

RECESSIVE LETHAL AMBER SUPPRESSORS IN YEAST

by

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ABSTRACT

Recessive Lethal Amber Suppressors in Yeast, by Marjorie C. Brandriss.
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Thesis Advisor: Dr. David Botstein, Associate Professor of Genetics

Suppressor mutations which reversed the phenotypes of two amber mutations were isolated in diploid strains of the yeast, Saccharomyces cerevisiae. These suppressor mutations differ from those isolated in haploid yeast since they are recessive lethal, i.e., they cannot be isolated or maintained in a haploid strain. The frequency of this type of mutation is very high; in fact, a majority of the suppressors isolated in diploids (using reversion of two different pairs of amber mutations) were recessive lethal.

Diploids carrying this mutation upon sporulation yield asci with only two live spores, both lacking the suppressor. At least two classes of recessive lethal suppressors exist. Aneuploid strains carrying one wild-type and one suppressor locus have been isolated and used in mapping studies: one suppressor mutation maps on chromosome III, the other does not.

One suppressor, SUP-RL1, was characterized further. Diploid strains were constructed which were heterozygous for thr4, MAL2 and SUP-RL1 in order to map the locus of the suppressor more precisely. Both tetrad and random spore analyses showed that the suppressor maps approximately 30 cm distal to the mating-type locus, between thr4 and MAL2, on chromosome III.

SUP-RL1 was introduced into diploid strains homozygous for an amber mutation corresponding to position 9 of iso-1-cytochrome c. The suppressed protein was isolated and analyzed; serine was found at position 9, replacing the wild-type residue, lysine.

It is believed that SUP-RL1 codes for an essential tRNA which is either a unique coding species or carries out another vital function possibly unrelated to protein synthesis. If SUP-RL1 codes for a serine tRNA, and not another tRNA mischarged with serine, it could be the serine tRNA which recognizes the codon UCG. There is an indication that, in yeast, this serine codon may be recognized by a single species of serine tRNA, which, when mutated to read the amber (UAG) codon, could result in recessive lethality.

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CHAPTER I
AN INTRODUCTION TO NONSENSE SUPPRESSION

Mutations which correct the phenotype of other mutations by affecting the fidelity of transmission of genetic information from DNA to protein have been called "informational suppressors." These suppressors correct mutational errors in the DNA which would otherwise result in mutant proteins or fragments of proteins. The errors in the DNA can be of several kinds: (1) missense mutations in which a codon specifying the correct amino acid has been mutated to another codon specifying another amino acid resulting in a partially or completely inactive protein (Yanofsky *et al.*, 1966); (2) frameshift mutations in which the triplet reading frame has been shifted by the addition or deletion of one or more bases in the DNA yielding severely altered proteins (Terzaghi *et al.*, 1966); or (3) nonsense mutations, in which a triplet coding for an amino acid has been mutated to one signalling chain termination, resulting in the formation of an incomplete polypeptide chain (Benzer and Champe, 1962; Garen and Siddiqui, 1962).

These primary mutations can, of course, be corrected by secondary intragenic mutations either in the affected codon or elsewhere within the gene. They can also be corrected by mutations in other genes called suppressor genes. The molecular basis for some suppressors of the types of mutations listed above have been found to be alterations in tRNA. The altered tRNAs misread the mutant codon as an acceptable amino acid or restore the reading frame. Most informational

suppressors respond to particular codons and not to specific loci. Missense suppressors (Carbon et al., 1966) insert an acceptable amino acid at a site which codes for an unacceptable amino acid. For instance, mutants in the glyU gene have been found which yield altered glycyl-tRNA and transfer glycine when the message reads AGA, a codon for arginine. Frameshift suppressors (Riddle and Roth, 1970; Roth, 1974) have been found which result in altered tRNAs with anticodons containing four bases instead of three. If the original mutation was an addition of G to a series of Gs, the frameshift suppressor restores the frame by reading the four bases at once. Nonsense suppressors result from mutated tRNAs whose anticodons read chain termination codons as if they were sense codons. When this occurs at a nonsense (mutant) codon in the middle of a gene, it results in the formation of a complete peptide and an active protein if the amino acid insertion was an acceptable one (Benzer and Champe, 1962; Garen and Siddiqi, 1962).

A fourth class of suppressors, ribosomal suppressors, can cause general misreading of the message. They lack codon specificity and usually act with low efficiency (Gorini, 1970).

The subject of this thesis concerns the isolation of a new class of nonsense suppressors in the yeast Saccharomyces cerevisiae. What follows is a brief review of nonsense suppression in the three organisms in which this phenomenon is best understood: Escherichia coli, Salmonella typhimurium, and Saccharomyces cerevisiae. Suppressors have been isolated in other organisms which are believed to be similar

to those described above, but they are as yet poorly characterized at the molecular level. These include mutants of Bacillus subtilis (Sherwin and Carlton, 1971), Schizosaccharomyces pombe (Hawthorne and Leupold, 1974), Neurospora crassa (Seale, 1972), and Drosophila melanogaster (Lindsley and Grell, 1968).

I. Nonsense suppression in *E. coli* and *S. typhimurium*

The phenomenon of nonsense suppression in bacteria has been well characterized and many of its aspects are well understood. Excellent reviews have been written on the subject which provide greater detail than this summary (Gorini and Beckwith, 1966; Garen, 1968; Gorini, 1970; Hartman and Roth, 1973).

A. Isolation of nonsense mutations and their properties in bacteria

Some of the earliest reports of suppressible mutations, i. e., ones which depended on a host function to yield wild-type or partially wild-type activity, came from work on T4rII mutants (Benzer and Champe, 1962) and on alkaline phosphatase mutants of *E. coli* (Garen and Siddiqi, 1962).

These mutations, as well as many others isolated since then, display an extreme negative mutant phenotype. This is evident when one looks for intragenic complementation or enzyme activity. Garen and Siddiqi (1962) showed that their nonsense mutants of alkaline phosphatase lacked enzymatic activity, immunological cross-reacting material (CRM), and the ability to complement intragenically. This led to the conclusion that probably only a fragment of the protein was being made. Generally, nonsense mutants are not leaky, temperature-sensitive, or osmotic remedial and are non-complementing. Often, however, large enough fragments of protein are made to show up as CRM. When intragenic complementation is found, it is usually with

mutants located to one side of the nonsense mutation (polarized complementation, Hartman et al., 1971).

A nonsense mutation early in an operon usually reduces (sometimes severely) the level of expression of "downstream" cistrons. This effect is called polarity and its biochemical basis is still not understood. The distance between the nonsense codon and the next reinitiation determines how severe the polarity is (Newton et al., 1965).

That nonsense mutations cause premature chain termination was shown by the isolation of protein fragments specified by mutant genes (Sarabhai et al., 1964; Fowler and Zabin, 1966; Zinder et al., 1966). The location of the nonsense mutation within the gene was shown to determine the length of the fragment.

Originally, the nonsense mutations fell into two classes, distinguished on the basis of phenotype reversal by particular suppressor mutations. The nonsense mutations were called "amber" and "ochre." Suppressors which reversed the phenotype of ochre mutants were also active on amber mutants. However, suppressors of amber mutations were not active on ochre mutations. When it was found that a single suppressor gene could reverse the phenotypes of different amber or ochre mutations present in the same cell, it became apparent that the suppressors were allele-specific but locus-non-specific.

B. Identification of the nonsense codons in bacteria

Support for the biochemical existence of nonsense codons came from studies with in vitro protein synthesizing systems in which

polynucleotides were used to direct the incorporation of amino acids into polypeptides or to bind specifically tRNA to ribosomes (Nirenberg et al., 1966). With the 64 possible triplets in the genetic code and 20 amino acids found in protein, many triplets were found to code for the same amino acid. This property is called degeneracy. Three triplets (UAA, UAG, and UGA) failed to code for any amino acid and these were suspected of being the punctuation marks which signalled the protein synthesizing machinery to stop at the end of a given protein. Table 1 gives the genetic code as it is usually presented.

Further support for the biochemical evidence that UAA, UAG, and UGA were the nonsense triplets came from reversion studies of mutations believed to be nonsense on the basis of suppression studies. Revertants of one class of nonsense mutations in the gene for alkaline phosphatase (Weigert and Garen, 1965; Weigert et al., 1966) and in the T4 head protein (Brenner et al., 1965) were isolated and the amino acid substitutions determined. In each case, the amino acid present was found to be either tryptophan, serine, tyrosine, leucine, glutamic acid, glutamine, or lysine, all a single base removed from UAG. These mutants correspond to the mutants called "amber." A second class of nonsense mutations reverted by substituting all of the above amino acids except tryptophan and these correspond to the ones called "ochre" (Weigert et al., 1967; Brenner et al., 1965). The existence of mutations which behaved like amber or ochre mutations but were not suppressed by either amber or ochre suppressors led to the prediction that a third nonsense codon existed in the genetic code. It

Table 1
The Genetic Code

First Letter	Second letter				Third Letter
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Ochre nonsense	Nonsense	A
	Leu	Ser	Amber nonsense	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

The abbreviated names of amino acids are given in the body of the table. For example, Met = methionine. Coding triplets (codons) on messenger RNA are read 5' to 3' using the first letter (left-hand column) and then the second letter (top heading) and thence the third letter (right-hand column). For example, the only codon for methionine is AUG. The three triplets that predominantly lead to polypeptide chain termination are shown; these are UAA ("ochre"), UAG ("amber"), and UGA. (Taken from Hartman and Roth, 1973)

was determined to be UGA on the basis of studies on chemical mutagenesis (Brenner et al., 1967).

The degeneracy of the genetic code led Crick (1966) to propose the Wobble Hypothesis to account for the codon-anticodon recognition. Since a given tRNA recognized more than one codon, more than the simple A-U, G-C base pairing was involved. Crick proposed that while the first two bases were paired strictly, the third codon base and first anticodon base could wobble to allow for greater flexibility. The rules for wobble are given in Table 2; additional rules are also listed (Nirenberg et al., 1966; Ohashi et al., 1970; Yoshida et al., 1971; Ishikura et al., 1971).

C. Properties of nonsense suppressors in bacteria

The first nonsense mutations isolated by Benzer and Champe (1962) and Garen and Siddiqi (1962) produced functional protein in some hosts and not in others. When these mutations were later tested in the same host, it was realized that T4 growth and production of alkaline phosphatase were both related to the presence of a suppressor (Garen, 1968). Since then, many more suppressible mutations and suppressors have been isolated. The bacterial suppressors fall into three classes: those which suppress only UAG (amber), those which suppress only UGA, and those which suppress both UAA and UAG (ochre). No suppressors have been found in E. coli or S. typhimurium which recognize only the UAA codon.

A sample of bacterial suppressors and their characteristics are listed in Table 3. The amino acid insertions were determined

Table 2
The Wobble Hypothesis

<u>First base in anticodon</u>	<u>Symbol</u>	<u>Third base in codon</u>
Uridine	U	$\begin{Bmatrix} A \\ G \end{Bmatrix}$
Cytidine	C	G
Adenosine	A	U
Guanosine	G	$\begin{Bmatrix} U \\ C \end{Bmatrix}$
Inosine	I	$\begin{Bmatrix} A \\ C \\ U \end{Bmatrix}$
Ribothymidine	rT	$\begin{Bmatrix} A \\ G \end{Bmatrix}$
2-thiouridine- 5-acetic acid methyl ester	S	A
Uridine 5-oxyacetic acid	V	$\begin{Bmatrix} U \\ A \\ G \end{Bmatrix}$

Table 3

Nonsense Suppressors in E. coli and S. typhimurium

<u>Gene</u>	<u>Map location</u>	<u>Amino acid inserted</u>	<u>Protein(s) Analyzed</u>	<u>tRNA altered</u>	<u>Approximate efficiency (%)</u>	<u>Nature of mutation</u>	<u>References</u>
<u>UAG suppressors</u>							
<u>Su1</u>	near <u>his</u>	ser	alkaline phosphatase; T4 head protein; f2 coat protein	--	6-63	--	Garen <u>et al.</u> , 1965; Signer <u>et al.</u> , 1965; Stretton and Brenner, 1965; Capecchi and Gussin, 1965; Engelhardt <u>et al.</u> , 1965
<u>Su2</u>	near <u>gal</u>	gln	alkaline phosphatase	--	2-30	--	Garen <u>et al.</u> , 1965; Signer <u>et al.</u> , 1965; Gesteland <u>et al.</u> , 1967
<u>Su3</u>	near <u>trp</u>	tyr	alkaline phosphatase	tyr _I UAU/C	15-67	anti-codon GUA → CUA	Garen <u>et al.</u> , 1965; Signer <u>et al.</u> , 1965; Goodman <u>et al.</u> , 1968; Andoh and Ozeki, 1968
<u>Su6</u>	F'14 region	leu	alkaline phosphatase	leu UUG	55	--	Chan and Garen, 1969; Gopinathan and Garen, 1970

Table 3
(continued)

<u>Gene</u>	<u>Map location</u>	<u>Amino acid inserted</u>	<u>Protein(s) analyzed</u>	<u>tRNA altered</u>	<u>Approximate efficiency (%)</u>	<u>Nature of mutation</u>	<u>References</u>
<u>UAG suppressors (continued)</u>							
<u>Su7</u>	near <u>ilv</u>	gln	trp synthetase	trp UGG	77	recessive lethal anticodon CCA→CUA	Soll and Berg, 1969a, b; Yaniv et al., 1974
<u>SU273</u>		lys	T4 head protein		8-10		Kaplan, 1971
<u>UAA-UAG suppressors</u>							
<u>Su4</u>	near <u>trp</u>	tyr	alkaline phosphatase	probably tyr _I UAU/C	4-12 UAA 1-16 UAG	--	Gallucci and Garen, 1965; Weigert and Garen, 1965
<u>Su5</u>	near <u>gal</u>	(lys)	--	--	3-6 UAA <2-5 UAG	--	Gallucci and Garen, 1965; Weigert and Garen, 1965
<u>Su8</u>	near <u>ilv</u>	--	--	--	5 (UAG)	recessive lethal	Soll and Berg, 1969

Table 3
(continued)

<u>Gene</u>	<u>Map location</u>	<u>Amino acid inserted</u>	<u>Protein(s) analyzed</u>	<u>tRNA altered</u>	<u>Approximate efficiency (%)</u>	<u>Nature of mutation</u>	<u>References</u>
<u>UGA Suppressors</u>							
<u>Su9</u>	near <u>ilv</u>	trp	alkaline phosphatase	--	5	--	Chan and Garen, 1970; Chan <u>et al.</u> , 1971
<u>Su</u> _{UGA}		trp	polytryptophan	trp UGG	50-60	G→A at residue 24	Hirsh, 1971; Hirsh and Gold, 1971
<u>Su</u> _{UGA}	= <u>Su7</u>	--	--	--	50	recessive lethal; in <u>S. typhimurium</u>	Miller and Roth, 1971
<u>SuK</u>	near lys and ser	--	--	--	10	recessive; in <u>S. typhimurium</u>	Reeves and Roth, 1971

by an amino acid sequence analysis of a suppressed protein. Each strain carried the given suppressor as well as a UAA, UAG, or UGA mutation in a gene whose protein product could be isolated and sequenced.

The column labelled "Approximate efficiency (%)" reflects the extent to which a complete protein is synthesized instead of a fragment. The efficiency of suppression was measured either as a ratio of suppressed protein (by CRM or enzyme activity) to the wild-type protein level or as the amount of peptides made distal to the nonsense mutation compared to the amount made which is proximal to the mutation.

The suppressors vary in their ability to suppress nonsense codons. Garen et al. (1965) found that for each allele tested Su3 was always more effective than Su1, and Su1 was more effective than Su2. They also found that particular alleles tend to be efficiently suppressed while others always show poor suppressibility with a variety of suppressors.

Studies on the suppression of nonsense mutations in the T4 lysozyme by Yahata et al. (1970) indicated that the efficiency with which a nonsense codon can be suppressed may be reduced when the succeeding base triplet resembles a nonsense codon. Similarly, Salser (1969) and Salser et al. (1969) have found that the reading context is responsible for the efficiency of nonsense suppressors as well as for phenotypic suppressors such as 5-fluorouracil and streptomycin. 5-fluorouracil is believed to cause suppression by being substituted into the message as U but then being translated as C. Streptomycin

is known to act on the ribosome leading to general misreading. Salser's group concludes that the effect of the reading context may be related to the process of chain termination.

Regardless of the mechanism determining suppressor efficiency, generally UAA suppressors have an efficiency of less than 20%, while UAA-UAG suppressors act with efficiencies often greater than 50%. The low efficiency of the UAA suppressors could be due to an intrinsic property of ochre suppressors or that high efficiency UAA suppression is lethal. This will be discussed further in section F., "Chain termination in vivo."

Since a mutation to nonsense suppressor is a gain of function rather than a loss of function, most suppressor mutations are dominant as expected, if they are alterations in tRNA genes. Reeves and Roth (1970) have isolated a recessive UGA suppressor which causes undermethylation of tRNA. Presumably a tRNA insufficiently methylated misreads UGA for sense. Undermethylation has been shown to alter the triplet recognition in ribosome binding studies (Littauer et al., 1966; Capra and Peterkofsky, 1968).

