature, functional tRNA molecules. Figure 1 depicts the mutants used in this study, their nomenclature, and their biochemical lesions as determined by Northern analysis (32, 47). The mutant tRNA genes used exhibited defects in transcription (*sup3-e* A16 or A19 and *sup9-e* A53), 5′-end maturation (*sup3-e* C2), and splicing (*sup3-e* A39 and *sup9-e* A37:13, C39, A46, Ai47:8, and A68 and *sup8-e*).

The mutant selection scheme is presented in Fig. 3. For the isolation of haplo-lethal mutants, a diploid strain, M8, was constructed. The strain was homozygous for two UGA nonsense mutants, *leu2-2*$_{UGA}$ and *his4-260*$_{UGA}$, and for one nonrevertible marker, *ura3-52*, for maintenance of the plasmid. A haploid strain, M1-rs29, was also used which carried the same markers. The inactive tRNA genes were cloned on yeast plasmids, either the centromeric (single-copy) YCp50 or the *ars* plasmid (multicopy) YRp17, carrying the *URA3* gene for plasmid selection. A His$^+$ Leu$^+$ phenotype in a mutagenized cell demonstrated restored opal suppressor activity, indicating that the inactive precursor tRNA had been processed to its active form. To verify that suppressor activity was due to the expression of the plasmid-borne tRNA gene mediated by the newly generated chromosomal mutant and not to the generation of a chromosomal opal suppressor tRNA or to the mutation of the plasmid-borne

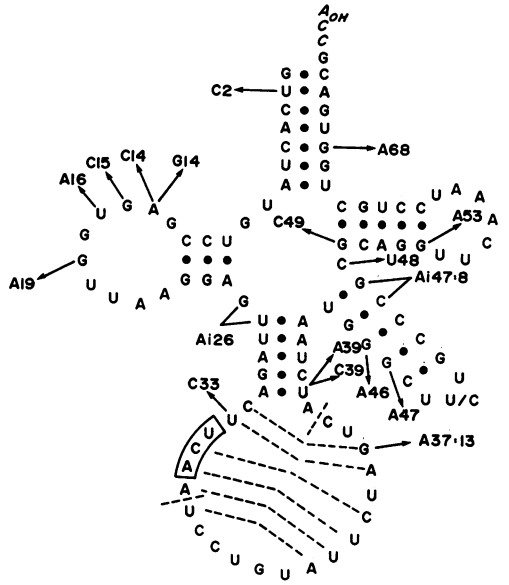

FIG. 1. Nucleotide substitutions in either *sup3-e* or *sup9-e* tRNA^{Ser}, shown on the tRNA cloverleaf form. The single-base mutations resulting in inactive tRNA genes used in this study are indicated. The nomenclature for mutants derives from the substituting nucleotide, followed by the position number of the substitution. An "i" is used to signify insertions which follow the position number given. The suppressor anticodon is boxed. Dotted lines indicate base pairing within the unspliced anticodon loop. The two dashed lines indicate the sites of intron cleavage. *sup3-e* and *sup9-e* differ in one base at the tip of the extra arm.

tRNA gene, Ura$^-$ colonies were selected on FOA plates (expected phenotype, Ura$^-$ His$^-$ Leu$^-$) and retransformed with the inactive tRNA gene construct (expected phenotype, Ura$^+$ His$^+$ Leu$^+$).

Isolation of mutants. Three mutants were isolated from separate mutageneses of approximately 10^9 cells each. These mutants facilitated suppression by splicing-deficient tRNAs only, suggesting that the mutations occurred in gene products involved in tRNA splicing. The mutants displayed no temperature sensitivity for growth or suppression, and their opal suppressor phenotypes were dominant as shown by crossing a mutant haploid derivative with a wild-type strain (data not shown). Dominance of the isolated mutants is inherent in this type of genetic selection which utilizes recovery of a function (recovery of suppression, in this case) and is, in fact, a major reason for employing this particular selection. Two mutants, CK104 and VC104, were isolated in the haploid strain M1-rs29 with *sup9-e* A46 cloned on YRp17. CK104 was allele specific in its suppression and exhibited suppression exclusively with *sup9-e* A46 on YRp17. VC104 exhibited suppression with several splicing-deficient tRNAs, but only when they were cloned onto YRp17. Both showed an increase in mature tRNA product as detected by Northern analysis (data not shown). Genetic analysis of these mutants revealed that the suppression phenotypes were due to two genes, each displaying partial suppression phenotypes. A third mutant, CK105, was isolated in the diploid strain M8 with *sup3-e* A39 cloned onto YCp50. Northern analysis of CK105 showed a significant increase in mature tRNA and a concomitant decrease in intron-containing precursor. Genetic analysis of CK105 demonstrated that a single gene was responsible for the

