The tRNA-Tyr Gene Family of *Saccharomyces cerevisiae*: Agents of Phenotypic Variation and Position Effects on Mutation Frequency

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ABSTRACT

Extensive phenotypic diversity or variation exists in clonal populations of microorganisms and is thought to play a role in adaptation to novel environments. This phenotypic variation or instability, which occurs by multiple mechanisms, may be a form of cellular differentiation and a stochastic means for modulating gene expression. This work dissects a case of phenotypic variation in a clinically derived *Saccharomyces cerevisiae* strain involving a *cox15* ochre mutation, which acts as a reporter. The ochre mutation reverts to sense at a low frequency while tRNA-Tyr ochre suppressors (*SUP*-**o**) arise at a very high frequency to produce this phenotypic variation. The *SUP*-**o** mutations are highly pleiotropic. In addition, although all *SUP*-**o** mutations within the eight-member tRNA-Tyr gene family suppress the ochre mutation reporter, there are considerable phenotypic differences among the different *SUP*-**o** mutants. Finally, and of particular interest, there is a strong position effect on mutation frequency within the eight-member tRNA-Tyr gene family, with one locus, *SUP6*, mutating at a much higher than average frequency and two other loci, *SUP2* and *SUP8*, mutating at much lower than average frequencies. Mechanisms for the position effect on mutation frequency are evaluated.

MEMBERS of clonal populations of bacteria and fungi exhibit frequent and significant instability or variation in their phenotypes (reviewed in Robertson and Meyer 1992; Soll 1992, 1997; Rainey *et al.* 1993; and Moxon *et al.* 1994). This phenotypic variation (also denoted adaptive evolution, intraclonal polymorphism, antigenic variation, and phase variation) plays a role in microbial adaptation to novel environments by generating genotypic and phenotypic diversity. For example, phenotypic variation plays an important role in bacterial pathogenesis by aiding in resistance to host defenses as well as in dissemination to and colonization of different ecological niches. The genotypic and phenotypic diversity generated by phenotypic variation can be viewed as a form of cellular differentiation in microorganisms and as a stochastic method for regulating gene expression.

There are a variety of phenotypic variation mechanisms. Some mechanisms for phenotypic variation, such as silencing (Pillus and Rine 1989; Gottschling *et al.* 1990) and [*PSI*$^+$] (True and Lindquist 2000), are epigenetic. However, other common mechanisms for phenotypic variation involve changes at the DNA sequence level, such as the expansion-contraction of highly mutable simple repetitive sequences (reviewed in Robertson and Meyer 1992; Rainey *et al.* 1993; Moxon *et al.* 1994). Where there are changes at the DNA sequence level, specific sequences in phenotypically variable genes can be thought of as being at the high-frequency end of the mutational continuum; that is, specific sequences in phenotypically variable genes are mutational hotspots.

As shown by extensive progress with silencing and [*PSI*$^+$], *Saccharomyces cerevisiae* is a particularly useful eukaryotic model for phenotypic variation. In addition to more recent studies with [*PSI*$^+$] and silencing, phenotypic variation has also been seen classically in *S. cerevisiae* (Winge 1944; Lindegren 1956; Skovsted 1956; Scheda and Yarrow 1966, 1968) but the descriptions were largely phenomenological, involving, for the most part, subjective assays, such as distinguishing among multiple colony morphologies in one strain. To avoid the phenomenology inherent in subjective assays, objective phenotypes, such as the conditional growth assays used to study silencing and [*PSI*$^+$], are extremely advantageous.

Phenotypic variation has been noted in clinically derived *S. cerevisiae* strains (McCusker and Davis 1991) and, given the relevance of bacterial phenotypic variation to pathogenesis, was an intriguing area for further investigation. This work focuses on one case of phenotypic variation in a clinically derived *S. cerevisiae* genetic background, which, in addition to having an objective conditional growth assay, displays intriguing effects on respiration and sporulation and presents an interesting genetic puzzle.