D. Biochemical basis of suppression in bacteria

The first evidence that tRNAs were responsible for nonsense suppression came when the presence of the Su1 gene in the E. coli cell was found to be associated with an altered species of serine tRNA (Capecchi and Gussin, 1965; Engelhardt et al., 1965; Andoh and Garen, 1967). Similar results were found with tyrosyl-tRNA Su3 (Wilhelm,

1966). When Su3 was incorporated into a $\phi 80$ transducing phage, it was possible to show specific hybridization between the DNA of this transducing phage and the tyrosyl-tRNA (Andoh and Ozeki, 1968; Landy et al., 1967). Therefore, the Su3 gene contains the genetic information for tyrosyl-tRNA. Gesteland et al. (1967) were able to show that a tRNA fraction from a strain carrying Su2 was responsible for suppression of lysozyme amber mutants of T4 in vitro.

The location of the mutations in the primary sequence of the suppressor tRNAs was determined by isolation, purification and base sequence analysis of the tRNAs. The tyrosyl-tRNA (Su3) was shown to be altered in its anticodon, having mutated from GUA \rightarrow CUA (Goodman et al., 1968; Goodman et al., 1970), gaining the ability to read UAG (amber) and losing the ability to read tyrosine (UAU/UAC). In the case of the UGA suppressor which inserts tryptophan (Hirsh, 1971), the anticodon was not found to be altered; instead, a base at position 24 (in the dihydro-uracil loop) in the tryptophan tRNA was mutated which somehow changed the codon-anticodon interaction. The recessive lethal amber suppressor Su7 (Soll and Berg, 1969a, b) has also been sequenced (Yaniv et al., 1974). The mutation was found in the anticodon CCA \rightarrow CUA of the tryptophanyl-tRNA. This single base change caused the misacylation of the tRNA with glutamine instead of tryptophan. This is the same tRNA which was mutated to read UGA isolated by Hirsh (1971). As anticipated by the Wobble Hypothesis, the Hirsh tRNA reads UGA as well as UGG (tryptophan) while Su7 can read only UAG (amber) having lost the ability to read UGG (tryptophan).

The possible suppressor anticodon-codon recognition patterns are listed in Table 4. As stated above, the mutations in tRNA causing suppression are not limited to the anticodon of the tRNA.

E. Duplication of genes for tRNA suppressors

Duplicate copies of tRNA genes have been found for several tRNAs. $\phi 80\text{pSu3}$ phage upon infection produce both Su3 and su⁻ tRNAs (Russell et al., 1970). Similarly, the $\phi 80\text{pSu3}$ DNA was found to be saturated with more than one tyrosyl-tRNA in hybridization experiments. In a strain carrying Su6, fractionation of tRNA species yielded one leucyl-tRNA whose binding to ribosomes was stimulated by the UAG (amber) triplet but not the leucine triplet, UUG. Another species of leucyl-tRNA eluting immediately after the new Su6 component bound UUG (leucine) like the parent su⁻ strain. This suggests that wild-type E. coli contains two very similar and probably duplicate genes for the UUG-binding leucyl-tRNA. In Su6 strains, one of these is altered to read UAG (Gopinathan and Garen, 1970).

It is possible that the presence of these duplicate tRNAs is required before the mutation to suppressor can be recovered. Suppressor mutations would be lethal if no compensating tRNA could translate the codons formerly recognized by the suppressor tRNA. This has been found to be the case for the tryptophan tRNA. First, Hirsh (1971) showed that when this tRNA was mutated to read the UGA codon by a mutation from G \rightarrow A at residue 24 of the tRNA, all the tryptophanyl-tRNA present in the cell displayed this mutation. Therefore, only one gene for the tryptophan tRNA exists in E. coli. This result,

Table 4

Suppressor anticodon-codon pairing

<u>Hypothetical suppressor anticodon</u>	<u>Triplet recognized</u>
CUA	UAG amber nonsense
UUA	{ UAA ochre nonsense UAG amber nonsense
IUA	{ UAA ochre nonsense UAC tyrosine UAU tyrosine
SUA	UAA ochre nonsense
UCA	{ UGA nonsense UGG tryptophan
ICA	{ UGA nonsense UGC cysteine UGU cysteine
SCA	UGA nonsense
VCA	{ UGA nonsense UGU cysteine UGG tryptophan
VUA	{ UAG amber nonsense UAA ochre nonsense UAU tyrosine

when considered with the codon-anticodon interactions allowed by the Wobble Hypothesis, lead to the expectation that a mutation to suppressor at the anticodon of the tryptophanyl-tRNA would not be recovered unless a compensating copy of the tRNA were present. This is precisely what Soll and Berg (1969) found. When they attempted to isolate suppressors in a merodiploid strain of E. coli, they found suppressors which displayed the recessive lethal phenotype, i. e., they could not be maintained in haploid strains. One suppressor, Su7, was later shown to be the tryptophanyl-tRNA which had been mutated at the anticodon (Yaniv et al., 1974). As discussed above, this mutation also changed the acylation of the tRNA from tryptophan to glutamine. Miller and Roth (1971) have isolated recessive lethal nonsense suppressors like Su7, in S. typhimurium. Therefore, if the mutation to suppressor causes the total loss of recognition of a particular codon in the bacterial cell, it results in lethality, unless another tRNA can compensate for it.

F. Chain termination in vivo

Since the nonsense codons cause premature termination and the production of protein fragments, they were implicated as the natural chain termination signals at the ends of genes. Two release factors, R_1 and R_2 , have been found in E. coli which are needed for the release of peptides upon chain termination (Scolnick et al., 1968). When combinations of R factors and nonsense codons were tested for their ability to release f-methionine from an f-met-tRNA (AUG)-ribosome

complex, R_1 worked with UAA or UAG to release f-met; R_2 worked with UAA or UGA.

Since either R_1 or R_2 can stimulate the release of coat protein and replicase of phage R17 and coat protein of phage f2, Capecchi and Klein (1970) and Beaudet and Caskey (1970) conclude that UAA, the only codon which both R factors recognize, must terminate these phage genes.

The presence of a UGA suppressor causes read-through of the $Q\beta$ coat protein resulting in a larger-than-normal protein. This led Weiner and Weber (1971) to conclude that UGA must be the termination signal for this gene.

Since highly efficient UAG and UGA suppressors have been isolated, it is tempting to speculate that UAG and UGA must be used only infrequently in natural messages. On the other hand, when UAA suppressors were derived from highly efficient UGA suppressors, Su1, Su2, and Su3, they were found to be inefficient and to retard growth (Ohlsson et al., 1968; Person and Osborn, 1968). This led to the speculation that UAA was the termination codon. This phenomenon could be explained by the fact that UAA suppressors in E. coli suppress both UAA and UAG and, if termination signals are frequently tandem UAA-UGA codons, then UAA but not UAG suppressors can cause read-through. Therefore, UAA but not UAG suppressors would be harmful to the cell (Lu and Rich, 1971).

Alternatively, if UAG and UGA but not UAA codons are placed in a reading context which favors termination over suppression, highly

efficient UAG and UGA suppressors would not be able to interfere with peptide release (Ganoza, 1966).

At present, it is evident that the differences in efficiencies of the two types of suppressors in E. coli is basically not understood.

The most direct proof that nonsense codons terminate genes comes from the sequencing of the RNA phages R17 and MS2. The coat protein of these phages is terminated by tandem codes UAA and UGA (Nichols, 1970; Min Jou et al., 1972). In MS2, the maturation protein is terminated by a single UAG triplet (Contreras et al., 1973). Recently, Platt and Yanofsky (1975) have reported that the nonsense codon UGA terminated the trpB message in E. coli.

II. Nonsense suppression in *Saccharomyces cerevisiae*

The study of nonsense suppression in yeast has not advanced as far as it has in *E. coli*, due in part to the lack of an in vitro assay for suppression. However, very recently, that hurdle was overcome and the long-held assumption that nonsense suppression in yeast was grossly analogous to that in *E. coli* has finally been substantiated. The following sections concentrate exclusively on suppression in *Saccharomyces cerevisiae*. Hawthorne and Leupold (1974) review suppression in *Schizosaccharomyces pombe* as well as in *S. cerevisiae*.

A. Isolation of nonsense mutations and their properties in yeast

The first nonsense mutations isolated in *S. cerevisiae* were isolated as nutritional requirements, the phenotypes of which were later found to be reverted to prototrophy by a single suppressor gene (Hawthorne and Mortimer, 1963).

Nonsense mutations in yeast behave similarly to those found in *E. coli* and *S. typhimurium*. Generally, they are not leaky, temperature-sensitive, or osmotic-remedial; they do not show intragenic complementation. The unusual mutations which do exhibit complementation show a polarized pattern, i. e., they complement with mutants which map only to one side of the nonsense mutation. This phenomenon is explained by the participation of incomplete polypeptide chains in the complementation reaction (Manney, 1964; Fink, 1966). A few exceptions to the above characteristics have been reported; they remain largely unexplained (Thuriaux et al., 1971; Jones, 1972).

It is difficult to assess the pleiotropic effects of nonsense mutations in yeast since so few potential operons have been found. Some suppressible mutations in the his4A region lack the function of his4B and his4C absolutely (Fink, 1966). In studies on the trp5 and his4 regions, the nonsense mutations lead to gene products whose molecular size is decreased compared to the wild-type enzyme (Fink, 1965, 1966; Manney, 1968; Shaffer et al., 1969). This could be evidence for premature chain termination, polarity, or protein-protein interactions in aggregates of different polypeptide chains.

B. Identification of the nonsense codons in yeast

As was the case in E. coli, the direct evidence that two of the nonsense codons were UAA and UAG came from reversion studies of mutants; in this case, in the iso-1-cytochrome c gene (Stewart et al., 1972; Stewart and Sherman, 1972; Stewart and Sherman, 1973). UAG mutants which reverted had replacements of glutamine, glutamic acid, lysine, leucine, serine, tyrosine, and tryptophan, all with codons one base change removed from UAG. UAA mutants had substituted all the above amino acids except tryptophan which is more than one base removed from UAA. Stewart and Sherman (1972) suggested that UGA must also be a nonsense codon, since UAA alleles, when mutated to UGA, should otherwise code for cysteine or tryptophan. Neither of these amino acid replacements was found. The nucleotide sequence for all of the first 44 base pairs of the gene has now been deduced, further confirming these codon assignments (Sherman and Stewart, 1973).

C. Properties of nonsense suppressors in yeast

Many nonsense suppressors have been isolated in S. cerevisiae. The large number of suppressor loci is a distinctive feature of suppression in yeast as compared with bacteria. The suppressors can be generally classified as to their efficiency of suppression, which is how they will be presented here.

Gilmore (1967) described eight classes of suppressors based on their ability to suppress five alleles now identified as UAA mutations. The most efficient suppressors, called the class 1, set 1, suppressors, have been the most thoroughly investigated to date. These include SUP2 to SUP8 and SUP11; they map in eight distinct loci on six different chromosomes and all insert tyrosine in response to a UAA mutation in iso-1-cytochrome c. Their properties are presented in Table 5. Liebman et al. (1976) have determined that several of the UAG-suppressor alleles of these UAA suppressors insert tyrosine as well, but are higher in their efficiency (close to 75% efficiency).

Other suppressors have been isolated and classified (Hawthorne, 1969a, b; Hawthorne and Mortimer, 1968; Hawthorne and Leupold, 1974). The most recent classification scheme (Hawthorne and Leupold, 1974) includes: (1) Gilmore's (1967) class 1, set 1 suppressors as well as others which also recognize only the UAA codon, (2) some which recognize only the UAG codon, (3) suppressors which are specific for the UGA codon, and (4) suppressors which recognize all three nonsense codons, UAA, UAG, and UGA ("omnipotent"). Many suppressors of these four types have been mapped and they are distributed over 11 chromosomes and two fragments (see Figure 1).

Figure 1

The genetic map of Saccharomyces cerevisiae

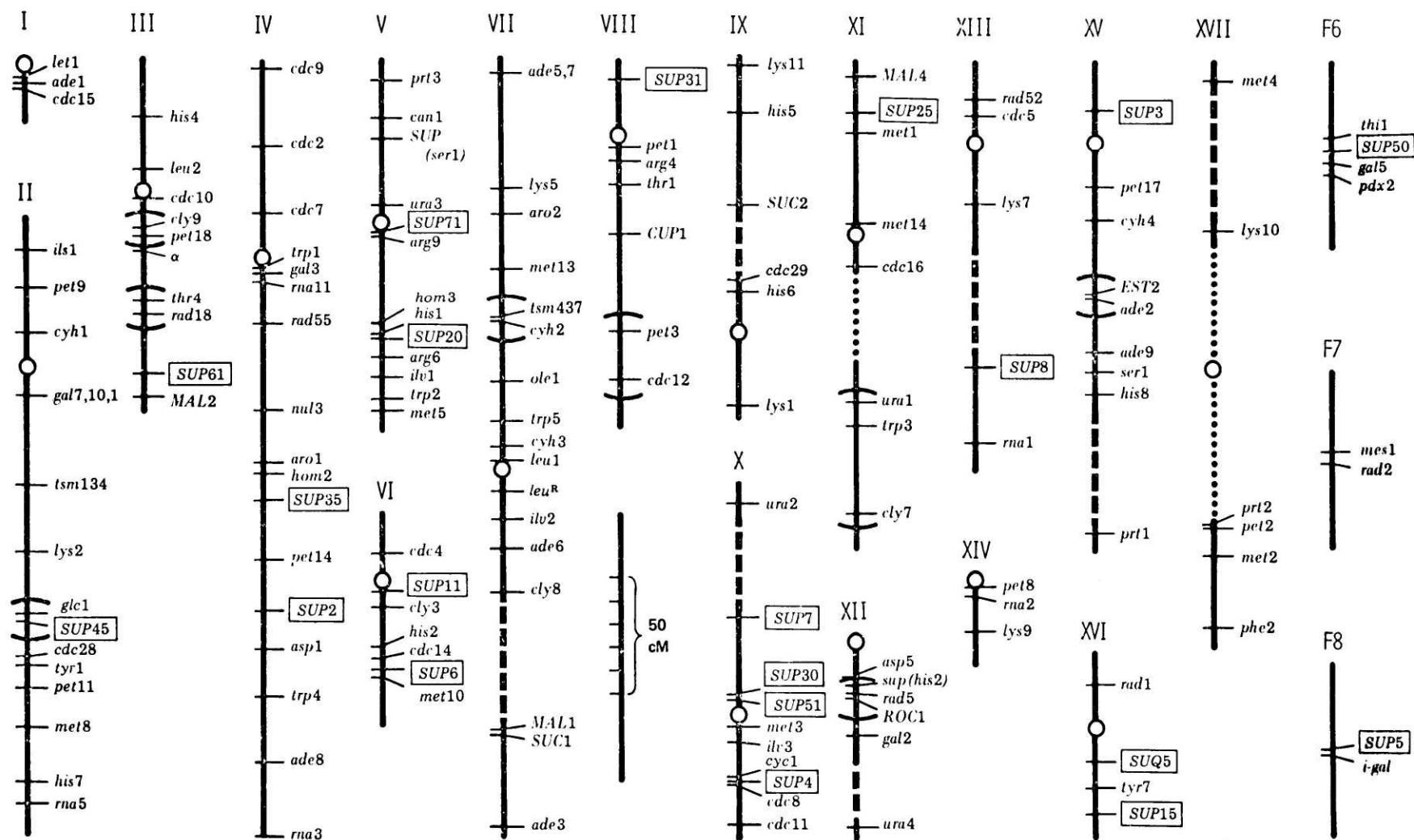


Table 5
Properties of the Class 1, Set 1 UAA Suppressors

<u>Gene</u>	<u>Map location^a (chromosome)</u>	<u>Amino acid inserted^b</u>	<u>Efficiency of suppression^b</u>
SUP2	IV	Tyrosine	5
SUP3	XV	Tyrosine	7
SUP4	X	Tyrosine	4
SUP5	fragment 8	Tyrosine	12
SUP6	VI	Tyrosine	6
SUP7	X	Tyrosine	9
SUP8	XIII	Tyrosine	7
SUP11	VI	Tyrosine	7

^aHawthorne and Leupold (1974)

^bGilmore et al. (1971)

The suppressors which recognize only one nonsense codon are dominant mutations believed to be altered tRNA. The "omnipotent" suppressors are recessive and are probably ribosomal mutations. There are no suppressors in yeast which correspond to the bacterial ochre suppressors which recognize both the UAA and UAG codons.

Cox (1965, 1971) has isolated another class of suppressors which require the presence of a non-Mendelian factor, psi^+ , for their expression. In cells carrying psi^+ , the UAA suppressor SUQ5 acts like one of the eight class 1, set 1 suppressors described above. In psi^- cells, no suppression by SUQ5 is seen. SUQ5 inserts serine into protein in psi^+ and psi^- strains (Liebman et al., 1975).

In diploid strains, when the psi^+ factor is present, the highly efficient ochre suppressors of Gilmore (1967) exhibit a recessive lethal phenotype. Only the two spores from each ascus which have failed to inherit the suppressor survive. Cox (1965, 1971) has proposed that the psi^+ factor enhances the efficiency of some ochre suppressors. Recently, Liebman et al. (1975) have found support for Cox's idea in that psi^+ strains carrying a UAA suppressor and a suppressible mutant of cytochrome c make enhanced levels of cytochrome c as compared to isogenic psi^- strains. Their results have led Liebman et al. (1975) to the idea that an excess of UAA suppression might in itself be lethal.