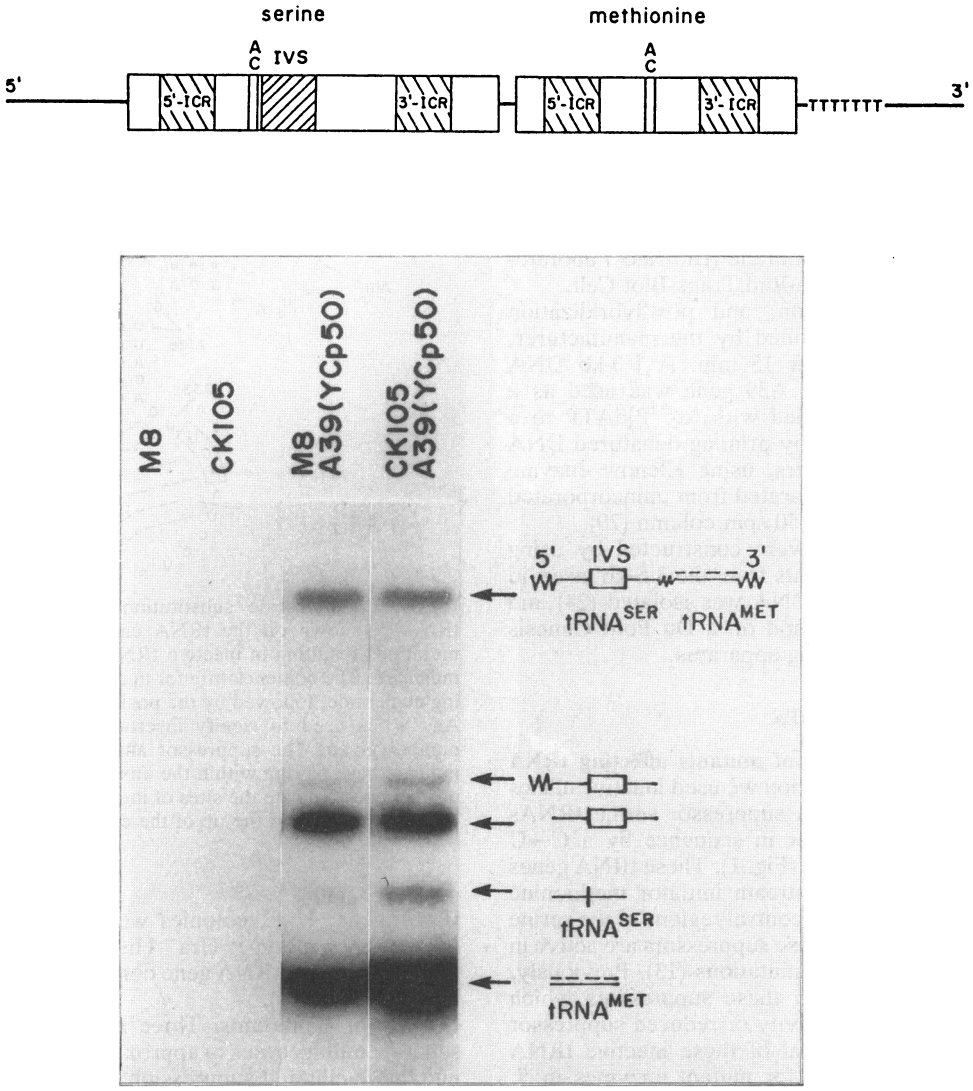

FIG. 2. (Top) Diagrammatic representation of the *sup3-e/sup9-e* dimeric gene. The anticodons of the serine and methionine tRNA genes are designated AC and the intervening sequence of the serine gene is designated IVS. The sequences corresponding to the 5′ and 3′ internal control regions (ICR) are hatched for each gene. RNA polymerase III transcription termination occurs in the oligo-T sequence. The two genes are separated by a 7-bp spacer. (Bottom) Expression of *S. pombe sup3-e* A39 in CK105 and M8. Northern hybridization analysis of RNA from CK105 and M8 strains, with and without *sup3-e* A39 (YCp50). The untransformed strains were grown in complete medium. The transformed strains were grown in minimal medium selective for plasmid-encoded *URA3*$^+$ expression. Diagrams depicting the unprocessed dimeric tRNA^{Ser}-tRNA^{Met} transcript, the intron-containing tRNA^{Ser} transcripts with and without 5′ flanking sequences, and the mature tRNA^{Ser} and tRNA^{Met} molecules are drawn on the right. The tRNA^{Ser} transcript is depicted as a solid line, and tRNA^{Met} is drawn as a combination solid/dashed line. The intervening sequence and 5′ and 3′ flanking regions of tRNA^{Ser} are indicated on the dimeric transcript. The spacer region between the two tRNAs is drawn as a small, wavy line. The RNA was probed with a 1.0-kb DNA fragment containing the *sup3-e* A39 gene containing the intron of tRNA^{Ser} and the tRNA^{Met}.

suppression phenotype, and the dominant mutant gene was designated *SPL1-1* (data presented below show that *spl1* maps to an open reading frame described previously by Oliver et al. [31] designated *nfs1*, and joint nomenclature will be used throughout Results). For the reasons given above, we continued the analysis only with *SPL1-1/NFS1-1*.