We show that this case of phenotypic variation is due to a *cox15* ochre mutation, which acts as a reporter and reverts to sense at a low frequency; it is suppressed by

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TABLE 1

***S. cerevisiae* strains**

Strain	Genotype	Reference
YJM421	*HO/HO MAT***a**/*MAT*α *cox15-421/cox15-421 SUP7-***o**/+	McCusker *et al.* (1994)
YSA3	*HO/HO MAT***a**/*MAT*α *cox15-421/cox15-421*	This study
V1-1	*MAT*α *ho*Δ::kanMX3 *cox15-421*	This study
V1-13−	*MAT*α *ho*Δ::natMX3 *cox15-421 leu2-5′*Δ::hygMX3 *ura3*Δ::kanMX3	This study
V1-13+	*MAT*α *ho*Δ::natMX3 *cox15-421 leu2-5′*Δ::hygMX3 *ura3*Δ::kanMX3 Pet+	This study
V1-22−	*MAT*α *ho*Δ::hygMX3 *cox15-421 trp1*Δ::kanMX3	This study
S1	*MAT*α *gal2 SUC2 CUP1*	This study
S95	*MAT***a** *lys2*	This study
S175	*MAT*α *ura3*Δ::SR[a] *fcy1*Δ::SR	This study
S182	*MAT*α *ura3*Δ::SR *fcy1*Δ::SR *cox15*Δ::Fcy1MX4	This study
S183	*MAT*α *cox15-421 ura3*Δ::SR *fcy1*Δ::SR	This study
S1201	*MAT*α *cox15-421 SUP4-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1202	*MAT*α *cox15-421 SUP5-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1203	*MAT*α *cox15-421 SUP6-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1204	*MAT*α *cox15-421 SUP7-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1205	*MAT*α *cox15-421 SUP11-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1219	*MAT*α *ura3*Δ::SR *fcy1*Δ::SR	This study
S1228	*MAT***a** *cox15-421 ura3*Δ::SR *fcy1*Δ::SR	This study
S1233	*MAT*α *cox15-421 SUP3-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1238	*MAT*α *ura3*Δ::SR *fcy1*Δ::SR	This study
S1252	*MAT*α *cox15-421 SUP11-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1253	*MAT*α *cox15-421 SUP11-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1254	*MAT*α *cox15-421 SUP6-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1255	*MAT*α *cox15-421 SUP7-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1256	*MAT*α *cox15-421 SUP4-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1257	*MAT*α *cox15-421 SUP5-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1258	*MAT*α *cox15-421 SUP8-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1259	*MAT*α *cox15-421 SUP8-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1260	*MAT*α *cox15-421 SUP2-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study
S1269	*MAT*α *cox15-421 SUP2-***o** *ura3*Δ::SR *fcy1*Δ::SR	This study

[a] SR, a *s*ingle *r*epeat left after the pop-out of an MX3 (Wach *et al.* 1994) cassette.

tyrosine-inserting ochre suppressors, which arise at a very high frequency. We also describe a novel phenotype associated with ochre suppressors, namely substantially reduced *PGK1* promoter activity; there is significant phenotypic variability in the effects of the tRNA-Tyr gene family on *PGK1* promoter activity. Therefore, this work provides an additional example of the pleiotropic impact of translational misreading on phenotype and of the relevance of translational misreading to phenotypic variation. Finally and most interestingly, within the eight-member tRNA-Tyr gene family there is a strong position effect on mutation frequency.

MATERIALS AND METHODS

Strains: All *S. cerevisiae* strains used in this study are shown in Table 1. Strains with names beginning with an "S" were isogenic with S288c, while strains with names beginning with a "V1-" were isogenic with the clinically derived strain YJM421 (McCusker *et al.* 1994).

Media: YEPD (Sherman *et al.* 1974) and YEPD containing G418 (Geneticin, GIBCO BRL, Gaithersburg, MD), hygromycin B (Calbiochem-Novabiochem, La Jolla, CA), and nourseothricin (clonNAT, Hans-Knoll Institute fur Naturstoff-Forschung, Jena, Germany) have been described previously (Wach *et al.* 1994; Goldstein and McCusker 1999). Osmotic sensitivity was tested on YEPD containing 1.5 or 1.75 M KCl. YEPEG contained 1% succinic acid, 1% yeast extract, 2% bacto-peptone, 2% glycerol (mixed, adjusted to pH 5.5 and autoclaved), and 2% ethanol (added after autoclaving). Strains carrying *cox15-421* were grown in medium containing antimycin A (Sigma, St. Louis; added after autoclaving to a final concentration of 1 μg/ml) when propagated long enough to potentially exhaust dextrose and select for ochre suppressors. Synthetic dropout media have been described previously (Rose *et al.* 1990). All types of solid media contained, 2% bacto-agar (Difco, Detroit).

Fcy1$^+$ Fcy1MX4-containing transformants were selected on synthetic minimal medium containing 1.7 g/liter yeast nitrogen base without amino acids or $(NH_4)_2SO_4$ (Difco), 20 g/liter dextrose (Sigma), 20 g/liter agar, and 1 mM cytosine (Sigma) as the sole nitrogen source (Erbs *et al.* 1997); medium used to select for loss of the Fcy1MX4 cassette was similar but lacked cytosine and contained 5 g/liter $(NH_4)_2SO_4$ and 10^{-4} M 5-fluorocytosine (Sigma). Cytosine and 5-fluorocytosine were added to the medium after autoclaving.