D. Biochemical basis of suppression in yeast

Until very recently, there was no direct experimental evidence that suppression in yeast was caused by altered tRNA genes as in E.

coli. The biochemical studies were held up since yeast tyrosyl-tRNA from suppressing strains would not suppress in an E. coli in vitro protein synthesizing system using f2 RNA as message (Kiger and Branter, 1973). Many other attempts were made using eukaryotic heterologous in vitro systems, but none succeeded.

Recently, Gesteland (personal communication) has found in vitro suppression using extracts of strains carrying several of the class 1, set 1 yeast suppressors. His system is composed of rabbit reticulocyte initiation factors, Krebs ascites cell ribosomes, Q β RNA and yeast tRNA (crude extract or purified). Using Q β RNA which contained a UAG mutation in the synthetase gene, both synthetase protein and fragment were made when the yeast tRNA fraction came from a UAG-suppressor-bearing yeast strain. When a more purified tRNA preparation from such a strain was added to a non-suppressing extract, suppression was evidenced by the production of synthetase protein. Experiments with the dominant UAA suppressors yielded similar results. Therefore, there is now good evidence to support the long-held assumption that nonsense suppression in yeast is due to changes in tRNA as in bacteria.

E. Redundant genes for suppressors in yeast

Gilmore et al. (1971) proposed that each of the class 1, set 1 suppressors was a tyrosyl-tRNA, and that eight redundant genes code for the single species of tyrosyl-tRNA which has been identified in yeast (Madison et al., 1966). Hybridization studies of yeast DNA

and tRNA estimate that 5-7 genes may code for each tRNA species (Schweizer et al., 1969), which is in good accord with the number of tyrosine-inserting suppressors found.

This thesis investigates the question of whether essential or single copy tRNAs exist in yeast as they do in bacteria. Any mutation in such a tRNA resulting in suppression would result in lethality and would be unrecoverable in haploid yeast strains. Therefore, suppressor mutations were isolated in diploid yeast strains where they would not be lethal. This thesis gives evidence that such tRNAs do exist in yeast, and a description of their isolation and characterization follows.

CHAPTER II
THE ISOLATION AND CHARACTERIZATION OF RECESSIVE LETHAL
AMBER SUPPRESSORS IN YEAST

I. Introduction

The study of nonsense suppression in yeast has been based primarily on suppressor mutations derived in haploid strains. These mutations represent a subset of suppressor types: those in which the mutation to ability to suppress does not alter any gene product whose wild-type function is essential to the viability of the organism. As described in Chapter I, yeast suppressors (Manney, 1964; Gilmore, 1967; Hawthorne and Mortimer, 1968; Gilmore et al., 1971; Stewart and Sherman, 1972; Stewart et al., 1972) are generally assumed to be analogous to E. coli suppressors, which have been shown to be altered tRNAs. The eight most efficient ochre suppressors are believed to be mutations in eight distinct loci coding for tyrosyl-tRNA (Gilmore et al., 1971).

The alteration of a unique coding species of tRNA to a form which recognizes a nonsense codon and fails to recognize the original sense codon would be lethal in a haploid strain of yeast. In an attempt to generate new classes of suppressors which insert different amino acids into protein, suppressor mutations were isolated in diploid yeast strains, where alterations lethal in haploid cells might still permit the diploid cells to survive and grow.

This chapter describes the isolation of a new class of suppressors which cannot be isolated or maintained as haploids. These suppressors are thus analogous to the recessive lethal suppressors isolated

in merodiploids of E. coli (Soll and Berg, 1969) and S. typhimurium (Miller and Roth, 1971).

II. Materials and Methods

A. Yeast strains and nomenclature: The heterothallic strains of Saccharomyces cerevisiae used in this study were derived from strains provided by Dr. F. Sherman and Dr. G. Fink. The symbols and nomenclature are those proposed at the IVth and Vth International Yeast Genetics Conference (von Borstel, 1969). The symbol "+" refers to the wild-type allele; capital letters indicate dominant alleles. "SUP" is the dominant suppressor mutation, and "sup+" refers to the wild-type suppressor-less condition. All other markers written in capital letters are wild-type alleles (THR4, MAL2, LEU2, CAN^S, HIS, CYC1, ADE, etc.). Phenotypes are denoted by a three-letter code in which the first letter only is capitalized, and the symbol is followed by either a "+" or "-". Each strain number is preceded by a letter which denotes its ploidy: either DB or MB followed by D for diploids, H for haploids, or A for aneuploids.

The genotypes of the strains used in this study are listed in Table 6. The parental diploid DBD195 was constructed by mating the two haploid strains DBH249 and DBH250. Other diploid strains containing a recessive lethal suppressor were constructed by a technique described under "D. Genetic Methods: Matings."

The his5-2, leu2-1, lys1-1, and lys2-1 alleles are acted upon by UAA but not UAG suppressors and are therefore referred to as ochre mutations (Gilmore, 1967; Hawthorne, 1969a, b). The ade3-26, met8-1, trp1-1, and tyr7-1 alleles are acted upon by UAG but not UAA suppressors and are thus called amber mutations (Hawthorne, 1969a, b;

Genotypes of Strains

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Table 6
Genotypes of Strains
(continued)

MBD86	<u>a</u>	<u>ade2</u>	<u>+</u>	<u>his1/5-2</u>	<u>+</u>	<u>+</u>	<u>+</u>	<u>thr4</u>	<u>trp1-1</u>	<u>+</u>	<u>mal2</u>	<u>SUP-RL1</u>
	α	ade2	ade3-26	+	his4	leu2-1	lys1-1	thr4	trp1-1	can ^r	+	+
MBD126	<u>a</u>	<u>adel</u>	<u>ade2</u>	<u>his1/5-2/4</u>	<u>lys1-1/2-1</u>	<u>trp1-1</u>	<u>tyr7-1</u>	<u>can^r</u>	<u>thr4</u>	<u>SUP-RL1</u>	<u>+</u>	
	α	+	+	+	+	trp1-1	+	+	+	+	+. mal2	

* MALX is an unknown maltose fermentation allele different from MAL2.

** Allele numbers indicated in this way were not distinguished by complementation tests.

Sherman *et al.*, 1973). The adel, ade2, his1, thr4, and can^r (resistance to canavanine) are recessive and are not suppressible by UAA or UAG suppressors. MAL2 is a wild-type dominant allele conferring ability to ferment maltose.

The iso-1-cytochrome c mutation, cycl-179, was originally induced with ultraviolet light and was isolated by the chlorolactate procedure (Sherman *et al.*, 1974). It has an amber codon (UAG) corresponding to amino acid residue 9 (normally lysine) (Stewart and Sherman, 1972) and is suppressible by tyrosine-inserting amber suppressors (Sherman *et al.*, 1973).

All strains are psi⁻; see Chapter I (Cox, 1965).

B. Media: The culture media used and their purposes are described below.

Rich medium (YEP glucose): 1% Difco Yeast Extract, 2% Difco Bacto Peptone, 2% glucose, 2% Difco Bacto-agar (omitted in liquid media). This is a complete medium and was used for propagation of most cultures, except when maintenance of aneuploidy was desired.

Minimal medium (SD): 0.67% Difco Yeast Nitrogen base (without amino acids), 2% glucose, 3% Difco Bacto-agar (omitted in liquid media). When required, the following concentrations of supplements were added: adenine sulfate, 20 mg/l; L-histidine-HCl, 20 mg/l; L-leucine, 30 mg/l; L-lysine-HCl, 30 mg/l; L-methionine, 20 mg/l; L-phenylalanine, 50 mg/l; L-tryptophan, 20 mg/l; L-tyrosine, 30 mg/l; L-canavanine sulfate, 60 mg/l. Supplemented minimal medium was used for the culture of aneuploids and as a control in scoring nutritional phenotypes.

C. Incubation conditions: All incubations were at 30°. For liquid cultures, flasks or tubes were aerated on rotary or reciprocating shakers.

D. Genetic methods:

Matings: Stationary phase cultures of haploid strains of opposite mating types were mixed together in a spot on YEP-glucose and replicated to selective plates, or spotted directly on selective minimal plates. In order to isolate progeny from rare matings between a/ α aneuploids and a or α haploids, 0.4 ml of the mating mixture was spread on appropriately supplemented selective minimal media. Diploids were purified by sub-cloning on selective media.

In order to transfer a recessive lethal mutation into a new genetic background, a modification of the above mating procedure was used. Spores which have inherited a recessive lethal mutation (SUP) germinate and proceed through several rounds of budding before growth ceases (Hawthorne and Leupold, 1974). If a SUP spore mates during these rounds of growth with another germinating spore which is sup⁺ or with a haploid sup⁺ tester strain which has been added to the sporulation mix, diploidy is restored and the SUP mutation is not lethal in a heterozygous state.

A diploid strain heterozygous for SUP-RL1 was sporulated (see D. Genetic methods: Sporulation) and the spores, still within the ascus, were mixed on a YEP-glucose plate with an excess of cells from a haploid strain. After a 2-3 day incubation at 30°, the mixture was subcloned on appropriately supplemented minimal medium to select SUP diploids.

It is not necessary to disperse the spores from the ascal sac before spreading on the YEP-glucose plate. When germination begins, the ascus ruptures and each spore comes in contact with sister and non-sister spores, and mating proceeds.

If the original SUP diploid is homozygous for one marker and its spores are mixed with a tester strain carrying a requirement complementary to that of the spores, progeny SUP diploids can be isolated by selection for prototrophy on minimal medium. In this way, diploids which have failed to sporulate and are present in the sporulation mixture, sup⁺ haploids and sup⁺ diploids arising from spores and spore-spore matings, respectively, will be counter-selected.

If the original diploid is heterozygous for a given marker and one wants progeny diploids which are homozygous for that marker, the mixture of spores is allowed to germinate without addition of a haploid tester strain. In this case, spore-spore matings will result and the desired progeny diploid will be formed with a certain frequency. Unfortunately, when the selection is made for SUP diploids, those diploids present in the sporulation medium which had failed to sporulate, can grow. These cells are identical to the original diploid and do not represent progeny in which genetic reassortment has occurred. After subcloning, the diploids must be screened for those which have inherited the desired markers, to distinguish them from the population of vegetative diploids.

Each new diploid was sporulated and tetrad analysis was performed to verify the presence of the recessive lethal suppressor (two live: two dead spores/ascus) and the homozygosity or hetero-

zygosity of outside markers. The diploids always yielded two live and two dead spores per ascus where all survivors were sup+.

Sporulation: Cultures were grown in YEP-glucose for 48 hours into stationary phase, washed once in water, resuspended in 0.3% potassium acetate and then diluted 1:10 into the same medium. They were then incubated with aeration at 30° until asci appeared (usually two days). The sporulated cultures could be stored in water at 4° indefinitely. Solid sporulation medium contained 0.98% potassium acetate, 0.5% glucose, 0.1% Bacto-yeast extract, and 1% Ionagar (Wilson Diagnostics, Inc.).

Tetrad analysis: After sporulation, the asci were dissected by the procedure of Johnston and Mortimer (1959) using Glusulase (Endo Laboratories) to digest the ascus walls. The sister spores of four-spored asci were then separated by micromanipulation on an agar slab where each tetrad of spores was aligned. The slab was transferred to a YEP-glucose plate and was incubated for two days. The spore colonies were picked and grown on a master YEP-glucose plate. The genotype of each spore was determined by replica-plating to appropriately supplemented minimal media. Complementation tests were used to determine the mating type of each spore and to discriminate markers of like phenotype.

Before the induction of suppressor mutations, the parent diploid DBD195 was subjected to tetrad analysis. Spore viability was greater than 90%. The heterozygous markers segregated 2+:2- and the homozygous markers segregated 0+:4-, as expected. After induction of a recessive lethal suppressor mutation, the diploids

yielded many asci with fewer than four live spores. The decrease in spore viability was attributed to segregation of the recessive lethal mutation. (Only those asci which had the four spores successfully dissected were used in genetic analysis.)

Random spore analysis: Diploids were analyzed by the random spore method of Gilmore (1967) with slight modifications. After sporulation, approximately 10^6 cells were treated with Glusulase for 1 hour and then diluted 1:50 in water. The suspension was sonicated using a Heat Systems Company-Ultrasonics, Inc. sonifier cell disrupter model W185 set at an output of 50-70 watts, using a standard microtip. Sonication time was 2 minutes (15-second intervals with 30-second cooling periods), and the spores were diluted and plated on appropriately supplemented minimal medium containing canavanine. Since resistance to canavanine is recessive, only haploids arising from can^r spores and rare can^r/can^r homozygotes could form colonies. For mapping purposes, the single colonies were picked and grown on a master YEP-glucose plate for two days at 30°. The genotype of each clone was determined by replica-plating to appropriately supplemented minimal media. Complementation tests were used to determine the mating type of each spore and to discriminate markers of like phenotype.

Mitotic segregational analysis: This test was performed to quantitate the instability of the SUP-bearing chromosome in putative aneuploid strains. Single colonies of the strains to be tested were

grown up in selective medium, diluted 100-fold into YEP broth and grown until stationary phase was reached. The cells were titered on YEP plates. 200 to 300 colonies were replica-plated and scored for loss of the suppressor and for mating type. The segregants which had lost the suppressor usually formed much larger colonies than their SUP/sup⁺ parents.

Aneuploid mapping: Trisomic analysis was used to determine which chromosomes were duplicated in the aneuploid SUP strains (Shaffer et al., 1971; Mortimer and Hawthorne, 1973). When haploid strains carrying mutations in mapped genes were mated to these aneuploids, the resulting trisomic progeny were expected to yield asci with $4^+:0^-$, $3^+:1^+$, and $2^+:2^-$ ratios for any gene located on the same chromosome as was duplicated in the aneuploid. In order to see the aberrant $4^+:0^-$ and $3^+:1^-$ tetrad ratios, the trisomic configuration for the mapped gene was $+/+/-$; both + alleles deriving from the aneuploid and the - allele from the tester haploid for the given tester mutation. The tester strain also carried trpl-1, a suppressible amber allele which permitted scoring for the presence of the suppressor mutation. The expected tetrads resulting from a trisomic strain are illustrated in Figure 2.

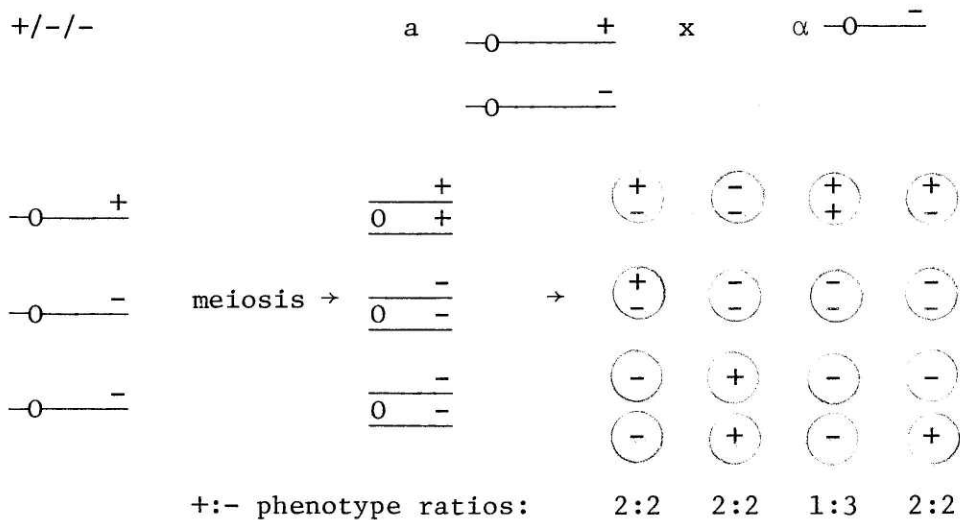
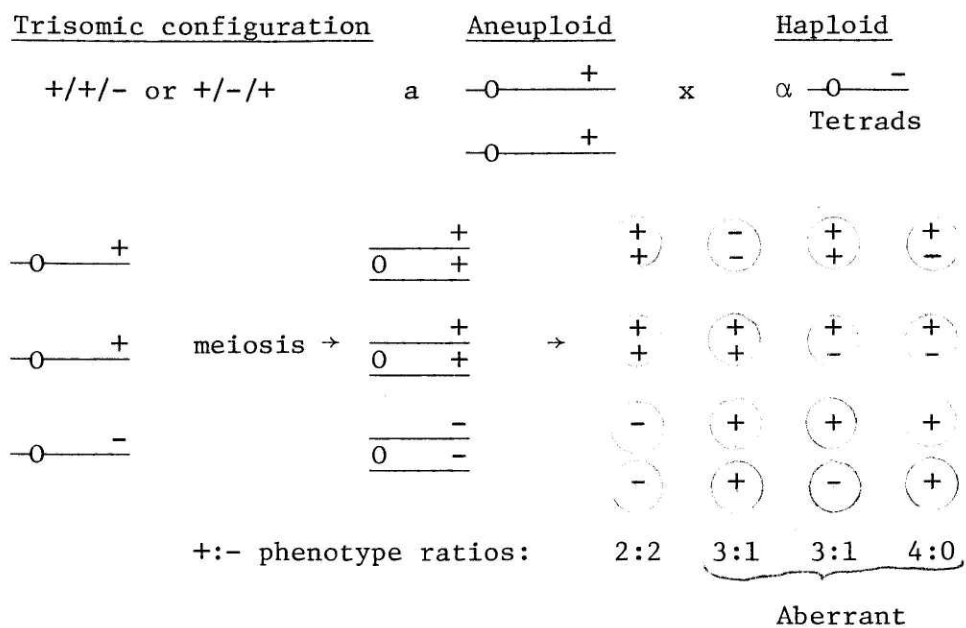
E. Determination of cytochrome c content: The segregation and suppression of the cycl-179 gene was scored from the levels of cytochrome c, estimated by spectroscopic examination of whole cells at low temperature (-190°C) (Sherman and Slonimski, 1964). Cells were streaked in a line on agar plates containing 1% Bacto-yeast

Legend to Figure 2

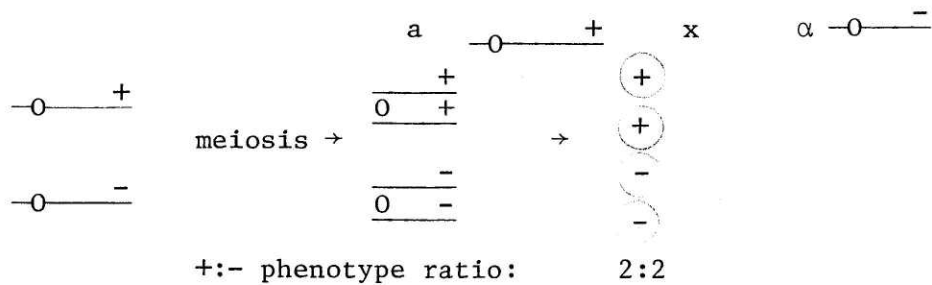
The horizontal lines with opened circles represent chromosomes and their centromeres. + and - are allelic forms of a given marker. If the trisomic configuration is $+/+/-$ or $+/-/+$, aberrant $3+:1-$ and $4+:0-$ tetrads will appear. A trisome containing two chromosomes with - alleles and one chromosome with a + allele will never yield $4+:0-$ or $3+:1-$ aberrant tetrads. In the lower part of the figure, the segregation of a marker in a diploid strain is given for comparison. The expected segregation for a Mendelian gene is 2:2.