Suppression with different inactive tRNA genes with *SPL1-1/NFS1-1*. To test whether the suppressibility of *SPL1-1/NFS1-1* was specific for *sup3-e* A39, the heterozygous mutant strain CK105 was transformed with other *S. pombe* tRNA genes defective in transcription, 5′-end maturation, and splicing. The ability of these tRNA constructs to suppress in wild-type and mutant strains is shown in Table 3. *SPL1-1/NFS1-1* only exhibited strong suppression with *sup3-e* A39, the inactive tRNA gene used in its isolation. The suppression observed with *sup9-e* C39 and *sup9-e* Ai47:8 was slightly enhanced. However, the weak suppression exhibited by wild-type strains transformed with C39 and Ai47:8 suggests that these precursor tRNAs are processed into functional tRNAs, but at a slower rate than the parent suppressor tRNA, *sup9-e*. Therefore, the longer generation time of the mutant strain (data not shown) may permit adequate concentrations of mature C39 and Ai47:8 tRNA^{Ser} to accumulate, yielding a stronger suppression phenotype

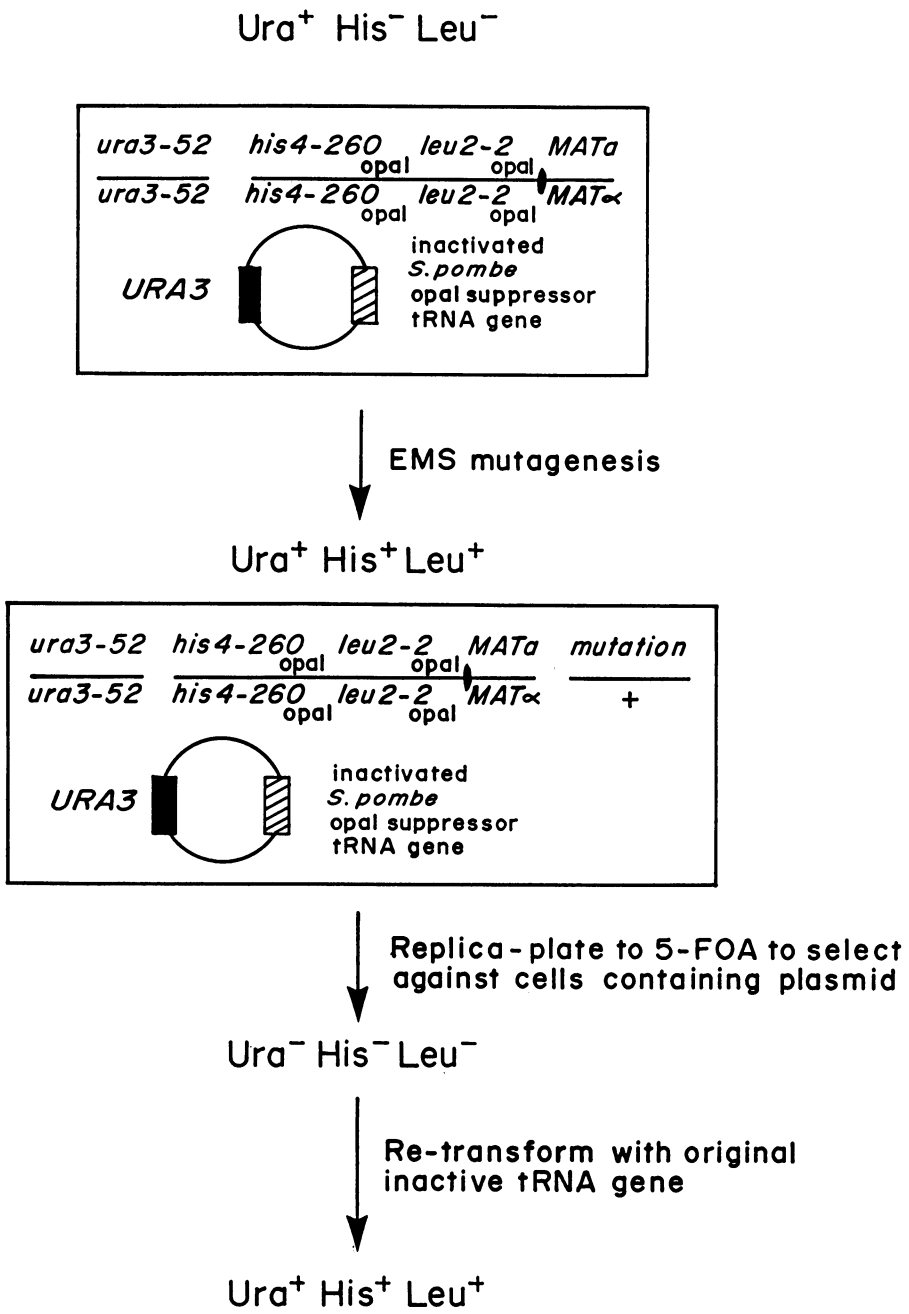

FIG. 3. Mutant selection scheme. The parental strain M8 was transformed with an inactive tRNA gene, resulting in a phenotype of Ura$^+$ Leu$^-$ His$^-$. Next, the transformed strain was mutagenized with ethyl methanesulfonate (EMS) and opal suppressing colonies were selected, i.e., selection for Ura$^+$ Leu$^+$ His$^+$ colonies. In the next step, the suppressing colonies were cured of the plasmid by growth on 5-FOA plates. Then the Ura$^-$ colonies were examined for the ability to grow on plates lacking leucine and histidine to determine whether suppressor activity was dependent on the presence of the plasmid; i.e., a phenotype of Ura$^-$ Leu$^-$ His$^-$ demonstrated co-segregation of suppressor activity and the plasmid-encoded tRNA. Finally, the cured colonies were retransformed with the inactive tRNA gene and assayed for suppressor activity as demonstrated by a phenotype of Ura$^+$ Leu$^+$ His$^+$.

than that exhibited by wild-type strains. Thus, it appears that *SPL1-1/NFS1-1* is allele specific for the *sup3-e* A39 suppressor tRNA gene.

Transcription and processing with *SPL1-1/NFS1-1*. Differences in processing of the splicing-deficient *sup3-e* A39 precursor by wild-type and mutant alleles of *spl1/nfs1* were examined by Northern blot analysis (Fig. 2, bottom). RNA from the wild-type strain showed accumulation of the intron-containing *sup3-e* A39 precursor tRNA and virtually no mature A39 tRNA^{Ser}. RNA from the *SPL1-1/NFS1-1*(*sup3-e* A39) transformant showed a significant increase in the amount of mature suppressor tRNA^{Ser} relative to the wild-type strain, consistent with the opal suppression phenotype of *SPL1-1/NFS1-1*. Quantitation of the differences in pro-