Preparation of genomic and plasmid DNA: Yeast genomic DNA was prepared as described previously (Ausubel *et al.* 2002). Plasmid DNA was isolated using QIAGEN (Valencia, CA) mini- and midi-kits. Operon (Alameda, CA) or IDT (Cornwallis, IL) synthesized oligonucleotides. Sequence analysis was

done by the Duke University Sequencing Core using an ABI prism sequencer.

PCR-mediated gene deletion construction: Using primers shown in Table 2, MX3 and MX4 cassettes were amplified by PCR for construction of insertion-deletion mutations as described previously (Wach *et al.* 1994; Goldstein and McCusker 1999; Goldstein *et al.* 1999). Colony PCR was used to confirm insertion-deletion mutations as described previously (Niedenthal *et al.* 1996; Goldstein and McCusker 2001).

Transformation of *Escherichia coli* and *S. cerevisiae*: DNA was introduced into DH10β cells (GIBCO BRL) via electroporation (Dower *et al.* 1988) with a Gene Pulser II (Bio-Rad). *URA3*-containing plasmids (0.1 μg) and PCR products [1.0 and 0.1 μg for PCR products with short (30–40 bp) and long (300–500 bp) regions of homology, respectively] were introduced into *S. cerevisiae* strains as described previously (Gietz *et al.* 1995). For PCR products with short regions of target homology, DMSO (10 μl) was added before heat-shocking the cells.

Cloning of *COX15*: A YCp50-based *S. cerevisiae* library (Rose *et al.* 1987), obtained from the American Type Culture Collection, was amplified and 0.5 μg of isolated plasmid DNA was transformed per reaction into the Pet$^-$ strain V1-13−. Each of 14 transformation reactions was plated onto three synthetic uracil dropout (SDC-URA) + antimycin A plates, which were incubated for 2 days at 30°; these plates were then replica plated to YEPEG plates, which were incubated for 3 days at 30°.

Introduction of the *cox15-421* mutation into the S288c background: The *cox15-421* ochre mutation was introduced into the S288c genetic background in a three-step process. First, the Fcy1MX4 cassette [the open reading frame (ORF) of the kanMX4 cassette (Wach *et al.* 1994) was replaced with the *S. cerevisiae FCY1* ORF] in plasmid pPH37 was amplified with primers PH127 and PH128 (with 5′ sequences up and downstream from the *COX15* open reading frame). The resulting PCR product was transformed into the *fcy1*Δ strain S175, selecting for the ability to utilize cytosine as the sole nitrogen source. The resulting *cox15*Δ::Fcy1MX4 mutation in S182 was confirmed by PCR (using primers PH164 and PH137 to assay for *cox15*Δ::Fcy1MX4), Fcy1$^+$ phenotype, and a ρ$^+$ Pet$^-$ phenotype.

Second, a PCR product containing the *cox15-421* ochre mutation was amplified from YJM421 genomic DNA using primers PH133 and PH134. Finally, the *cox15-421*-containing PCR product was cotransformed with the *URA3*-containing plasmid pRS316 (Sikorski and Hieter 1989) into strain S182, selecting for Ura$^+$. The resulting Ura$^+$ colonies were replica plated to 5-fluorocytosine-containing medium to screen for replacement of *cox15*Δ::Fcy1MX4 by *cox15-421*. Replacement of *cox15*Δ::Fcy1MX4 by *cox15-421* was confirmed by PCR using primers PH136 and PH139 (to assay for the *COX15* ORF) and primers PH164 and PH137. The *ura3*Δ *fcy1*Δ *cox15-421* strain S183 was used for further analysis.

Library construction for cloning *SUP7*-o from YJM421: Genomic DNA from YJM421, which was completely digested with *Hin*dIII or *Xba*I, was ligated into 0.5 μg of appropriately digested pRS316 (Sikorski and Hieter 1989). For each digest, three ligation reactions were incubated for 16 hr at 15° and then diluted to 100 μl with H_2O. Each ligation reaction was split into 50-μl aliquots for direct transformation into strain V1-13−. Each of 14 yeast transformation mixes was plated onto three SDC-URA + antimycin A plates. An estimated 140,000 and 60,000 Ura$^+$ colonies were recovered from the *Hin*dIII- and *Xba*I-digested DNA ligations, respectively. After being grown for 2 days on SDC-URA + antimycin A, Ura$^+$ transformants were replica plated to YEPEG to screen for Pet$^+$.