Figure 2

Aneuploid Mapping



In a haploid x haploid cross:



extract, 2% Bacto-peptone and 1% sucrose, and, after three days, were scraped from the agar, blotted with paper towels, and pressed between two plexiglass sheets. The cells were then cooled in liquid nitrogen and were quickly subjected to examination using a Hartree low-dispersion spectroscope. At -190°C , the cytochrome c α absorption band is at 547 nm. The blackness of the cytochrome c band permits an estimate of the amount of cytochrome c present in the cells.

III. Results

A. Isolation of amber suppressor mutations in diploids:

Single clones of the diploid DBD195, $\frac{a}{\alpha} \frac{his1}{+} \frac{leu2-1}{adel} \frac{trp1-1}{trp1-1} \frac{tyr7-1}{tyr7-1}$ $\frac{+}{can^r} \frac{sup^+}{sup^+}$, were inoculated into rich medium. After growth to stationary phase, about 4×10^7 cells from each independent culture were spread on plates lacking tryptophan, tyrosine, and phenylalanine (an additional requirement of tyr7 mutants) to select simultaneous reversion of the amber (trp and tyr) markers. When ethyl methane sulfonate (EMS) was used to induce mutations, one drop was placed in the center of each plate.

Colonies appearing were assumed to bear suppressors since the frequency was far greater than that expected for simultaneous reversion to wild-type of both trp1-1 and tyr7-1. These strains do, in fact, carry suppressors since tryptophan and tyrosine-requiring cells segregate from suppressor-bearing aneuploid strains derived from the diploids. These aneuploid strains will be described more fully in a later section.

B. Determination of recessive lethality by random spore

screening: A random spore analysis was carried out to determine whether the suppressors were lethal or non-lethal in haploid progeny. The method takes advantage of the heterozygosity of the diploid for a recessive can^r marker: CAN^s/can^r diploids which have failed to sporulate and all CAN^s spores fail to form colonies in the presence of canavanine. Only can^r spores and rare can^r/can^r diploids can grow on the canavanine plates.

The diploid SUP colonies were purified, cultured, and sporulated. Mixtures of spores were sonicated and plated on two kinds of canavanine-containing minimal media: selective minimal medium containing no tryptophan, tyrosine, or phenylalanine (selects SUP can^r) and supplemented medium (permits the growth of both SUP can^r and sup⁺ can^r). A comparison was made between the number of colonies appearing on the two types of plates. If a non-lethal suppressor is segregating, SUP can^r spores will appear on the minimal plates lacking aromatic amino acids and both SUP can^r and sup⁺ can^r spores will grow on the fully supplemented plates. If the diploid contains a recessive lethal suppressor, only sup⁺ haploids will be viable and such cells will grow only on the fully supplemented medium. Minimal plates lacking aromatic amino acids will show no spore growth if a recessive lethal suppressor is segregating.

C. Frequency of recessive lethal suppressors in diploid strains:

From each independent sup⁺ parent diploid culture, 2-4 single SUP colonies were examined for recessive lethality. Among 33 cultures containing suppressor mutations which arose spontaneously, 90% of all the suppressors were recessive lethal. From 47 suppressor-bearing cultures which had been treated with EMS, 67% of the colonies tested had recessive lethal suppressors. A similar frequency of recessive lethality among suppressors was found in diploids carrying another pair of amber mutations, his4-580 and met8-1. In diploid strains, it seems that the recessive lethal class of suppressor is far more frequent under our conditions, than the non-lethal

class. Soll and Berg (1969a) reported a similar high frequency of recessive lethal suppressors in merodiploids of E. coli.

Asci from diploids which random spore analysis indicated carried a recessive lethal suppressor were dissected. Each ascus bore two live and two dead spores. Rarely, an ascus with three viable spores occurred; without exception, all three spores lacked the suppressor mutation. The surviving spores were all leu2-1 trp1-1 tyr7-1 sup⁺. The adel, his1, and can alleles segregated independently. Data for a representative sample of SUP diploid isolates is shown in Table 7.

D. Isolation of aneuploid strains carrying recessive lethal suppressors: Haploid spores which have inherited a recessive lethal suppressor from a heterozygous diploid do not grow. However, a spore which contains such a suppressor as well as a sup⁺ copy of the suppressor locus should survive. Such rare spores can arise from meiotic non-disjunction (aneuploids), translocation, or genetic duplication, and might be detected in the random spore analysis of a diploid carrying a recessive lethal suppressor. The only colonies which can grow on the selective minimal canavanine plates are diploids which have converted or mutated the CAN^S locus to become can^r/can^r, and "haploids" which have become heterozygous for the recessive lethal suppressor locus.

The number of can^r SUP spores (possible aneuploids) on the minimal plate was usually less than 0.1% of the number of colonies appearing on the supplemented plate. Such colonies were purified by subcloning, cultured, and tested for the ability to sporulate; those

Table 7
Segregation of Markers from Diploids
Carrying Recessive Lethal Suppressors

SUP Diploid Isolate	Number Asci*	Number of spores:					
		live: dead	α :a	ade ⁺ : adel	his ⁺ : his1	leu ⁺ : leu2-1	sup ⁺ : SUP [†]
339	6	12:12	7:5	7:5	5:7	0:12	12:0
340	9	17:19	6:11	11:6	10:7	0:17	17:0
341	10	14:26	8:6	6:8	7:7	0:14	14:0
342	11	23:21	11:12	11:12	10:13	0:23	23:0
343	10	20:20	9:11	9:11	9:11	0:20	20:0

The genotype of the diploid is $\frac{a}{\alpha} \frac{his1}{+} + \frac{leu2-1}{leu2-1} \frac{trp1-1}{trp1-1} \frac{tyr7-1}{tyr7-1} \frac{CAN^S}{can^r} + \frac{SUP}{SUP}$

Each ascus yielded two live and two dead spores, with one exception, in strain DBD342. Here, one ascus gave 3 viable spores, all of which lacked the suppressor mutation. No spores carrying the suppressor mutation were ever detected in tetrad analysis. The actual tetrad viability rates were:

Strain	Total*	Live:Dead				
		4:0	3:1	2:2	1:3	0:4
339	6	0	0	6	0	0
340	9	0	0	8	1	0
341	10	0	0	6	2	2
342	11	0	1	10	0	0
343	10	0	0	10	0	0

*Some asci gave only one viable spore, a few gave no viable spores.

[†]The suppressor mutation is scored by counting Trp⁺ Tyr⁺ spores.

which sporulated were presumed to be diploids and were discarded. The remaining strains were subjected to further genetic analysis.

Among the original recessive lethal suppressor-bearing diploids, two groups were seen. One type of diploid generated, when sporulated, "aneuploid" progeny which either displayed a single mating type (a or α) or were non-mating, indicating disomy for chromosome III. The other type generated only aneuploids which displayed a single mating type (a or α).

E. A recessive lethal amber suppressor on chromosome III:

Strain DBA309 was isolated by the random spore technique from SUP diploid DBD339, as a single colony growing on a minimal plate containing canavanine. It did not sporulate and was further characterized.

Spot-mating tests indicated that strain DBA309 would not mate with sup⁺ trp1-1 tyr7-1 tester strains of either mating type to produce tryptophan and tyrosine-independent (i. e., SUP/sup⁺) diploids. When strain DBA309 was grown to stationary phase in rich medium, where the SUP allele is not required, sup⁺ cells (Trp⁻Tyr⁻) appeared at a frequency of 0.4% and these sup⁺ segregants all displayed the α mating type. No colonies were seen which could mate and were still SUP, or which became sup⁺ and failed to mate as α . This suggests that strain DBA309 is aneuploid for both the mating-type locus (chromosome III) and the suppressor locus. The observed cosegregation further suggests that strain DBA309 is aneuploid for

all or part of chromosome III and that the suppressor (called SUP-RL1) is included in the duplicated region. Note, however, that no linkage of SUP to mating type was detected in the tetrad analysis (Table 7). To confirm the aneuploidy of chromosome III, strain DBA309 was forced to mate with strain DBH49, a his4-280 trp1-1 CAN^s sup⁺, with selection to maintain the suppressor mutation. The plate matings yielded recombinant progeny at a frequency of 10^{-5} - 10^{-6} . If the resulting progeny are trisomic for one or more linkage groups, then heterozygous markers on the affected chromosomes should frequently segregate aberrantly during meiosis. Tetrad analysis of one such strain is presented in Table 8; the result demonstrates that his4, mating type and the SUP locus all segregate aberrantly.

His4 is located on chromosome III, and the fact that 6 out of the 11 tetrads which had more than two viable spores gave $4^{+}:0^{-}$ and $3^{+}:1^{-}$ ratios for histidine implies that the strain is a $+/+/-$ trisome for this locus. Mating type and leu2-1 are also located on chromosome III. The presence of non-mating spores indicated that more than 2 copies of the mating-type locus were present. The trisomic configuration $\alpha/\alpha/\underline{a}$ is suggested by the appearance of many more spores which mated as α (either α or α/α) than those mating as \underline{a} . This further suggests that the putative trisomic strain arose from the mating of DBH49 with a variant of the aneuploid DBA309 which had undergone a conversion or mutation from α/\underline{a} to α/α . The leu2-1 allele was donated by the aneuploid resulting in a trisomic configuration $-/-/+$. Rarely should this configuration result in aberrant tetrad ratios; only $2^{+}:2^{-}$

Table 8

Aneuploidy of Chromosome III in Strains DBA309 and DBA338

	Fraction of Tetrads Segregating Aberrantly (4:0 + 3:1)	
	<u>DBA309 x DBH49</u>	<u>DBA338 x DBH49</u>
Asci with more than 2 live spores:*	11/22	22/54
<u>his</u> ⁺ : <u>his4-280</u>	6/11	0/22
<u>leu</u> ⁺ : <u>leu2-1</u>	0/11	0/22
Asci with non-mating spores [†]	6/11	0/22

The spore progeny of a rare mating between DBA309 or DBA338 and DBH49 were dissected and characterized. Trp⁺ spores indicated the presence of SUP-RL. Markers not listed segregated 2:2 as expected. Complementation tests were performed to distinguish his4 from his1.

DBA309: $\frac{a \text{ leu2-1 } \text{SUP-RL1 } \text{trp1-1 } \text{tyr7-1 } \text{can}^r}{\alpha \text{ leu2-1 } +}$

DBH49: $a \text{ his4-280 } \text{trp1-1 } \text{CAN}^s \text{ sup}^+$

DBA338: $\alpha \text{ adel } \text{his1 } \text{leu2-1 } \text{trp1-1 } \text{tyr7-1 } \text{can}^r \frac{\text{SUP-RL2}}{+}$

*A $\frac{\text{SUP-RL}}{+}$ diploid will yield 2 live: 2 dead spores per ascus.

[†]The mating-type locus is located on chromosome III. An extra third chromosome will yield spores of $\frac{a}{\alpha}$ genotype which will fail to mate.

tetrads were seen. Alternatively, it is possible that leu2-1 is not included in the duplicated region.

Trisomy for the SUP locus was established by the finding that many tetrads (11 out of 22) contained more than two live spores. If the hybrid had contained only two copies of this locus, then each ascus should yield two live and two dead spores. When DBA309 was mated with an a tester strain under conditions not requiring retention of the suppressor, the progeny all yielded 4 viable sup⁺ spores per ascus. This suggests that in the aneuploid DBA309, the SUP allele is present on the copy of chromosome III carrying the a mating-type allele. I conclude that DBA309 is aneuploid for chromosome III and that the SUP-RL1 locus resides on this chromosome.

F. SUP-RL1 lies between thr4 and MAL2 on chromosome III:

To map the position of SUP-RL1 on chromosome III more precisely, the suppressor was moved into a new genetic background in diploid strains heterozygous for thr4 and MAL2, genes which have been mapped previously (Hawthorne and Mortimer, 1960; Mortimer and Hawthorne, 1965).

Two diploid strains were constructed such that the arrangement of thr4 and MAL2 with respect to SUP-RL1 was reciprocal, to control for any differential viability of recombinant phenotypes. Their genotypes for chromosome III markers were: MBD80 $\frac{a}{\alpha} \frac{+}{thr4} \frac{SUP-RL1}{+} \frac{mal2}{+}$ and MBD126 $\frac{a}{\alpha} \frac{thr4}{+} \frac{SUP-RL1}{+} \frac{+}{mal2}$.

Figure 3 details the genealogy of both strains. Each mating step involved either the germination of spores en masse and selection

for SUP diploids resulting from spore-spore matings (steps 2 and 4) or the germination of spores when mixed with an excess of cells of a haploid tester strain and selection for SUP diploids resulting from spore-haploid matings (steps 1, 3, 5, 6, and 7). The rationale and details of this technique for moving a recessive lethal mutation from one genetic background to another are outlined in II. D. Genetic Methods: Matings.

Figure 3 lists only those markers important in the construction of the strains MBD80 and MBD126. The complete genotypes for the strains are listed in Table 6. In each step of Figure 3, the inheritance of the suppressor was selected. In crosses 1, 3, 5, 6, and 7, it was possible to obtain progeny diploid strains which resulted from a SUP-spore X sup⁺ haploid cross by selection for prototrophy of a given marker that was homozygous recessive in the parent diploid. For example, in step 1, all the spores and vegetative diploids (unsporulated DBD339 cells) were Leu⁻; the tester strain was Leu⁺. The desired diploid progeny was selected as Trp⁺ Leu⁺ having inherited the SUP-RL1 gene from one of the spores and the Leu⁺ character from the haploid. Similarly, when MBD16 was screened, it was found to be histidine-requiring so prototrophy for histidine was used in the next step (3) to isolate the desired progeny.

Cross 2 in the construction requires further explanation. The desired diploid was to be homozygous for an amber mutation cycl-179 and heterozygous for the recessive lethal amber suppressor as well. The assumption was made that this diploid would show a level of cytochrome c below that of SUP diploids carrying either $\frac{CYC1}{CYC1}$ or

Legend to Figure 3

The complete genotypes of the strains used are listed in Table 6. Only those markers of importance in each step are noted here. Allele names listed in upper-case letters are dominant (usually wild-type); allele names listed in lower-case letters are recessive (usually mutant). Phenotypes are indicated by an initial upper-case letter followed by two lower case letters and either + or -.

Figure 3

Construction of Mapping Strains

<u>Cross No.</u>		<u>Parents</u>
1	DBD339 spores	<u>a leu2 SUP-RL1 mal2 trp1 tyr7 MALX CYC1</u> <u>α leu2 + mal2 trp1 tyr7 MALX CYC1</u>
	DBH422	<u>α sup⁺ mal2 lys1 trp1 tyr7 malx cycl-179</u>
		Phenotype selected: Leu ⁺ Trp ⁺ Tyr ⁺ (i. e., <u>SUP</u>)
		Yields new strain: <u>MBD15</u>
2	MBD15 spores	<u>a leu2 SUP-RL1 mal2 trp1 tyr7 malx cycl-179</u> <u>α + + mal2 trp1 tyr7 + +</u>
		selfed
		Phenotype selected: Trp ⁺ Tyr ⁺
		Phenotype screened: Cyc ⁻
		Yields new strain: <u>MBD16</u>
3	MBD16 spores	<u>a THR4 SUP-RL1 mal2 trp1 tyr7 his5 cycl-179</u> <u>α THR4 + mal2 trp1 tyr7 his5 cycl-179</u>
	DBH466	<u>a thr4 sup⁺ mal2 trp1 HIS5</u>
		Phenotype selected: Trp ⁺ His ⁺
		Phenotype screened: Mal ⁻
		Yields new strain: <u>MBD27</u>
4	MBD27 spores	<u>a + SUP-RL1 mal2 trp1 his5-2</u> <u>α thr4 + mal2 trp1 +</u>
		selfed
		Phenotype selected: Trp ⁺
		Phenotype screened: Thr ⁺ His ⁻
		Yields new strain: <u>MBD64</u>

Figure 3

(continued)

Cross No.