TABLE 3. Suppression by *SPL1-1/NFS1-1* with different inactive tRNA genes[a]

tRNA gene	Defect[b]	Vector	Growth by strain: M8 (*spl1/spl1*)	Growth by strain: CK105 (*SPL1-1/spl1*)
C2	p	YCp50	−	−
C14	s	YCp50	−	−
G14	s	YCp50	−	−
C15	s	YCp50	−	−
A19	t, p	YCp50	−	−
Ai26	s	YCp50	−	−
C33	s	YCp50	−	−
A37:13	s	YCp50	−	−
A37:13	s	YRp17	−	−
A39	s	YCp50	−	+++
C39	s	YCp50	+	++
A46	s	YCp50	−	−
A46	s	YRp17	−	−
A47	s	YCp50	−	−
Ai47:8	s	YRp17	+	++
U48	t	YCp50	−	−
C49	t, p	YCp50	−	−
A53	t	YCp50	−	−
A68	s	YCp50	−	−
sup8-e	s	YCp50	−	−

[a] Growth on plates lacking leucine by strains transformed with different tRNA genes. +++, colony growth in 1 day; ++, 2 days; +, 3 days; −, no growth.

[b] t, transcriptional defect; p, 5′ processing defect; s, splicing defect.

cessing of the *sup3-e* A39 precursor by the wild-type and mutant strains was determined by scintillation counting of the relevant radioactive bands cut from a Northern blot. The amounts of mature tRNA and intron-containing precursor were standardized relative to the amount of primary transcript which was equivalent in both wild-type and mutant strains. With this protocol, a 300% increase in mature $tRNA^{Ser}$ and a 20% decrease in intron-containing precursor were exhibited by the mutant strain relative to the wild-type strain. The increase in mature product and concomitant decrease in precursor suggest that increased processing of precursor tRNA into mature $tRNA^{Ser}$ is occurring, thus implying an effect on tRNA splicing by *SPL1-1/NFS1-1*.

An increase in the amount mature $tRNA^{Met}$ is also observed in the mutant transformant (Fig. 2, bottom). However, it is known from previous Northern blot analyses that the wild-type $tRNA^{Met}$ is more stable than the mutant $tRNA^{Ser}$ and that its hybridization consistently exceeds the combined hybridization of the $tRNA^{Ser}$ intermediates and mature product (47). This may be because the DNA probe used for hybridization was perfectly homologous to $tRNA^{Met}$ but contained the intron of $tRNA^{Ser}$, making hybridization to mature $tRNA^{Ser}$ less efficient. Furthermore, the longer generation time of the mutant strain may contribute to the increased accumulations of the more stable $tRNA^{Met}$.