Determination of mutation frequencies and rates: To estimate mutation frequencies and rates, six independent colonies of each strain were grown overnight at 30° in 5 ml of YEPD containing 1 μg/ml of antimycin A. After overnight growth, cells were pelleted, washed twice with water, resuspended in water, and counted. Aliquots of ~100 cells were inoculated into 50 ml of YEPD medium containing 5% dextrose and grown at 30°. The cell densities of all cultures were determined by cell counts to estimate the number of cell divisions each culture had undergone since inoculation for subsequent mutation rate determinations. Cells were then harvested in log phase and washed with water.

To measure the Pet$^+$ mutation frequency in each *cox15-421* culture, ~2×10^8 cells were plated onto each of two YEPEG plates. To measure forward mutation frequencies at the *CAN1* and *LYP1* loci in each culture, ~3×10^7 cells were plated onto synthetic arginine dropout medium containing 40 μg/ml canavanine (Sigma) and synthetic lysine dropout medium containing 100 μg/ml of *S*-2-aminoethyl-L-cysteine (Sigma), respectively. Simultaneously, appropriately diluted cells of each culture were plated onto YEPD to estimate the number of viable cells. The plates were incubated at 30° for 2–3 days after which colonies were counted. The mutation frequencies were calculated for each culture by dividing the average number of colonies on selective media by the number of cells plated; these frequencies were then averaged. Mutation rates were calculated using the formula $\mu = (f_2 - f_1)/\ln(N_2/N_1)$, where μ is the mutation rate, f_1 is the initial mutation frequency in culture, f_2 is the final mutation frequency in culture, N_1 is the initial number of cells in culture, and N_2 is the final number of cells in culture (Drake 1991); these rates were then averaged.

Isolation of independent Pet$^+$ mutants: For each of the *cox15-421* strains, one Pet$^-$ colony was streaked (separately) for single colonies on YEPD. After incubation at 30° for four days, the YEPD plates, which contained multiple single Pet$^-$ colonies, were replica plated to YEPEG. After incubation at 30° for 2–3 days, Pet$^+$ papilli appeared from many (but not all) of the replica-plated Pet$^-$ colonies. To ensure that Pet$^+$ mutants were independent, a single Pet$^+$ papilla was picked from each Pet$^-$ colony and purified by streaking on YEPD for single colonies for subsequent genotyping purposes.

Genotyping of tRNA-Tyr: The genotypes of tRNA-Tyr loci in Pet$^+$ *cox15-421* strains were first assayed by determining the presence (in *sup*$^+$) or absence (in *SUP*-**o**) of the *Hpy*CH4III site in the anticodon region of the tRNA-Tyr genes. Using the sets of primers shown in Table 2 and genomic DNA as templates, PCR products containing each of the eight tRNA-Tyr loci were obtained from each of the Pet$^+$ strains. PCR products were ethanol precipitated, digested with the restriction enzyme *Hpy*CH4III, and electrophoresed in 4% agarose gels. In Pet$^+$ derivatives of haploid *cox15-421* strains, the PCR products of a given tRNA-Tyr locus were either *Hpy*CH4III sensitive or *Hpy*CH4III resistant. In diploid *cox15-421* strains, the PCR products of *sup*$^+$/*sup*$^+$ tRNA-Tyr loci were *Hpy*CH4III sensitive while *SUP*-**o**/+ loci yielded a mixture of *Hpy*CH4III-sensitive and -resistant PCR products.

For all putative *SUP*-**o** mutants (tRNA-Tyr loci with *Hpy*CH4-III-resistant PCR products), *m*utant-*a*llele-specific *a*mplification (MASA; Takeda *et al.* 1993; Hasegawa *et al.* 1995) PCR was performed to confirm the *SUP*-**o** genotypes. Genomic DNAs were used as a template in conjunction with primers with different 3′ sequences (Table 2, SA86–SA90) in combination with locus-specific primers (Table 2, SA2, SA4, SA5, SA8, SA9, SA12, SA14, and SA16). MASA-PCR reactions were initiated at 95° for 5 min followed by 35 amplification cycles (95° for 30 sec, 52° for 30 sec, and 72° for 2 min) and terminated with a 10-min extension at 72°. The reaction mixtures were then electrophoresed in 4% agarose gels. To be deemed a *SUP*-**o** mutant, a *Hpy*CH4III-resistant tRNA-Tyr locus in Pet$^+$