Parents

5A

MBD64 spores

a + SUP-RL1 mal2 trp1 his CAN^S

α thr4 + mal2 trp1 his can^r

DBH467

α thr4 sup⁺ MAL2 trp1 HIS CAN^S

Phenotype selected: Trp⁺ His⁺ Thr⁺

Phenotype screened: Mal⁺

Yields new strain: MBD80

a + SUP-RL1 mal2 trp1 + can^r

α thr4 + MAL2 trp1 his CAN^S

5B

MBD64 spores

a + SUP-RL1 mal2 trp1 his CAN^S

α thr4 + mal2 trp1 his can^r

DBH467

α thr4 sup⁺ MAL2 trp1 HIS CAN^S

Phenotype selected: Trp⁺ His⁺

Phenotype screened: Mal⁺ Thr⁻

Yields new strain: MBD86

6

MBD86 spores

a thr4 SUP-RL1 mal2 trp1 ade2 CAN^S

α thr4 + + trp1 ade2 can^r

MBH468

α THR4 sup⁺ mal2 trp1 ADE2 can^r

Phenotype selected: Trp⁺ Thr⁺ Ade⁺

Phenotype screened: Mal⁺ Can^S

Yields new strain: MB126

a thr4 SUP-RL1 + trp1 ade2 CAN^S

α + + mal2 trp1 + can^r

$\frac{\text{CYC1}}{\text{cycl-179}}$, since the suppressor was likely to suppress with an efficiency less than 100%. Therefore, only those diploid subclones were examined further which showed a drop in cytochrome c level. Of $\frac{\text{SUP-RL1}}{+}$ diploids, the $\frac{\text{cycl-179}}{\text{cycl-179}}$ genotype is expected at a frequency of 1/4. However, the sporulation mix which is plated on YEP-glucose contains the asci of diploids which have sporulated as well as vegetative diploids, which have failed to undergo sporulation. When the spores are allowed to germinate and mate among themselves, these vegetative diploids also grow and there is no way to distinguish the progeny diploids from the vegetative diploids already present in the mix. These vegetative diploids have a cytochrome c content characteristic of the $\frac{\text{SUP-RL1}}{+} \frac{\text{CYC1}}{\text{cycl-179}}$ diploid. The presence of these diploids greatly reduces the percentage of $\frac{\text{SUP-RL1}}{+} \frac{\text{cycl-179}}{\text{cycl-179}}$ progeny diploids in the screening. In the group examined of which MBD16 was a member, a total of 3 out of 43 had the desired phenotype ($\frac{\text{SUP-RL1}}{+} \frac{\text{cycl-179}}{\text{cycl-179}}$). Therefore, homozygous cycl-179 diploids could be distinguished from the heterozygotes as predicted if the suppressor is less than 100% efficient. The possibility of isolating a mutant suppressor with a lower efficiency of suppression than the original isolate, or of overlooking diploids containing a high efficiency SUP-RL1 seems remote, in a population containing this frequency of the desired genotype.

After the selection process (subcloning on appropriately supplemented minimal medium), the progeny diploids were then screened for the inheritance of non-selected markers by replica-plating. Thirty to sixty clones were usually examined. When all requirements were met,

the diploid was sporulated and the tetrads were analyzed as a final check of the genotype. Only then were the spores used for the next cross. The tetrad data for the seven diploid strains in the genealogy is given in Table 9. (Data for DBD339 is listed in Table 7.)

Diploid strains MBD80 and MBD126 were sporulated and both tetrad and random spore analyses were performed in order to localize SUP-RL1 (Table 9). For both strains, a recessive lethal suppressor segregated and all the survivors were sup⁺. For mating type, thr4 and MAL2, the variance from +:- ratios of 1:1 is considerable, thereby showing linkage to the suppressor. In MBD80, lys2-1 and leu2-1 were segregating 1:1 and the strain is TYR7. It is also heterozygous for can^r but the +:- ratio deviates from 1:1. The gene for canavanine resistance lies on chromosome V, so this deviation is not due to linkage to SUP-RL1 and remains unexplained. Two adenine and two histidine alleles here segregated as well. Strain MBD126 is heterozygous for tyr7-1, can^r, and a histidine and a lysine allele. Two adenine alleles were segregating and the strain is LEU2. Strain MBD80 showed poorer spore viability than its original parent, DBD339; about half the tetrads had only one live spore. MBD126, on the other hand, had excellent spore viability. One tetrad yielded three live spores, all sup⁺. In this case, SUP-RL1 must have been lost through gene conversion or mutation. This tetrad was omitted from the analysis described below when map distance with respect to the suppressor was calculated.

Tetrad analysis was done on the basis of the complete half-tetrads which resulted from each ascus. The distance between SUP-RL1

Table 9
Tetrad Analysis of Strains Used

Strain	Total asci	No. of spores: (Live:Dead)				
		4:0	3:1	2:2	1:3	0:4
MBD15	7	0	0	6	0	1
MBD16	9	0	0	4	5	0
MBD27	8	0	0	5	3	0
MBD64	9	0	0	7	1	1
MBD80	54	0	0	35	19	2
MBD86	7	0	0	3	3	1
MBD126	12	0	1	10	1	0

	<u>MBD15</u>	<u>MBD16</u>	<u>MBD27</u>	<u>MBD64</u>	<u>MBD80</u>	<u>MBD86</u>	<u>MBD126</u>
Ade ⁺ :Ade ⁻	4:8	7:6	2:11	5:10	21:68	0:9	7:17
His ⁺ :His ⁻	0:12	0:13	0:13	0:15	22:67	2:7	14:10
Leu ⁺ :Leu ⁻	7:5	4:9	6:7	15:0	46:43	5:4	24:0
Lys ⁺ :Lys ⁻	8:4	7:6	13:0	15:0	44:45	6:3	9:15
Trp ⁺ :Trp ⁻	0:12	0:13	0:13	0:15	0:89	0:9	0:24
Tyr ⁺ :Tyr ⁻	0:12	0:13	10:3	15:0	89:0	9:0	12:12
Can ^s :Can ^r	7:5	0:13	8:5	9:6	59:30	6:3	10:14
Thr ⁺ :Thr ⁻	NT ^a	NT ^a	3:10	1:14	13:76	0:9	19:5
sup ⁺ :SUP-RL1	12:0	13:0	13:0	15:0	89:0	9:0	24:0
Mal ⁺ :Mal ⁻	9:3 ^b	NT	13:0	15:0	23:66	4:5	19:5
a:α	2:8 ^c	5:8	10:3	9:6	29:60	2:7	7:17
Cyc ⁺ :Cyc ⁻	6:6	0:13	4:9	NT	NT	NT	NT

^aThe diploid was known to be $\frac{THR4}{THR4}$.

^bThe MAL allele segregating here is not MAL2.

^cThe mating type of two spores was not determined.

and any other marker could be determined unambiguously since all survivors were sup⁺ and ascal type was clearly either parental ditype (P), tetratype (T), or non-parental ditype (N). Some uncertainty did arise, however, in assigning ascal types where the other marker pairs were concerned, due to the absence of two of the four spores in each tetrad. The data for these markers, therefore, is not presented. These map distances have been published previously (Mortimer and Hawthorne, 1965). The data from the analysis of half tetrads is presented in Table 10. The formula used for map distance was:

$$\text{distance (centimorgans)} = 100 \frac{T + 6(N)}{2(P + T + N)} \quad (\text{Perkins, 1949})$$

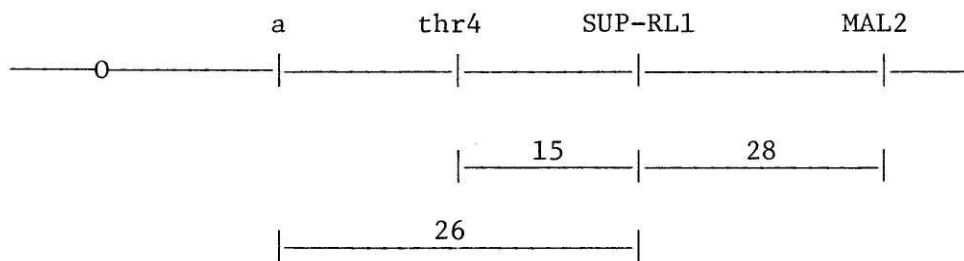
SUP-RL1 maps about 28 cm distal to the mating-type locus between thr4 and MAP2 on chromosome III.

Random spore analysis has an advantage over tetrad analysis for mapping SUP-RL1, since there is no uncertainty in the data concerning marker pairs which do not include the suppressor. Random spore analysis was therefore carried out as well, and the results correspond well to published data for chromosome III markers. Table 11A lists the classes of spores found and their frequencies. The order which best fits the data according to the smallest number of cross-overs necessary is a-thr4-SUP-RL1-MAL2.

Table 11B lists the fraction of total spores carrying a particular gene. If there were no lethal mutation segregating, one would expect equal frequencies for the + and - alleles of the genes.

Table 10
Half Tetrad Mapping Data

<u>Marker pair</u>	<u>No. of asci</u>			<u>Map distance</u>	<u>Average</u>
	<u>P</u>	<u>T</u>	<u>N</u>		
<u>a-SUP-RL1</u>	12	23	0*	33	26
	6	4	0	20	
<u>thr4-SUP-RL1</u>	24	11	0	16	15
	7	3	0	15	
<u>MAL2-SUP-RL1</u>	18	16	0	31	28
	5	5	0	25	



*The first line of numbers is data from MBD80; the second line is from MBD126.

Table 11

A. Spores Classes from Random Spore Analysis

<u>Genotype</u>	<u>MBD80</u>		<u>MBD126</u>	
	<u>No. of spores</u>	<u>No. of crossovers if order is thr4-SUP-RL1-MAL2</u>	<u>No. of spores</u>	<u>No. of crossovers if order is thr4-SUP-RL1-MAL2</u>
α <u>thr4</u> <u>MAL2</u>	148	0	3	3
α <u>thr4</u> +	79	1	0	2
<u>a</u> + <u>MAL2</u>	65	1	10	2
<u>a</u> <u>thr4</u> <u>MAL2</u>	45	1	9	2
<u>a</u> + +	26	2	49	1
<u>a</u> <u>thr4</u> +	11	2	13	1
α + <u>MAL2</u>	10	2	98	1
α + +	<u>3</u>	3	<u>164</u>	0
TOTAL	388		346	

Order deduced: a - thr4 - SUP-RL1 - MAL2

B. Distribution of Alleles in Spore Classes

<u>Strain</u>	<u>Fraction of total spores:</u>					
	<u>a</u>	<u>α</u>	<u>thr4</u>	<u>THR⁺</u>	<u>MAL2</u>	<u>mal⁺</u>
MBD80	.38	.62	.73	.27	.69	.31
MBD126	.23	.77	.07	.93	.35	.65

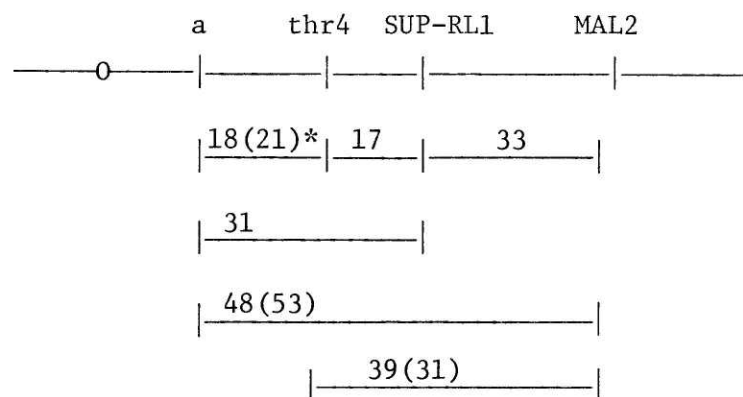
With a lethal mutation segregating, however, a bias appears depending upon the distance between a given allele and the lethal mutation. By using reciprocal diploids, it is possible to find preferential viability among the progeny spores. For example, the maltose fermentation allele which is trans to the lethal SUP-RL1 appears among the progeny 2/3 more frequently than the allele which was cis to SUP-RL1. The reciprocal diploid shows this effect in the opposite direction, as expected. For the mating type locus, however, a was cis to SUP-RL1 in both diploids and therefore a progeny appeared $\frac{1}{2}-\frac{1}{4}$ as frequently as α progeny. The thr4 locus showed slight preferential viability for THR4 progeny in strain MBD126. In its reciprocal, where thr4 was trans to SUP-RL1, $-:+$ progeny were found in the expected 3:1 ratio based on map distance. The reverse 1:3 was not found when thr4 was cis to SUP-RL1; in this case, considerably more than 75% of the survivors were THR4. There may be a reduction in recombination in the region between thr4 and SUP-RL1. However, this effect was not seen in the tetrad analysis where both diploids indicated a thr4-SUP-RL1 distance of 15 cm, and so perhaps should be discounted as due to unrelated random spore effects, or since such a small number of spores is involved, to the laws of small numbers.

Table 12 lists the mapping data based on the random spore analysis. The map distances given here are in excellent agreement with those obtained from tetrad analysis.

Table 12

Random Spore Mapping Data

<u>Marker Pair</u>	<u>MBD80:</u>		<u>MBD126:</u>		<u>Average map distance</u>	<u>Published map distance</u>
	<u>Number of recombinants</u>	<u>% recombination</u>	<u>Number of recombinants</u>	<u>% recombination</u>		
<u>a-thr4</u>	69	18	62	18	18	21
<u>a-SUP-RL1</u>	147	38	81	23	31	--
<u>a-MAL2</u>	192	50	163	47	48	53
<u>thr4-SUP-RL1</u>	104	27	25	7	17	--
<u>thr4-MAL2</u>	165	43	121	35	39	31
<u>MAL2-SUP-RL1</u>	119	31	120	35	33	--
TOTAL	388		346			



*Numbers in parentheses are the published values for these map distances (Mortimer and Hawthorne, 1965).

G. A recessive lethal amber suppressor not on chromosome III:

Strain DBA338 was isolated by the random spore technique from SUP diploid DBD341 in the same manner as strain DBA309. Strain DBA338 differed from DBA309 in that it always displayed the α mating type. Sup⁺ segregants occurred at a frequency of about 1% in vegetative growth (Table 13).

In tetrad analysis of crosses between DBA338 and a a trp1-1 tyr7-1 tester strain, equal numbers of a and α spores appeared, and no non-mating spores were detected. Table 14 contrasts results found in similar crosses of DBA309 and DBA338 to tester haploid strains.

Crosses to strain DBH49 (his4-280) confirmed that DBA338 is not disomic for chromosome III (Table 8). Of 22 asci with more than 2 viable spores, none gave aberrant tetrad ratios for the his4 locus. This strongly suggests that the suppressor in DBA338 is not on chromosome III and thus not identical to the one present in DBA309.

Table 13

Segregation of SUP and Mating-Type in Aneuploid Strains

<u>Phenotype</u>	<u>Inferred genotype</u>	<u>Percentage of colonies which grew on rich plates</u>	
		<u>DBA309 (SUP-RL1)</u>	<u>DBA338 (SUP-RL2)</u>
' α ' Trp ⁻ Tyr ⁻	α <u>trp1-1</u> <u>tyr7-1</u> <u>sup</u> ⁺	0.4	0.8
non-mater Trp ⁺ Tyr ⁺	$\frac{a}{\alpha}$ <u>trp1-1</u> <u>tyr7-1</u> <u>SUP-RL1</u> <u>sup</u> ⁺	99.6	0
non-mater Trp ⁻ Tyr ⁻	$\frac{a}{\alpha}$ <u>trp1-1</u> <u>tyr7-1</u> <u>sup</u> ⁺	0	0
' α ' Trp ⁺ Tyr ⁺	α <u>trp1-1</u> <u>tyr7-1</u> <u>SUP-RL</u> <u>sup</u> ⁺	0	99.2

Strains DBA309 and DBA338 were grown to stationary phase in YEP-glucose, a rich medium which does not select for maintenance of suppressors. Dilutions were plated on YEP-glucose plates for single colonies. When grown up, these colonies were tested by replica plating for their ability to grow in the absence of tryptophan, tyrosine, and phenylalanine and for mating with standard a and α haploid tester strains.

Table 14
Analysis of Spore Progeny from Crosses of Aneuploids
to Tester Haploid Strains

	<u>DBA338 X DBH299</u>	<u>DBA309 X DBH87</u>
Number of tetrads	15	9
ade ⁺ :ade1	21:16	14:14
his ⁺ :his ⁻	13:24*	15:13
leu ⁺ :leu2-1	0:37	17:11
lys ⁺ :lys1-1	20:17	--
met ⁺ :met8-1	31:6 [†]	--
CAN ^S :can ^r	14:23	14:14
sup ⁺ :SUP-RL	23:14	18:10
α:a:a/α	16:21:0	12:3:10 [‡]

DBA338: α adel his1 leu2-1 trp1-1 tyr7-1 can^r SUP-RL2
sup⁺

DBH339: a his4ABC66A4-580 leu2-1 lys1-1 trp1-1 tyr7-1 met8-1
CAN^S sup⁺

DBA309: a leu2-1 SUP-RL1 trp1-1 tyr7-1 can^r
α leu2-1 sup⁺

DBH87: a adel his1 trp1-1 tyr7-1 CAN^S sup⁺

*Two his⁻ markers are segregating.