To determine whether *SPL1-1/NFS1-1* exerted an influence over the synthesis of *S. cerevisiae* tRNAs, the expression of six *S. cerevisiae* tRNA genes (the intron-containing tRNA genes for $tRNA^{Ser}_{CGA}$, $tRNA^{Trp}$, $tRNA^{Pro}$, $tRNA^{Leu}_{UAG}$, and $tRNA^{Ile}$ and the intron-less tRNA gene for $tRNA^{Arg}$) and the 5.8S rRNA gene was studied. Oligonucleotides homologous to the introns and the splice junctions of the tRNAs and to an internal sequence of 5.8S rRNA served as probes in Northern blots. Expression of these genes was studied with and without the plasmid-borne *S. pombe sup3-e* A39 gene to determine whether the *SPL1-1/NFS1-1* gene product reacted differently in the presence of its preferred substrate, *sup3-e* A39. Overall, there was no difference between the wild-type and mutant strains in their synthesis of any of the six tRNAs or 5.8S rRNA (data not shown). Therefore, the synthesis of *S. cerevisiae* tRNAs (with and without introns) and 5.8S rRNA does not seem to be affected by *SPL1-1/NFS1-1*.

TABLE 4. Tetrad analysis of strain CK105 (*SPL1-1/spl1*): segregation of spore viability

Ratio of viable/nonviable spores[a]	No. of tetrads
$0^+:4^-$	4
$1^+:3^-$	11
$2^+:2^-$	13
$3^+:1^-$	0
$4^+:0^-$	0

[a] All surviving spore colonies were incapable of suppression when transformed with *sup3-e* A39.

Genetic analysis of *SPL1-1/NFS1-1*. Tetrad analysis revealed the presence of a haplo-lethal mutation in the original mutant strain CK105 (Table 4). All surviving spores were incapable of suppression, suggesting that a single mutation was responsible for the suppression phenotype and for the haplo-lethal phenotype. The wild-type gene was named *spl1*, for splicing.

The nonrandom segregation of *MAT* and *HIS4* in the surviving spores (MATα His^+, 27; MAT**a** His^+, 6; MATα His^-, 4; MAT**a** His^-, 0) suggested that *spl1/nfs1* was linked to these loci on chromosome III. Because spores containing *SPL1-1/NFS1-1* were nonviable, the lack of MAT**a** His^- spores implied that *SPL1-1/NFS1-1*, *MAT***a**, and *his4-260* mapped to the same copy of chromosome III.

Since the region between *MAT* and *HIS4* had been mapped and cloned by Newlon et al. (27), we obtained 11 subclones spanning this 140-kb region. Transformants of the clone carrying *spl1/nfs1* should complement the lethality of *SPL1-1/NFS1-1* and lead to the random segregation of *MAT* and *HIS4*. The different subclones were transformed into CK105 and maintained by integration into homologous sequences on chromosome III or by autonomously replicating sequences present in the insert DNA. It was found that clone G4B complemented the lethality of *SPL1-1/NFS1-1*. Table 5 shows the distribution of spore phenotypes, using a random spore analysis, of three transformants with plasmids containing contiguous DNA fragments covering *CEN3* to *LEU2*. Transformation with 10 of the plasmids maintained the preponderance of His^+ MATα spores originally seen when

TABLE 5. Complementation of the haplo-lethality of *SPL1-1* by a 7.5-kb DNA insert of G4B

Strain	Segregation of markers (% with given phenotype) in viable haploid spores from CK105 (*SPL1-1/spl1*) transformants[a]: His^+ : *MAT*α	His^- : *MAT*α	His^+ : *MAT***a**	His^- : *MAT***a**	Total spores
CK105:G4B	25	19	19	38	16
CK105:D8B	96	4	0	0	24
CK105:C2G	78	6	13	3	31

[a] Random spore analysis was performed.

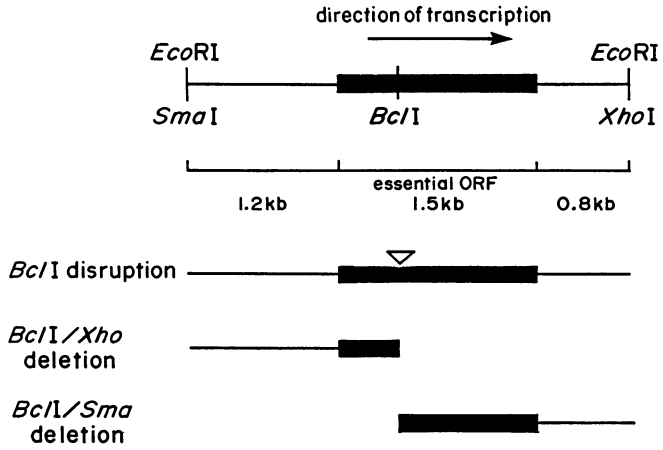

FIG. 4. Disruption and deletions of *nfs1* in the 3.5-kb insert of pSPL3.5. Three subclones were constructed which created one *URA3* disruption at the *Bcl*I site and two deletions on either side of the *Bcl*I site in *nfs1*. The top drawing shows the pertinent restriction endonuclease sites and a black box depicting the *nfs1* gene. The line immediately below details the sizes of the gene and the flanking regions. ORF, open reading frame.

spores from the untransformed strain were analyzed (data shown for two representative plasmids). Transformation with G4B, however, resulted in the random segregation of *MAT* and *HIS4* alleles, demonstrating that G4B carried a copy of *spl1/nfs1*.