[†]Excess of prototrophs not understood; however, commercial preparations of leucine are often contaminated with methionine.

[‡]Three prototrophic spores: mating-type determination not performed.

IV. Discussion

This chapter described the isolation of recessive lethal amber suppressor mutations in diploid strains of yeast. The frequency of this type of mutation is very high; in fact, a majority of the suppressors isolated in diploids (using reversion of two different pairs of amber mutations) were recessive lethals. At present, the number of loci capable of mutation to recessive lethal suppressor is unknown. On the basis of segregation of suppressor-bearing aneuploids, it is clear that at least two such loci exist. One locus is on chromosome III while another, as yet unmapped, is not on this chromosome.

Isolation of nonsense suppressors in yeast has heretofore been achieved almost exclusively in haploid strains. The best-studied group, the eight class I set 1 ochre suppressors, inserts tyrosine into protein (Gilmore et al., 1971). A common interpretation of this finding is that eight redundant genes exist for the coding of tRNA^{Tyr} in Saccharomyces cerevisiae, and that each of the suppressors is an alteration in coding specificity at one of these genes. There is proof that tyrosine-inserting amber suppressors in E. coli are alterations in one of several redundant tRNA^{Tyr} genes (Russell et al., 1970; Squires et al., 1973). Recently, Gesteland (personal communication) has shown that the tyrosine-inserting suppressors in yeast are also alterations in tRNA.

There are two possible explanations for recessive lethality of nonsense suppressors: (1) over-suppression or (2) the involvement

of an essential tRNA. A discussion of these two models for recessive lethality follows.

A. Over-suppression as an explanation for recessive lethality

A possible explanation for the recessive lethality of these amber suppressor mutations involves the efficiency of suppression. The suppressors might be so efficient in substituting an amino acid at the site of an amber codon that the physiology of the haploid cell is disrupted, resulting in failure to grow. In this case, it would not be the absence of an essential function but the over-suppression which is lethal to the haploid cell. Some effect on cell growth when suppression efficiency is enhanced was suggested by Gilmore (1967). By generating a very efficient suppressor mutation, it can be imagined that a diploid could be produced with a recessive lethal phenotype, yielding two live spores and two dead spores. On the over-suppression hypothesis, if the suppressor mutations are in the tRNA genes, the tRNAs need not be unique coding species represented by a single gene copy in the genome of yeast.

This explanation has been advanced in the case of certain ochre suppressors (class 1, set 1 of Gilmore, 1967) in the presence of the non-Mendelian factor, psi (Cox, 1971). Cox proposed that the psi factor enhances suppression efficiency to a level lethal to the haploid cell. Evidence consistent with Cox's idea was provided by Liebman et al. (1975), who found an increase in suppressed iso-1-cytochrome c carrying an ochre mutation in psi⁺ cells compared to

psi- cells containing the ochre suppressor SUQ5. Psi has no detectable effect on amber suppressors.

B. The essential tRNA hypothesis

Alternatively, a recessive lethal mutation could involve an essential gene represented in a single copy in the genome, the absence of which leads to lethality. If the suppressor mutation codes for a tRNA, then the tRNA may be a unique coding species or carry out another vital function possibly unrelated to protein synthesis. In this model, the wild-type form of the mutated gene plays a critical role.

The recessive lethality of SUP-RL1 seems to be explained best by the essential tRNA hypothesis. Since every aneuploid strain (SUP/sup⁺) generated was disomic for chromosome III, one is forced to conclude that the wild-type locus of the suppressor gene is necessary to the cell's survival. Aneuploidy might also be predicted in the case of over-suppression; however, it is likely that duplication of a number of genes on many different chromosomes would serve to lower the efficiency of the suppressor and therefore restore viability (e. g., a larger dose of termination factors, ribosomal proteins, etc.).

The recessive lethal amber suppressor, Su7, isolated in a mero-diploid of E. coli by Soll and Berg (1969), is an example of a mutation in an essential tRNA. The mutation involved a single base change in the anticodon of the tryptophan tRNA altering codon recognition from UGG to UAG and simultaneously causing aminoacylation of

glutamine instead of tryptophan (Soll, 1974; Yaniv et al., 1974). Since E. coli is known to have only one gene coding for tRNA^{Trp} (Hirsh, 1971), the loss of the ability to translate UGG as tryptophan is believed responsible for the recessive lethality.

By analogy to E. coli, one type of recessive lethal suppressor in S. cerevisiae could be an altered tRNA^{Trp}. Yeast appears to have a single species of this tRNA (two forms exist which differ only in that one U is modified to ψ) (Keith et al., 1971), although the number of genes which code for tRNA^{Trp} is unknown.

C. SUP61: Another recessive lethal amber suppressor

Hawthorne and Leupold (1974) mention the isolation of a recessive lethal suppressor, SUP61, from a diploid strain of yeast. All asci analyzed gave two live spores per ascus. The spores carrying the recessive lethal suppressor mutation were rescued by spore mating shortly after germination and the suppressor recovered. Analysis was performed on a trisome. SUP61 has been mapped on chromosome III (Mortimer and Hawthorne, 1973), about 30 cm distal to the mating-type locus. The suppressor (SUP-RL1) in DBA309 could well be identical to SUP61.

CHAPTER III

DETERMINATION OF THE AMINO ACID INSERTION OF SUP-RL1I. Introduction

Numerous nonsense suppressors in the yeast Saccharomyces cerevisiae have been systematically characterized with respect to their ability to suppress nutritional mutants and iso-1-cytochrome c (cycl) mutants which contain defined UAA (ochre) and UAG (amber) mutations. In addition, the efficiencies of suppression have been determined from the levels of iso-1-cytochrome c in suppressed cycl mutants. These studies revealed that eight suppressors, each at a distinct locus on six different chromosomes, acted efficiently on UAA cycl mutants and that all of them caused the insertion of tyrosine at UAA sites (Gilmore, 1967; Gilmore et al., 1971). Another UAA suppressor, SUQ5-1, was found to insert serine with a low efficiency and was found to become highly efficient with the non-Mendelian genetic factor, psi⁺, which also increases the efficiencies of the UAA suppressors that insert tyrosine (Liebman et al., 1975). In an extensive search for UAG suppressors that acted efficiently on cycl mutants, again only eight suppressors were uncovered, and these were allelic to the eight UAA suppressors that insert tyrosine (Liebman et al., 1976). Thus, so far the only efficient suppressors that have been reported are those which insert tyrosine at UAA and UAG sites and the one which inserts serine at UAA sites when the efficiency is increased by the action of the psi⁺ determinants. While it has not been excluded that other

efficient UAA suppressors may be uncovered in psi⁺ strains, it does not appear as if any other efficient UAG suppressors could be normally obtained in haploid strains (Liebman et al., 1976).

This situation contrasts with that in E. coli where high efficiency suppressors have been found which insert serine (Capecchi and Gussin, 1965), glutamine (Garen et al., 1965; Kaplan et al., 1965), tyrosine (Garen et al., 1965; Goodman et al., 1968; Gallucci and Garen, 1966), and leucine (Chan and Garen, 1969; Gopinathan and Garen, 1970).

This difference can be explained in several ways. It is possible that tyrosine is the only compatible replacement for the ochre and amber alleles which were used. Alternatively, lower efficiency suppressors whose amino acid insertions require greater effort to determine have not been studied extensively. These suppressors may insert amino acids other than tyrosine. Similarly, mutations which result in high efficiencies of suppression incompatible with cell survival would not appear under the isolation conditions used. The insertion of tyrosine may be the result of a combination of amino acid compatibility requirements and a critical range of suppression efficiency. Finally, suppressors which may have arisen from essential tRNAs would not be detected in these studies, since the suppressors were derived in haploid yeast strains.

The determination of amino acid insertions of suppressors generated under less restrictive conditions should indicate the existence of suppressors carrying all the amino acids with codons one base removed from the amber codon. The analysis of SUP-RL1, the

recessive lethal amber suppressor, presents another opportunity to broaden the spectrum of nonsense suppressors in yeast.

The amino acid insertion of SUP-RL1 was determined by the analysis of a suppressed protein, iso-1-cytochrome c, in a strain in which SUP-RL1 was coupled to a known amber mutant of iso-1-cytochrome c. SUP-RL1 is shown to act efficiently on the UAG mutant and to cause the insertion of serine into position 9 (normally occupied by a lysine residue) of iso-1-cytochrome c.

II. Materials and Methods

A. Yeast strains: The construction of strain MBD16

$\frac{a \text{ ade3-26 } \underline{\text{his1}} \text{ his5-2 } \underline{\text{leu2-1}} \text{ } \underline{\text{lys1-1}} \text{ } \underline{\text{trp1-1}} \text{ } \underline{\text{tyr7-1}} \text{ } \underline{\text{can}}^r \text{ SUP-RL1 } \underline{\text{cycl-179}}}{\alpha \quad + \quad + \quad \underline{\text{his5-2}} \quad + \quad + \quad \underline{\text{trp1-1}} \text{ } \underline{\text{tyr7-1}} \text{ } \underline{\text{can}}^r \quad + \quad \underline{\text{cycl-179}}}$

was described in Chapter II. (See Figure 2 and section F. of Results in that chapter.) Additional strains used are listed below:

DBA309 $\frac{a \text{ leu2-1 } \text{ SUP-RL1 } \underline{\text{trp1-1}} \text{ } \underline{\text{tyr7-1}} \text{ } \underline{\text{can}}^r}{\alpha \text{ leu2-1} \quad +}$

DBA311 $\frac{a \text{ leu2-1 } \text{ SUP-RL1 } \underline{\text{his1}} \text{ } \underline{\text{trp1-1}} \text{ } \underline{\text{tyr7-1}} \text{ } \underline{\text{can}}^r}{a \text{ leu2-1} \quad +}$

DBA323 $\frac{\alpha \text{ SUP-RL1 } \underline{\text{ade3-26}} \text{ } \underline{\text{lys1-1}} \text{ } \underline{\text{trp1-1}} \text{ } \underline{\text{tyr7-1}} \text{ } \underline{\text{cycl-179}}}{\alpha \quad +}$

DBH251 $\alpha \text{ } \underline{\text{his1}} \text{ } \underline{\text{trp1-1}} \text{ } \underline{\text{tyr7-1}}$

SL158-23B $\alpha \text{ } \underline{\text{ade3-26}} \text{ } \underline{\text{his5-2}} \text{ } \underline{\text{lys1-1}} \text{ } \underline{\text{met8-1}} \text{ } \underline{\text{trp1-1}} \text{ } \underline{\text{tyr7-1}} \text{ } \underline{\text{cycl-179}}$

B. Media: Media, incubation conditions, genetic methods, and the determination of cytochrome c content were as described in Chapter II.

C. Growth of yeast and preparation of iso-1-cytochrome c:

Twelve-liter batches of the suppressed strains were grown under de-repressed conditions by the following procedure. Precultures were prepared by growing each strain to stationary phase with vigorous shaking for three days in a 6-liter Erlenmeyer flask which contained 1 liter of the following medium: 1% Bacto-yeast extract, 2% Bacto-peptone, and 3% dextrose. The 1-liter preculture was added to a model MA1401 fermentor (Fermentation Design, Inc., Allentown, Pa.)

that contained 2 liters of the following medium: 2.7% Ardamini-Z yeast extract (Yeast Products, Inc., Paterson, N. J.), 2.7% technical grade Bacto-peptone, 4% dextrose, 1% (v/v) of 95% ethanol, 0.01% streptomycin, 10^5 units of penicillin, and 20 ml of the anti-foam P-2000 polypropylene glycol (Dow Chemical Company). The fermentor was operated at 30°C, an air flow of 30 liter/min., and an agitation speed of 500 revs/min. After 1 to 2 hours, an additional 9 liters of the same medium was pumped (model RRP2G20 Lab pump, Fluid Metering, Inc., Oyster Bay, N. Y.) into the fermentor at approximately 400 ml/hour. The fermentor was run for approximately an additional 2 hours after the pumping was completed. This growth procedure, entailing high aeration and slow feeding of rich medium, results in particularly high yields of cells that are derepressed and therefore contain high amounts of cytochrome c. The cells were collected in a Lourdes CFR-1 rotor (Vernitron Medical Products, Inc., Carlstadt, N. J.) that holds a maximum of approximately 800 g wet cells, an amount which is lower than the yield of cells from the culture. The cells were stored frozen until iso-1-cytochrome c was extracted and purified.

Approximately 2 kg, wet weight, of washed yeast suspended in 1 liter of 1.0 M NaCl and 500 ml of ethyl acetate were shaken gently for about 18 hours at 10-18°. Five liters or more of distilled water and approximately 100 g of a relatively coarse (100-200 mesh) Amberlite CG-50 (Na^+) cation exchange resin (Mallinkrodt) were added to the resulting autolysate. (The resin had been prepared

by alternate washing with NaOH and HCl (Hirs, 1955), and the sodium form was treated with sodium hypochlorite (Hagihara *et al.*, 1956). Finally, the resin was equilibrated against 0.1 M sodium phosphate buffer, pH 7.2.) After shaking for 30 minutes, an additional 50 g of resin were added to the mixture; this process was repeated once and then the solution was shaken for a final 30 minutes. At this point, the cytochrome c was bound to the resin. After washing the resin with deionized water, it was packed in a column and the cytochrome c was eluted with 2M NaCl in 0.1 M sodium phosphate buffer, pH 7.2. The cytochrome c, identified by its red color, was collected and dialyzed at 4°C overnight against two changes of a solution which was 0.1% by weight $(\text{NH}_4)_2\text{CO}_3$ and 0.005% by volume mercaptoethanol. Several drops of mercaptoethanol were added to each dialysis bag. The sample was then bound to the top of a 1.5 cm x 20 cm column of fine CG-50 cation exchange resin (200-400 mesh) which was prepared as described above. A small amount of mercaptoethanol was then passed through the column and a linear gradient established between 400 ml of a solution containing 0.1 M sodium phosphate buffer, pH 7.2, and the same volume of a 0.8 M NaCl solution in the same buffer was used to elute the cytochrome c. A drop of mercaptoethanol was added to each reservoir. The mercaptoethanol was used to minimize the amount of the disulfide dimer of iso-1-cytochrome c (Motonaga *et al.*, 1965). Usually, two distinct bands (the iso-1 and iso-2-cytochromes c) could be seen travelling down the column. The first to be eluted, the iso-1-cytochrome c, was collected and bound to a small amount of fine resin from which it was eluted with a minimal amount of 2 M NaCl

in 0.1 M sodium phosphate buffer, pH 7.2, The sample was then dialyzed as described above and was rechromatographed on a 1.5 cm x 20 cm column of CG-50 precisely as described above. Also as described above, the pink eluate was concentrated on a small fine resin column. The rechromatographed and concentrated sample was dialyzed in the cold against a 0.1% $(\text{NH}_4)_2\text{CO}_3$ solution and was applied to a 45 cm x 2.5 cm column of superfine G-75 Sephadex with $\frac{1}{2}$ drop of mercaptoethanol. The Sephadex had been equilibrated with 0.4 M NaCl in a pH 7.5 sodium phosphate buffer, 60 mM (Flatmark, 1964), to which 1 ml thiodiglycol/liter and 10 mg cycloheximide/liter had been added. The sample was eluted at 4°C with a flow rate of 15 ml/hr, and 2 ml fractions were collected. The fractions which contained the central portion of the red peak were determined visually, and pooled. The purified cytochrome c was concentrated with the use of fine resin as described above, was dialyzed at 4°C against $(\text{NH}_4)_2\text{CO}_3$, and was freeze-dried.

D. Identification of structural changes in iso-1-cytochrome c:

This work was performed by Dr. John Stewart at the University of Rochester.

The methods used for enzymic digestion, peptide mapping, sequential Edman degradation and chromatographic identification of the phenylthiohydantoin and dansyl derivatives have been described (Stewart et al., 1971). Cytochrome c was digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin and with chymotrypsin

in 0.03% ammonium bicarbonate (w/v). The enzymic digests were lyophilized and were subjected first to electrophoresis on Whatman No. 3 MM paper wet with pyridine acetate, pH 6.5, and then to chromatography, in the second dimension, with n-butanol, pyridine, acetic acid, water (15:10:3:12 v/v). Peptide maps were developed sequentially with a ninhydrin, collidine reagent, with an Ehrlich reagent, and with a Pauly reagent.

Samples of 0.3 mole of cytochrome c were sequentially degraded. After cleavage of the thiazolinones, trifluoroacetic acid was removed by lyophilization and the thiazolinones were extracted into ethyl acetate and converted to phenylthiohydantoins. These were identified by one-dimensional chromatography on thin layer plates of fluorescent silica gel. Dansyl NH₂-terminal analysis was performed according to Gros and Labouesse (1969).

III. Results

A. Suppression of the UAG mutant cycl-179 by SUP-RL1: A diploid strain MBD16 which was heterozygous for SUP-RL1 and homozygous for cycl-179, a UAG mutation corresponding to lysine 9 of iso-1-cytochrome c, was constructed as described in Chapter II (see Figure 1 and Results, section F., page 62). Tetrad analysis (Table 9) revealed its genotype as $\frac{a}{\alpha} \frac{ade3-26}{+} \frac{his1}{+} \frac{his5-2}{his5-2} \frac{leu2-1}{+} \frac{lys1-1}{+} \frac{trp1-1}{trp1-1} \frac{tyr7-1}{tyr7-1} \frac{SUP-RL1}{+} \frac{can^r}{can^r} \frac{cycl-179}{cycl-179}$. It was found to have a level of cytochrome c that was intermediate to the level found in heterozygous ($\frac{cycl-179}{CYC1}$) and homozygous ($\frac{cycl-179}{cycl-179}$) diploids, suggesting that SUP-RL1 was suppressing the amber mutation. Approximately 10-20% of the normal level of total cytochrome c was made.

In addition to MBD16, a strain disomic for chromosome III was constructed which carried SUP-RL1 and the cycl-179 allele. Strain DBA311, a leu2-1 SUP-RL1 his1 trp1-1 tyr7-1 can^r CYC1, was derived as a segregant from a cross between DBA309 (described in Chapter II) and DBH251 α his1 trp1-1 tyr7-1. Strain DBA311 was crossed to strain SL158-23B, α ade3-26 his5-2 lys1-1 met8-1 trp1-1 tyr7-1 cycl-179 and the diploid was sporulated and dissected. One of the segregants was DBA323 $\frac{\alpha}{\alpha} \frac{SUP-RL1}{+} \frac{ade3-26}{+} \frac{lys1-1}{+} \frac{trp1-1}{+} \frac{tyr7-1}{+} \frac{cycl-179}{+}$. By low temperature spectroscopy, this aneuploid was found to contain about 50% of the wild-type level of cytochrome c. However, after extensive growth in nutrient medium, the amount of cytochrome c dropped to about 10% of the wild-type level. Only 7% of the cells had actually lost the suppressor-bearing chromosome, III, which fails to

account for the drop in cytochrome c level. Most of the cells retained the suppressor since the nutritional markers trp1-1 and tyr7-1 remained suppressed. This appearance of strains with lowered efficiencies of suppression is similar to the effect extensively studied in haploid strains, where the inhibition of growth by highly efficient UAG suppressors results in selection of strains with secondary mutations that cause partial inactivation of the suppressors (Liebman and Sherman, 1976).

Other diploid strains which were genotypically $\frac{\text{SUP-RL1}}{+} \frac{\text{cycl-179}}{\text{cycl-179}}$ like MBD16, but which contained relatively high levels of cytochrome c, displayed the same drop in cytochrome c content after growth in nutrient medium as described for the aneuploid strain DBA323. However, these strains failed to sporulate, behaving similarly to those strains having highly efficient UAG suppressors described by Liebman and Sherman (1976). Since strain MBD16 was relatively stable in its cytochrome c content (10-20%) and it sporulated well, it was grown in large quantities from which suppressed iso-1-cytochrome c was isolated.

B. Altered iso-1-cytochrome c from the cycl-179 mutant suppressed by SUP-RL1: Chromatographic analysis of cytochromes c from strain MBD16 revealed fractions corresponding in position to iso-1-cytochrome c and iso-2-cytochrome c. Thus, both the increased total amount of cytochrome c observed in intact cells and the presence of cytochrome c at the elution position of iso-1-cytochrome c established that SUP-RL1 was suppressing cycl-179.

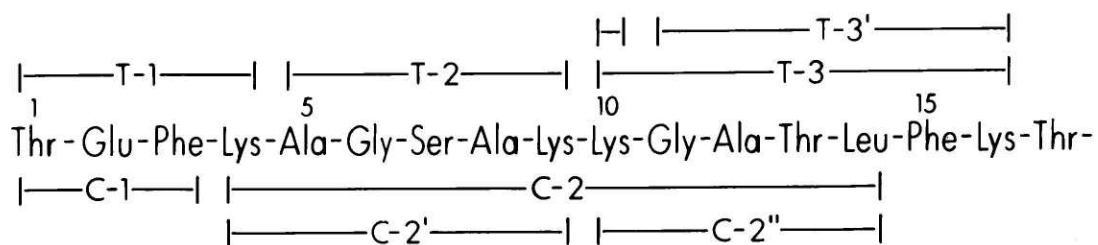
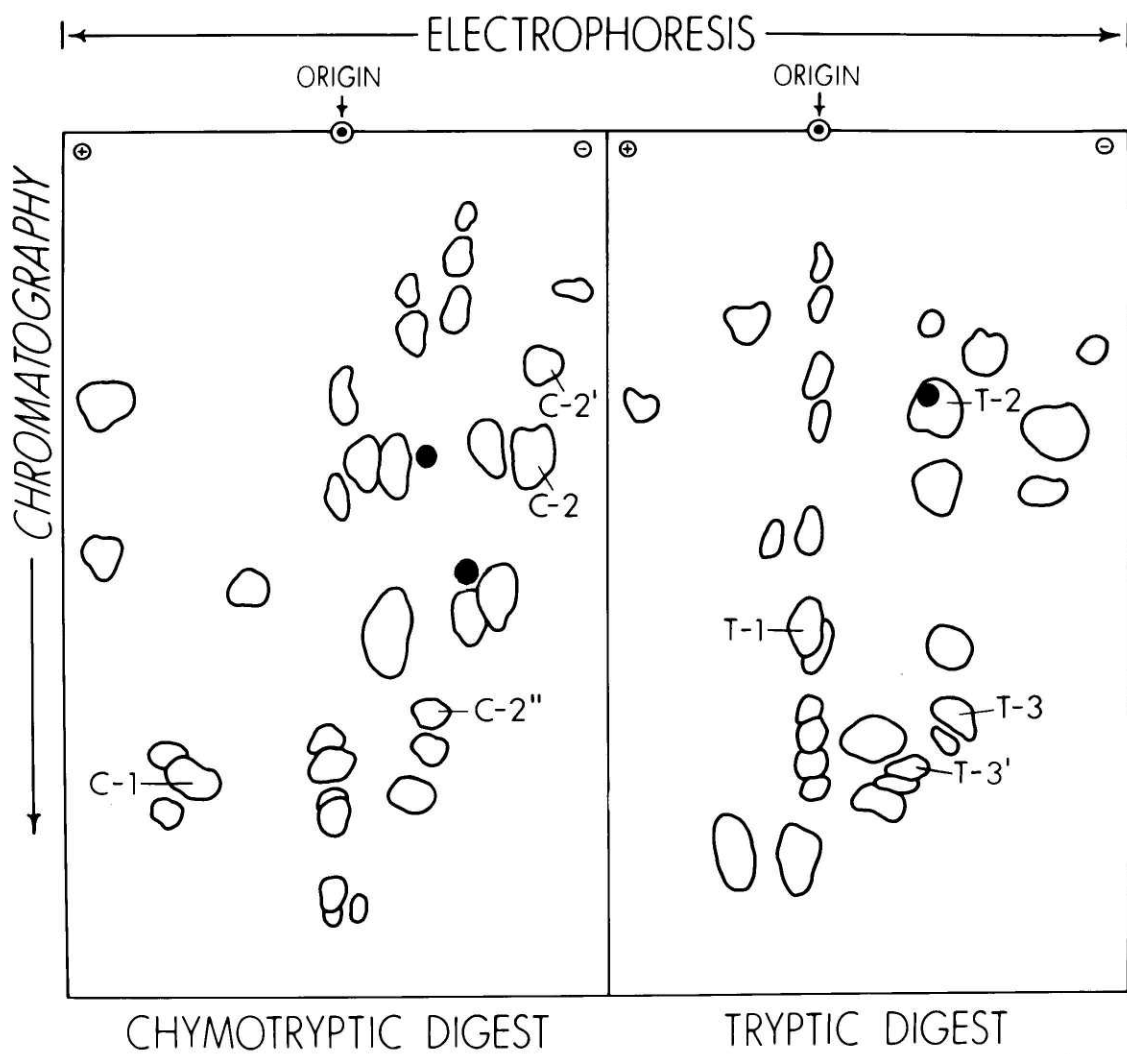
Peptide maps of tryptic and chymotryptic digests of the suppressed iso-1-cytochrome c demonstrated that a residue other than the normal residue of lysine occupied position 9 which corresponds to the site of the UAG mutation. The distribution of sites of enzymic cleavage near residue 9 and the mobilities of the resulting peptides make peptide maps reliable indicators for replacements of lysine 9 (Stewart and Sherman, 1972). The peptide maps of the iso-1-cytochrome c from MBD16 were identical or very similar to those of the proteins have either serine or glutamine replacing lysine 9 as the sole change (see Figure 4). The NH₂-terminal region of the iso-1-cytochrome c was subjected to sequence analysis through position 10, which revealed a serine residue replacing the normal lysine 9. The NH₂ terminal sequence was established as Thr-Glu-Phe-Lys-Ala-Gly-Ser-Ala-Ser-Lys.

Figure 4

Peptide map of iso-1-cytochrome c

Outlined areas represent normal spots on ninhydrin-stained peptide maps of iso-1-cytochrome c, from wild-type S. cerevisiae. The maps of MBD16 lacked the normal spots C-2, C-2', T-2, and T-3 and contain only three-quarters of the normal intensity of the spot to the right of T-2.

The filled circles represent centers of new ninhydrin-positive peptides containing a serine residue at position 9 replacing the original lysine.



IV. Discussion

A. The action of SUP-RL1: The action of the recessive lethal amber suppressor mutation, SUP-RL1, has been shown to cause the insertion of serine corresponding to the site of an amber codon in the gene coding for iso-1-cytochrome c. The suppressed protein contained serine instead of the normal lysine 9, at the residue position controlled by the amber triplet. The suppressor can cause the production of approximately 50% of the normal amount of iso-1-cytochrome c in disomic strains heterozygous for the suppressor ($\frac{\text{SUP-RL1}}{+}$). This level of suppression nearly corresponds to the level observed with the tyrosine-inserting UAG suppressors which can cause the production of approximately 75% of the normal level in haploid strains (Liebman and Sherman, 1976; Liebman et al., 1976).

There is evidence that SUP-RL1 determines a mutated tRNA. In a heterologous in vitro protein-synthesizing system, crude yeast supernatant made from a $\frac{\text{SUP-RL1}}{+}$ disomic strain successfully translated Q β RNA containing a UAG mutation in the Q β synthetase gene (R. Gesteland, personal communication). Suppression was also observed with a tRNA fraction prepared from this supernatant. A high degree of suppression was observed with the in vitro system, where approximately equal amounts of the amber fragment and the completed synthetase were synthesized. This 50% of in vitro suppression corresponds to the 50% of in vivo suppression of the cycl-179 mutant. mutant.

The simplest interpretation of the data is that SUP-RL1 is an altered serine-tRNA. There are six serine codons which in different

organisms are read by a variable number of tRNAs. In yeast, ribosome binding studies have shown that the codons UCU, UCC, and UCA are read by a set of tRNAs (Ser_1 and Ser_2) which have the anticodon IGA, but differ from one another in positions outside the anticodon (Caskey *et al.*, 1968). Another set of tRNAs (Ser_{3a} and Ser_{3b} , Ishikura *et al.*, 1971) reads the codons AGC and AGU with the anticodon GCU. The last codon, UCG, which is the only one related to the amber codon by a single base change ($\text{UCG} \rightarrow \text{UAG}$) is believed to be recognized by a tRNA bearing the anticodon CGA. If this species of tRNA is coded for by a single gene in haploid yeast, its loss of function would lead to recessive lethality. The cell could then be unable to put serine into protein where UCG was coded.

B. A comparison of seryl-tRNAs: Kruppa and Zachau (1972) have reported the isolation of a $\text{tRNA}_{\text{x}}^{\text{Ser}}$ in yeast which could be the UCG-responding tRNA. It is present in very small amounts.

The anticodon CGA was determined from a partial nucleotide sequence of a minor species of serine tRNA from rat liver (Rogg and Staehelin, 1971 a, b). A single base change of G to U in the anticodon of such a tRNA that recognizes UCG would result in a tRNA that recognizes the amber codon UAG. If a similar tRNA exists in yeast, a UAG suppressor which inserts serine could arise from a $\text{G}\cdot\text{C} \rightarrow \text{T}\cdot\text{A}$ transversion.

It is noteworthy that while yeast, guinea pig and rat liver, and Drosophila use a similar arrangement of tRNAs for the recognition

of serine codons (Caskey et al., 1968; Rogg and Staehelin, 1971; White et al., 1975), E. coli has a different pattern. AGU and AGC are read by one tRNA species carrying the anticodon GCU as in the higher organisms, but the codons UCU, UCA, and UCG are probably read by one tRNA species, and UCU and UCC by another tRNA species (Caskey et al., 1968; Ishikura et al., 1971). Apparently the codon with the 3' modified base, uridine 5-oxyacetic acid (V) is capable of reading UCA, UCG, and, less efficiently, UCU (Ishikura et al., 1971).

The possibility remains as in the case of the E. coli recessive lethal suppressor, Su7 (Soll and Berg, 1969) that the mutation in SUP-RL1 has led to mischarging of another tRNA with serine. The mutation in Su7 involved a single base change in the anticodon of the tryptophan tRNA altering codon recognition from UGG to UAG simultaneously causing aminoacylation of glutamine instead of tryptophan (Soll, 1974; Yaniv et al., 1974). Until the SUP-RL1 tRNA is purified and sequenced, its identity will remain unknown.

The insertion of serine by another nonsense suppressor from yeast has recently been reported (Liebman et al., 1975). This suppressor, SUQ5, originally isolated by Cox (1965), is clearly unrelated to SUP-RL1. The SUQ5 suppressor which maps on chromosome XVI, acts only on UAA and not UAG codons and is efficient only in the presence of the psi⁺ genetic factor (Liebman et al., 1975). It was suggested that SUQ5 originated by mutation of a gene controlling a serine tRNA with a UGA anticodon; the mutated anticodon UUA, would be enzymatically

converted to SUA, which could be the anticodon of the tRNA determined by SUQ5 (Liebman et al., 1975).

In yet another case, a weak amber suppressor, SUPG, isolated by Liebman (1973), has been found to insert leucine (unpublished results). The spectrum of amino acid insertions by yeast nonsense suppressors has begun to broaden now that different types of suppressors (diploid-derived instead of haploid-derived, low efficiency instead of high efficiency) are coming under study.

APPENDIX

A. The attempt to isolate recessive lethal ochre suppressors

Two haploid strains DBH271 and DBH272 were crossed to form a diploid of genotype $\frac{a \text{ leu2-1 lys1-1 trp1-1}}{\alpha \text{ leu2-1 lys1-1 trp1-1}} + \frac{\text{adel his1 CAN}^S}{\text{tyr7-1 adel his1 can}^r}$, containing the two ochre alleles, leu2-1 and lys1-1. In the same manner as DBD195, this diploid was found to have good spore viability (over 90%) and behaved genetically as expected. Twenty-five independent cultures were grown to stationary phase in rich medium and approximately 4×10^7 cells were spread on plates containing adenine, histidine, and tryptophan, to select for $\text{Leu}^+ \text{Lys}^+$ revertants. Some plates were treated with one drop of EMS. After six days at 30° , two single colonies were picked from each plate and were purified by sub-cloning. Each was then sporulated and a random spore analysis was performed as described in Chapter II for the $\text{Trp}^+ \text{Tyr}^+$ revertants of DBD195.

In every case, the growth pattern of spores on the plates containing canavanine indicated that a non-lethal suppressor was segregating. The results are shown in the table below. Recessive lethal ochre suppressors were never found.

<u>Mutagenic treatment</u>	<u>No. of clones containing non-lethal suppressors</u>	<u>No. of clones containing recessive lethal suppressors</u>
None	10/10	0/10
EMS	35/40*	0/40
Total	45/50*	0/50

*The suppressors in five revertants were not classified due to poor growth or lack of sporulation.

It is possible that a recessive lethal ochre suppressor does exist in yeast but that its amino acid insertion is incompatible with the function of either the leu2-1 or the lys1-1 locus. Other ochre alleles would have to be used to determine whether this is an explanation for the negative result.

Alternatively, the tRNAs which can be mutated by a single base change to recognize the ochre codon are not unique coding species, present in the DNA in a single gene copy, and therefore, are not essential. Their mutation to suppressor form would not result in recessive lethality.

Further, if many of the messages in the yeast cell use the ochre codon as a termination signal, the generation of a highly efficient ochre suppressor might be a lethal event for a diploid cell as well as for a haploid cell. Therefore, recessive lethal suppressors of the over-suppressing variety would not be found.

However, it is clear that too small a number of colonies was examined to permit a definitive statement concerning the existence of recessive lethal ochre suppressors in yeast.

B. Studies on SUP-RL2

The isolation and original characterization of DBD341, which contained SUP-RL2, has been described in Chapter II. The aneuploid strain, DBA338, derived from it was shown to segregate SUP-RL2 at a measurable frequency and the duplicated chromosome was shown to be one other than chromosome III.

The original DBD341 in vegetative culture was lost and all attempts to reconstruct it from asci preserved in water failed. Every putative diploid which was found from the sister spore matings when sporulated yielded some tetrads with more than two live spores, indicative of a trisome ($2n+1$).

However, DBA338 remained intact and was used in crosses with haploid strains containing cycl-179 in order to couple SUP-RL2 and cycl-179.