The clone G4B contained a 7.5-kb *Bam*HI DNA fragment located approximately 15 kb centromere proximal from *HIS4*. Symington and Petes had identified an essential 1.2-kb region on this fragment on the basis of the inviability of spores containing DNA insertions in this region (42). DNA sequencing of chromosome III revealed that this region encoded a 1.5-kb open reading frame containing the proper signals for transcription and translation initiation and termination, and the gene was designated *nfs1* (31).

To verify that *spl1* and *nfs1* were the same gene, we demonstrated that *nfs1* was able to complement the haplo-lethal phenotype of *SPL1-1/NFS1-1*. Subclones were constructed that disrupted or deleted portions of the *nfs1* gene cloned onto pSPL3.5 (a complementing 3.5-kb subclone of the G4B insert) (Fig. 4). Four clones, including intact pSPL3.5, were transformed into CK119, a heterozygous mutant strain, and tetrad analysis was performed. Only intact pSPL3.5 complemented the haplo-lethal phenotype (Table 6). However, as a centromeric plasmid, it did not rescue every *SPL1-1/NFS1-1* spore, but resulted in increased 3:1 and 4:0 segregation for viability. In the tetrads with four viable spores, 2:2 segregation of FOA^s:FOA^r was observed, implying that the *URA3*$^+$ −pSPL3.5 clone segregated with the *SPL1-1/NFS1-1* allele in the two FOA^s spores and was, therefore, necessary for survival. The untransformed strain and the deletion and disruption subclone transformants displayed a maximum of 2:2 segregation for viability and also displayed the nonrandom segregation of *MAT* and *HIS4* alleles, demonstrating their inability to complement the haplo-lethal phenotype. Since none of the disruption or deletion *nfs1* subclones was able to complement the haplo-lethality of *SPL1-1/NFS1-1*, we report that *spl1* and *nfs1* (31) are the same gene and will henceforth be referred to as *nfs1*.

To verify that the suppression phenotype of mutant strain

TABLE 6. Tetrad analysis of CK119 (*SPL1-1/spl1*) transformants with *nfs1* subclones: segregation of spore viability

Strain	No. of tetrads with given ratio of viable/nonviable spores				
	$0^+:4^-$	$1^+:3^-$	$2^+:2^-$	$3^+:1^-$	$4^+:0^-$
CK119[a]	2	1	9	0	0
CK119:pSPL3.5, intact *nfs1*[b]	1	1	2	3	7
CK119:pSPL3.5, *Bcl*I disruption[a]	3	5	6	0	0
CK119:pSPL3.5, *Bcl*I/*Xho*I disruption[a]	2	6	7	0	0
CK119:pSPL3.5, *Bcl*I/*Sma*I disruption[a]	2	4	8	0	0

[a] The phenotypes of the viable spores from these strains displayed the nonrandom *MAT* and *HIS4* segregation typical of *SPL1-1/spl1* strains.
[b] The phenotypes of spores from CK119 transformed with pSPL3.5 showed random segregation of the *MAT* and *HIS4* alleles.

CK105 also mapped to *nfs1*, a *URA3*$^+$ disruption of *nfs1* was constructed to gene-convert the homologous sequence on chromosome III, *NFS1-1* or *nfs1*, in the heterozygous mutant strain CK115. This experiment was intended to test whether the conversion of the *NFS1-1* allele by the *URA3*$^+$-disrupted *nfs1* gene would also abolish suppression. Since the mutant strain CK115 carried only single copies of *nfs1* and *NFS1-1*, conversion of the wild-type *nfs1* allele would result in an inviable cell. Therefore, only conversion of the mutant *NFS1-1* allele would be isolated. The mutant strain, with a stable *URA3*$^+$ integration, was assayed for opal suppressor activity by transformation with *sup3-e* A39 on YCp50 and growth on plates lacking adenine and histidine. In CK115 (*SPL1-1/spl1*), white colonies on limiting adenine formed in 2 days, and there was growth in 4 days on plates with no histidine. In CK115 (*SPL1-1*::*URA3*$^+$/*spl1*), red colonies formed on limiting adenine, and there was no growth on plates with no histidine. The disrupted strain was shown to be incapable of suppressing the *ade1-UGA* and *his4-260* mutations, suggesting that the plasmid-borne *nfs1* disruption had gene-converted the *NFS1-1* allele, resulting in elimination of the suppression due to *NFS1-1* expression. Demonstrating the role of *nfs1* in tRNA splicing, both the haplo-lethal phenotype and the opal suppression phenotype of the mutant strain CK105 map to the newly described *nfs1* gene.