DBA338 α ade1 his1 leu2-1 trp1-1 tyr7-1 can^r SUP-RL2 was crossed to SL210-3A a cycl-179 ade3-26 his5-2 leu2-1 met8-1 ilv1-1 lys1-1 trp1-1 tyr7-1. No disomic segregants were found from the spores of this diploid in which SUP-RL2 was coupled to cycl-179. The two genes acted as if they were linked and a crude calculation based on 18 tetrads showed them to be 8-19 map units apart, on chromosome X. The uncertainty is due to the tetrads in which only two spores germinated which sometimes could not be definitely assigned an ascus type (P, T, N).

By spore-haploid matings similar to those described in Chapter II, a diploid was constructed which was derived from the spores of DBD341 and a haploid DBH325 α ade3-26 his5-2 lys1-1 trp1-1 tyr7-1 cycl-179. MBD14 was shown to be $\frac{\text{SUP-RL2}}{\text{sup}^+} \frac{\text{CYC1}}{\text{cycl-179}}$; all half-tetrads which arose from the dissection of 11 tetrads were cycl-179, indicating, again, a tight linkage to the cycl locus. MBD22 and MBD23, other $\frac{\text{SUP-RL2}}{\text{sup}^+}$ diploids similarly derived, indicated that SUP-RL2 was between 0-14 map units from cycl.

These diploids all displayed another property which served to prevent further characterization of SUP-RL2: the suppressor segregated out at a very high frequency and was lost from a population in growth to stationary phase. This instability precluded attempts to isolate a strain which had either a meiotic or mitotic crossing over between cycl-179 and SUP-RL2 or to obtain kilogram quantities of fermentor-grown yeast in order to isolate a suppressed iso-1-cytochrome c.

These derived diploids were not trisomes ($2n+1$) since only tetrads with two live spores were seen and never any SUP-RL2 disomic spores, which would be expected if chromosome X were duplicated. Therefore, the instability of the locus cannot be explained by instability of an extra chromosome.

A single mutational event, from SUP-RL2 to sup⁺, followed by selection can account for this instability in the same manner Liebman *et al.* (1976) found. Alternatively, if the locus of SUP-RL2 were a hotspot for recombination or mitotic crossing over, then only those recombinants yielding $\frac{\text{sup}^+}{\text{sup}^-}$ progeny would survive, since $\frac{\text{SUP-RL2}}{\text{SUP-RL2}}$ is a lethal condition. SUP-RL2 could be bounded on both sides by a direct repeated sequence or by a sequence with partial homology (a cluster of tRNA genes); a recombinational event followed by selection might well account for the SUP-RL2 instability.

A non-lethal suppressor which inserts tyrosine, SUP4, has been found to be very closely linked to the cycl locus (Hawthorne and Mortimer, 1968). It is difficult to imagine how SUP-RL2 might be

allelic to a non-lethal suppressor. Given the map distance found, SUP-RL2 and SUP4 are probably not allelic.

C. Studies on SUP61

Hawthorne reported the isolation of a recessive lethal amber suppressor SUP61 which, when sporulated, yielded only two live spores per ascus and mapped between thr4 and MAL2 on chromosome III (Hawthorne and Leupold, 1974; Mortimer and Hawthorne, 1973). He has also succeeded in converting SUP61 to an ochre suppressor and a UGA suppressor.

Hawthorne generously gave the strain to David Botstein with SUP61 in the ochre suppressor form, and I constructed a strain containing SUP61 in a diploid background homozygous for cycl-72, an ochre mutation corresponding to position 66 of iso-1-cytochrome c. The procedure was completely analogous to that used for SUP-RL1.

Hawthorne's strain, which was called MBD89, had the genotype

$$\frac{a \text{ SUP61 } leu2-1 \text{ trp1-1 } met8-1 \text{ ura4 } his5 \text{ lys1-1 } arg4 \text{ isol } ade}{\alpha \quad + \quad leu2-1 \text{ trp1-1 } met8-1 \text{ ura4 } + \quad + \quad + \quad + \quad ade} \cdot \frac{Leu2-1}{\alpha}$$
Leu2-1 and lys1-1 are the suppressed UAA mutations, trp1-1 and met8-1 are UAG mutations, ura4 is a non-suppressed UAA mutation, and the mutations his5, his7, arg4, isol, and ade were not specified by Hawthorne.

The spores of MBD89 were mated to strain B047-7B $\alpha \frac{his5-2 \text{ leu2-1}}{\alpha}$ lys1-1 met8-1 tyr7-1 can1-100 cycl-72, to form MBD88, which was homozygous for met8-1 and leu2-1, and heterozygous for SUP61, cycl-72, and the rest of the markers. The spores of MBD88 were allowed to mate among themselves and a diploid isolated which was $\frac{a \text{ SUP61 } leu2-1}{\alpha \quad + \quad leu2-1}$ cycl-72 met8-1 lys1-1. All the surviving spores had the level of

cytochrome c corresponding to that expected for an UAA mutation in iso-1-cytochrome c. By low temperature spectroscopy, the diploid MBD88 had 50% of the wild-type level of iso-1-cytochrome c.

This strain has been sent to Drs. Fred Sherman and John Stewart for isolation and analysis of the suppressed protein to determine the amino acid insertion of SUP61.

The question of interest is, are SUP-RL1 and SUP61 allelic? Whether the amino acid insertions are alike or different does not constitute proof that the suppressors are allelic or not.

One crude test has been performed and others can be performed which can give a better indication of their allelism.

The spores from DBD339 ($\frac{a \text{ SUP-RL1 } leu2-1 \text{ trp1-1 } tyr7-1}{\alpha} + \frac{leu2-1 \text{ trp1-1 } tyr7-1}{\alpha}$) were mixed with the spores of MBD89 ($\frac{a \text{ SUP61 } leu2-1 \text{ trp1-1 } met8-1 \text{ ura4}}{\alpha} + \frac{leu2-1 \text{ trp1-1 } met8-1 \text{ ura4}}{\alpha}$) on rich medium and allowed to germinate for 2-3 days at 30°. The mixture was subcloned on minimal medium. Since a diploid containing both SUP-RL1 and SUP61 will be independent of tryptophan and leucine, it is possible to determine if any prototrophic progeny result from such a cross.

After one week at 30°, the plate contained a heterogeneous streak of very small colonies. The heterogeneity, small size, and slow growth rate of the colonies which appeared were not typical of developing wild-type diploid colonies, leading to the suspicion that either mutations were occurring during the subcloning or aneuploid progeny were being selected.

Two putative $\frac{\text{SUP61}}{+} \frac{\text{SUP-RL1}}{+}$ subclones were purified and analyzed genetically. Using the standard sporulation procedure, each isolate

was grown to stationary phase on a rich medium and then transferred to sporulation medium. One (MBD85) of the two isolated became Trp^- after growth on rich medium; presumably it lost SUP-RL1. The other remained $\text{Trp}^+ \text{Leu}^+$ (MBD134).

Tetrad dissection of MBD85 and MBD134 yielded viability ratios listed below.

	No. of asci:					
	Live: Dead					
	4:0	3:1	2:2	1:3	0:4	Total asci
MBD85	0	0	2	7	2	11
MBD134	0	0	3	2	5	10

Tetrad analysis indicated that both diploids were heterozygous for ura4, met8-1, and tyr7-1, showing genetic input from both parents, MBD89 and DBD339. All surviving spores were leu2-1 and trp1-1, so no suppressor-bearing spores appeared. Every survivor was mating type a, indicating very tight linkage of SUP to mating type. Such tight linkage is not seen in either diploid parent containing only one suppressor.

Two possibilities exist: allelism or non-allelism. If SUP61 and SUP-RL1 are allelic, the expectation is that no diploid colonies would have been formed from the spore-spore mating. However, it is known that aneuploid spores are formed at a certain frequency in yeast (see Chapter II). The mating of a spore carrying $\frac{\text{SUP61}}{+}$ with one carrying SUP-RL1 would yield a trisomic strain with one wild-type

copy of the suppressor locus which is possibly sufficient for growth (albeit slow). The formation of trisomic strains could explain the heterogeneity, slow growth, and small colony size as well as the genetic instability of the suppressor mutation.

Alternatively, it is possible that SUP-RL1 and SUP61 are not allelic but the presence of two efficient suppressors in the cell retards cell growth. The viability data however, is not consistent with this hypothesis in that one expects predominantly dead spores from a diploid containing two recessive lethal suppressors which are within 30 map units of, and in trans to, each other.

Too few subclones were analyzed to be able to clarify the question of allelism. Obviously, the possible formation of aneuploid strains confuses the outcome of such a crude allelism test. There are two possible solutions to the problem of allelism, tetraploid analysis and a modified disomic analysis. Unfortunately, to some extent one must rely on counting dead and live spores. This weakens the analysis somewhat since spore viability varies greatly and often does not approach 100%, even in the absence of the segregation of a recessive lethal mutation.

Tetraploid analysis, in theory, yields clear differences if two recessive lethal suppressors are allelic or not.

It is necessary first to construct two diploid strains $\frac{a \text{ SUP-RL1 leu2-1 trp1-1}}{a + \text{leu2-1 trp1-1}}$ and $\frac{\alpha \text{ SUP61 leu2-1 trp1-1}}{\alpha + \text{leu2-1 trp1-1}}$, mitotic recombinants of the parental strains. These are then crossed and the spore progeny analyzed. If SUP61 is allelic to SUP-RL1 and both are considered

virtually unlinked to their centromere, then about 5/6 of the spore progeny survive and 1/6 dies. Three types of asci are found: Type I has four live spores, two with SUP61 and two with SUP-RL1. Type II has two live spores, both sup⁺. Type III has three live spores, one with SUP61, one with SUP-RL1, and one sup⁺. These would occur with a frequency of 4:1:4. This is based on an analysis by Roman et al. (1955).

If SUP61 and SUP-RL1 are not allelic but are linked, there is no loss in spore viability expected. One type of ascus (I) should contain two SUP-RL1 spores and two SUP61 spores. Type IV has one sup⁺, one SUP-RL1, one SUP61, and one SUP-RL1 SUP61 spore. A third type (V) has two SUP61 SUP-RL1 and two sup⁺ spores.

Non-allelism and non-linkage is indicated by a loss of spore viability as well as the appearance of tetrad types not seen under other conditions. Besides the appearance of types I, IV, and V, there is type VI, which has one SUP61, two sup⁺, and one dead spore. Type VII has one SUP-RL1, two sup⁺, and one dead spore. Type VIII has two SUP61, one sup⁺, and one dead, and type IX is identical to type VII with SUP-RL1 replacing SUP61. Finally, type X has three sup⁺ spores and one dead spore.

This allelism test depends on stable suppressors, viability, and sporulation of the SUP61 +/+ +/+ SUP-RL1 /+ + tetraploid and viability of the $\frac{\text{SUP-RL1}}{+} \frac{\text{SUP61}}{+}$ diploid. It can be expanded to look at the allelism of any two suppressors as long as they can be differentiated by suppression pattern and the double heterozygote is viable.

A modification of the tetraploid analysis involves the use of trisomes to determine allelism. One must convert the chromosome III amber suppressor to its ochre form in a disomic strain and then compare it to a series of recessive lethal amber suppressors. This is done by mixing the disome (either $\frac{a}{a}$ or $\frac{\alpha}{\alpha} \frac{SUP-RL1}{+} oc$) with spores of each of the diploids on rich medium in order to isolate a $\frac{SUP_{oc}}{SUP_{am}}/+$ trisome using counterselecting markers. The disomes and trisomes must be stable and sporulate well if this technique is to have validity. One must first show that SUP-RL1am and SUP-RL1oc in a trisome behave as alleles are expected to, and that the control matings spore ($\alpha \underline{SUP}_{am}$) X disome ($\frac{a \sup^+}{a \sup^+}$) and spore ($\alpha \underline{SUP}_{am}$) X haploid ($\underline{a \sup^+}$) occur and the progeny behave as expected.

The segregation patterns of the spores will distinguish between suppressors which are allelic, non-allelic but linked, or non-allelic and unlinked.

If the suppressors are allelic, only asci with viability ratios (live:dead) of 2:2 and 1:3 are expected. No 4:0 or 0:4 asci should appear, nor any spores with both suppressors.

If the suppressors are not allelic and unlinked, no spores are recovered which have the amber suppressor since this marker is segregating as it would in a diploid. Some 0:4 asci are also predicted, as well as 2:2 and 1:3 asci.

Trisomes containing two suppressors which are not allelic but linked, yield asci which have viability ratios of 4:0, 3:1, and 2:2 only and some spores should contain both suppressors.

Such allelism tests which can distinguish different recessive lethal amber suppressors make it possible to identify new classes of suppressors when they are isolated.

CHAPTER IV

SUMMARY AND CONCLUSIONS

This thesis has described the isolation and characterization of a new class of nonsense suppressors in Saccharomyces cerevisiae, recessive lethal suppressors which cannot be isolated or maintained in haploid strains of yeast. One suppressor, SUP-RL1, maps on chromosome III about 30 cm distal to the mating-type locus and has been shown to insert serine into iso-1-cytochrome c at the site of a UAG mutation.

A. Analyzing recessive lethal mutations

Genetic analysis of a recessive lethal mutation is more difficult than analysis of conditional lethals. By necessity, one must assume the inheritance of a trait by its lack of appearance -- negative evidence which is weak as a form of proof. In addition, tests of allelism are impossible unless one resorts to higher ploidies, as described in the Appendix, section C. However, yeast is an ideal organism in which to study recessive lethal mutations since it has a stable haploid phase in which it can be manipulated with the ease of bacteria and it also has a stable diploid phase with the advantage of a simple and easily reversible conversion to diploidy.

That recessive lethal nonsense suppressor tRNAs exist in yeast suggests that these tRNAs are unique species which are present in

a single copy in the yeast genome. In the case of the tyrosyl-tRNAs, there are probably eight redundant genes (Gilmore et al., 1971). The serine-inserting UAA suppressor SUQ5 (Liebman et al., 1975) is not recessive-lethal, so there must be compensating tRNAs which exist in the haploid yeast strain. It is possible that SUQ5 is an altered tRNA, from the set of iso-accepting seryl-tRNAs (Ser₁ or Ser₂ of Caskey et al., 1968) which read the codons UCU, UCC, and UCA with the anticodon IGA. The existence of iso-accepting tRNAs which can compensate could explain why SUQ5 is non-lethal. In E. coli, too, gene duplications for SU3 and probably for SU6, have been found. Since in E. coli, S. typhimurium, and now S. cerevisiae, there are tRNA genes which are essential, the question remains as to why the cell leaves itself unprotected from the effects of mutation by keeping particular genes as single copies whose alteration would result in self-destruction. One explanation could be that the number of tRNA genes is a function of the need for particular tRNAs in the cell which in turn would relate to the frequency of each amino acid in cell proteins.

B. Other classes of recessive lethal suppressors

One expects another class of recessive lethal nonsense mutants which are not due to the loss of an essential tRNA but instead are harmful to the cell through over-suppression. However, since UAG suppressors in yeast have been found which act with 50-75% efficiency in terms of their cytochrome c production, it is perhaps unlikely that recessive lethal (by reason of over-suppression) UAG suppressors

will be isolated. Perhaps the cell can tolerate very high levels of UAG suppression without harm.

With UAA suppression, however, it seems more likely that recessive lethality due to over-suppression would be found. Yeast, like E. coli, is more sensitive to suppression of UAA mutations, showing lowered efficiency of UAA suppression and retarded growth rate. As mentioned in the Appendix, Section A, a limited search for recessive lethal UAA suppressors was carried out in which none was found.

From diploid strains carrying recessive lethal suppressors of the over-suppressing variety, in theory, one should be able to derive aneuploid strains carrying different duplicated chromosomes. Different chromosomes, when duplicated, could provide compensation by the yeast cell for the imbalance in the protein synthesizing system caused by the over-suppression. As an example, one might expect disomic strains duplicated for the appropriate release factors which would result in an increase in the dosage of these genes which might compete more successfully in favor of termination over suppression. However, tracking down duplicated chromosomes might be very difficult in yeast, even when every chromosome is marked by mutations.

The possibility of selection of aneuploid strains suggests a novel use for recessive lethal suppressors. By growing such hetero-allelic disomic strains (SUP/sup⁺) on medium requiring the presence of the suppressor, one can maintain by selection the extra chromosome

within the cell. This is useful in cases where two or perhaps three copies of a given chromosome are needed in order to obtain proportionally more DNA of that chromosome, or when more copies of a gene product whose gene maps on the chromosome of interest are desired.

In strains carrying recessive lethal suppressors which are essential tRNAs, it would be of interest to find yeast mutants which still show suppression but whose suppressors are no longer lethal. Theoretically, this could happen if another non-essential tRNA mutated to read the codon which was no longer read by the original suppressor (a form of missense suppression). Alternatively, one might find mutations allowing greater wobble in the anticodon.

C. The identity of the SUP-RL1 tRNA

Since the characterization of Su7 (Soll and Berg, 1969; Yaniv et al., 1974) in which the tryptophan tRNA was mutated in the anticodon to read the UAG (amber) codon instead of the UGG (tryptophan) codon with the concurrent misacylation of glutamine instead of tryptophan, the amino acid insertion is no longer useful as a definitive criterion for the identity of the suppressing tRNA. For this reason, assignment of a specific tRNA to the suppressor mutation, SUP-RL1, cannot be made until the tRNA responsible for suppression has been purified and its complete sequence determined. Without the help of a yeast transducing phage to magnify the amount of SUP-RL1 tRNA within the yeast cell, it may prove difficult to isolate the tRNA, particularly if it represents, as I suspect, a minor species of tRNA.

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