DNA sequence and amino acid homology of *nfs1*. We determined the nucleotide sequence of *nfs1* (GenBank accession number M98808), which was also accomplished when the yeast chromosome III was sequenced (31). The gene sequence showed good homology to the *nifS* genes in the nitrogen-fixing bacteria *Azotobacter vinelandii* and *A. chroococcum*, *Anabaena* sp., and *Klebsiella pneumoniae* (31). A data base search also showed that the dimorphic yeast *Candida maltosa* contained a highly homologous gene located at the same chromosomal position as *nfs1*, i.e., adjacent to *LEU2* (31, 44).

An alignment of the amino acid sequence of the deduced *S. cerevisiae* NifS protein product of 497 amino acids with that of *C. maltosa* and *Anabaena* sp. (Fig. 5) reveals a very high level of amino acid identity even though the two yeasts are evolutionarily quite distant (2). These NifS proteins represent a distinct class of highly conserved proteins in which not only is the amino acid sequence conserved across the divide between prokaryotes and eukaryotes, but the

```
          1                                                                    80
C. mal    .......... .......... .......... .......... .......... .......... .......... ..........
S. cer    MLKSTATRSI TRLSQVYNVP AATYRACLVS RRFYSPPAAG VKLDDNFSLE THTDIQAAAK AQASARASAS GTTPDAVVAS
Anabae    .......... .......... .......... .......... .......... .......... .......... ..........

          81                                                                   160
C. mal    .......... .......... .......... .......... .......... .......... .......... ..........
S. cer    GSTAMSHAYQ ENTGFGTRPI YLDMQATTPT DPRVLDTMLK FYTGLYGNPH SNTHSYGWET NTAVENARAY VAKMINADPK
Anabae    .......... ......MSVI YLDNNATTKV DPDVVEAIMP YLTDYYGNP. SSMHTFGGQL GKAVRTAREQ VAALLGAD.E
                               * ***  ***   ** *        *  ****  *  *  *      **  **   **    **

          161                                                                  240
C. mal    .......... .......... .......... .......... .......... .......... .......... ..........
S. cer    .EIIFTSGAT ESNNMVLKGV PRFYKKTKKH IITTRTEHKC VLEAARAMMK EGFEVTFLNV DDQGLIDLKE LEDAIRPDTC
Anabae    SEIVFTSCGT EGDNAAIRAA LLAQPA.KRH IITTQVEHPA VLNVCKQLET QGYTVTYLSV NSHGQLDLDE LEASLTGNTA
           ** ***  * *  *              * * ***   **   **          *  ** * *    * *** * **      *

          241                                                                  320
C. mal    .......... .......... .......... .......... .......... .......... .......... ..........
S. cer    LVSVMAVNNE IGVIQPIKEI GA.ICRKNKI YFHTDAAQAY GK.IHIDVNE MNIDLLSISS HKIYGPKGIG AIYVRRRPRV
Anabae    LVTIMYANNE TGTVFPIEEI GK.RVKERGA IFHVDAVQAV GK.IPLNMKT STIDMLTISG HKIHAPKGIG ALYVRRGVRF
          **  *  ***  *   ** ** *           ** ** **  ** *       * ** * **  **   * * * * ****  *

          321                                                                  400
C. mal    ..DPIITGGG QERGLRSGTL APPLVAGFGE AARLMKQESA FDKKHIEKLS TKLKNGLLSI PSTQFNGCNN PAYQYPGCVN
S. cer    RLEPLLSGGG QERGLRSGTL APPLVAGFGE AARLMKKEFD NDQAHIKRLS DKLVKGLLSA EHTTLNGS.. PDHRYPGCVN
Anabae    R..PLLIGGH QERGRRAGTE NVPGIVGLGK AAELELIHIE TAIKKETRLR DRLEQTLLAK IPDCE.VNGD ITQRLPNTTN
          *  * * **  **** * **    *   * *  ** *                        **                 **   *

          401                                                                  480
C. mal    VSFAYIEGES LLMALK..DI ALSSGSACTS ASLEPSYVLH ALGADDALAH SSIRFGIGRF TTEAEVDYVI QAINERVHFL
S. cer    VSFAYVEGES LLMALR..DI ALSSGSACTS ASLEPSYVLH ALGKDDALAH SSIRFGIGRF STEEEVDYVV KAVSDRVKFL
Anabae    IGFKYIEGEA ILLSLNKYGI CASSGSACTS GSLEPSHVLR AMGLPYTTLH GSIRFSLCRY TTEAQIDRVI EVMPEIVERL
              * ***   *  *    *   ********  ***** **  * *      *  **** * *   **   * *        * *

          481
C. mal    RKKTPLWEMV QEGIDLNSIE WSGH
S. cer    RELSPLWEMV QEGIDLNSIK WSGH
Anabae    RALSPFKNDE AGWLQAQEQT LAHR
          *   *
```

FIG. 5. Alignment of *nifS*-like sequences. The alignment was created with the Pileup routine of the GCG sequence analysis software package (8). An asterisk denotes positions of identical amino acids. The references for the sequences are (GenBank/EMBL accession no.) as follows: C. mal, *C. maltosa* (44), X05459; S. cer, *S. cerevisiae* (31), Swissprot no. P25374 and GenBank no. M98808; Anabae: *Anabaena* sp. (25, 26). For maximum homology of the *C. maltosa* protein, two frameshifted regions of the published nucleotide sequence (reference 44, X05459) were corrected, inserting a G after A223 and deleting G425. The known nucleotide sequence contains only the carboxy-terminal half of the *nifS*-like open reading frame.

relative position of the *nifS* homolog is completely conserved in the genomes of two unrelated yeasts (2).

DISCUSSION

The results presented here demonstrate that extragenic activation of inactive mutant suppressor tRNA genes can be used to isolate genes which affect tRNA splicing. Such an approach is complementary to biochemical strategies. Presumably, the mutant *NFS1-1* activates the mutant precursor tRNA by, directly or indirectly, splicing the tRNA into its mature form. While the nature of the *nfs1* gene product is not known, it is evident from the sequence and size of *nfs1* that this gene cannot encode the splicing ligase or the two smaller subunits associated with the splicing endonuclease. The 1.5-kb open reading frame of *nfs1* would encode a protein of 55 kDa and, therefore, may encode the large subunit of the endonuclease complex (molecular weight, 51,000 [35]). Alternatively, *nfs1* may not directly encode any of the enzymatic activities of the splicing process but may encode a protein which facilitates the formation of the correct RNA structure required for splicing or recognition of this structure, or *nfs1* may affect the formation or activity of the correct multiprotein splicing complex and its location in the nuclear membrane. The relation of *nfs1* to the *nifS* genes of nitrogen-fixing bacteria, involved in a very different metabolic pathway, may provide insight into the role of *nfs1* in yeasts (see discussion below).

The allele specificity of *NFS1-1* may suggest potential sites of recognition on the tRNA by *nfs1*. *NFS1-1* imparts full opal suppressor activity exclusively with *sup3-e* A39 and not with tRNAs carrying mutations at other sites or even with a substitution at the same site, i.e., *sup3-e* C39. The precise splicing problem that occurs with *sup3-e* A39 or C39 is not known except that the accumulation of intron-containing precursor with both tRNAs as shown by Northern analysis (data not shown) implies failure to cleave the precursor at all as opposed to cleavage at the incorrect

position. The ability of *NFS1-1* to distinguish between *sup3-e* A39 and *sup3-e* C39 may highlight a specific functional group on *sup3-e* A39 with which *NFS1-1* interacts or it may be due to slightly different tertiary conformations assumed by the two mutant precursor tRNAs.

NFS1-1 recognizes the tRNA mutation which occurs one nucleotide after the 3′ splice site, i.e., position 39. This may implicate *NFS1-1* in 3′ splice site cleavage. Although only limited features of pre-tRNA sequence and structure are thought to be recognized by the splicing complex, two general requirements are maintenance of the mature tRNA cloverleaf structure and exposure of the 3′ splice site in a single-stranded region (11, 19, 41, 43). *nfs1* may have a role in maintaining the positioning and/or structure surrounding the 3′ splice site for recognition.

Many loss-of-suppression mutants isolated in other studies were ultimately found to be nuclear transport or nuclear membrane stability mutants (1, 14). While the mutants exhibited in vivo accumulation of intron-containing precursor tRNAs, cell extracts showed no defect in splicing activity. This is not likely to be the case with *NFS1-1* because in vivo and in vitro analyses of the inactive tRNA gene used in the isolation of *NFS1-1*, *sup3-e* A39, both demonstrated that this tRNA was specifically deficient in splicing. Removal of the nuclear transport step, as provided by cell extracts, does not change the processing pattern of *sup3-e* A39. Therefore, the ability of *NFS1-1* to activate *sup3-e* A39 is unlikely to be linked to nuclear transport or membrane stability.

The high degree of amino acid sequence homology exhibited by the *nifS*-like proteins, including *nfs1* from *S. cerevisiae* and the *C. maltosa* homolog, suggests a similar level of functional homology. Recently, it was shown that the purified *Anabaena nifS* gene possesses a new enzymatic activity, cysteine desulfurase, which is thought to be involved in the formation of Fe-S clusters in enzymes (5a). Thus, the requirement of NAD^+ for the phosphatase step of tRNA splicing (23) and the requirement for functional Fe-S clusters in nitrogenase may be the common thread running through these seemingly disparate reactions.

An impressive collection of tRNA splicing mutants has been generated and characterized in order to study the intricate and interrelated steps of tRNA processing in *S. cerevisiae*. *NFS1-1* joins this collection of mutants which encode different gene products involved in tRNA maturation. Genetic selections of the type described here continue to be invaluable for identifying the total complement of such proteins and elucidating their relationship to one another.

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