TABLE 2

Primer sequences

Primer	Sequence (5′–3′)	Position[a]	Primer function[b]
JM37	CCTCGACATCATCTGCCC	+116[c]	*COX15*; A
PH108	TCGCTTCGCTACTTGG		YCp50 vector; F
PH109	GATGTCGGCGATATAGG		YCp50 vector; F
PH127	TATAATAGTGCACAGCACTGAAGTTGTAACTACTGTACGTCAGCTGAAGCTTCGTACGC[d]	−41	*COX15*; B
PH128	TGTCAATTCTCATAAGAATACCTTTATCCATTATAATGCATAGGCCACTAGTGGATCTG[d]	+30	*COX15*; B
PH132	AAATGGACGGATGCTC	174	*COX15*; C
PH133	ATGTACTCCTCCTAGC	−332	*COX15*; C
PH134	ATTTTCTCCGTATAGGTC	+329	*COX15*; A, C
PH136	GTAATCATTACCTTGCC	1375	*COX15*; D
PH137	AAGTCCCTAATACACTC	+894	*COX15*; D
PH139	TAAGTCATCGGTTTTGAG	+1609	*COX15*; D
PH158	GAAACATAGAAGTGGGC	11	*COX15*; C
PH164	TATGGTATTCCACGCTG	301	*FCY1*; D
PH198	CAAGTTTTTTGGGGTCGAG		pRS316 vector; F
PH199	CTCGGAATTAACCCTCAC		pRS316 vector; F
SA1	CAACACCTCGCAAGAAATCG	−21	*SUP2*; C
SA2	CGAATGTGTCATTGACTATCC	+100	*SUP2*; C, E
SA3	GCAATCTCTTTGAAATGTTCCT	−117	*SUP11*; C
SA4	GAGATTTAAGAAATACTTGTTCTG	+87	*SUP11*; C, E
SA5	GTCACAGAAGTCACGTATATAA	+94	*SUP6*; C, E
SA6	AAGACGAATAACTCCTCCGC	−105	*SUP6*; C
SA7	CAATTCACTCAAAGTCCTTTCA	−99	*SUP7*; C
SA8	CTTTACAATCACTTATTGATCAG	+123	*SUP7*; C, E
SA9	GTCACAATTTGATAATAACTCTTC	+95	*SUP4*; C, E
SA10	GTCAGTATTCTCTTCCATCCC	−138	*SUP4*; C
SA11	CCGAGAGGTTACGACAACAT	−74	*SUP5*; C
SA12	TCTACTCAATTTACTTTAAAGGC	+112	*SUP5*; C, E
SA13	GACAACAGGTCACTTCTGCA	−95	*SUP8*; C
SA14	GAATGTCATTTGGTAGCTACC	+89	*SUP8*; C, E
SA15	CTTCTTCGAGCCAGGCCG	−74	*SUP3*; C
SA16	ATACTTGTTCATTCTTGGCAGA	+84	*SUP3*; C, E
SA86	GTTTAAGGCGCAAGACT**G**[e]	19	tRNA-Tyr wild-type anticodon; E
SA87	GTTTAAGGCGCAAGACT**TT**[e]	19	tRNA-Tyr ochre suppressor; E
SA88	GTTTAAGGCGCAAGACT**C**[e]	19	tRNA-Tyr amber suppressor; E
SA89	GTTTAAGGCGCAAGACT**TC**[e]	19	tRNA-Tyr opal suppressor; E
SA90	GTTGGTTTAAGGCGCAAGAC	15	Common to all tRNA-Tyr; E
SA92[f]	CCCAAGCTTCCACGTTGAGTGCACA	+430	*SUP2*; E
SA98[g]	TCAAGGAATATTTCTACCATCAGA**C**AACAAATCAGAAAGACTCAACTATA	91	*COX15* (TAA to Gln); G
SA99[g]	TCAAGGAATATTTCTACCATCAGATA**C**CAAATCAGAAAGACTCAACTATA	91	*COX15* (TAA to Tyr); G
SA100[g]	TCAAGGAATATTTCTACCATCAGATA**T**CAAATCAGAAAGACTCAACTATA	91	*COX15* (TAA to Tyr); G

(*continued*)

TABLE 2

(Continued)

Primer	Sequence (5′–3′)	Position[a]	Primer function[b]
SA101[g]	TCAAGGAATATTTCTACCATCAG**A**A**A**ACAAATCAGAAAGACTCAACTATA	91	*COX15* (TAA to Lys); G
SA102[g]	TCAAGGAATATTTCTACCATCAG**G**A**A**CAAATCAGAAAGACTCAACTATA	91	*COX15* (TAA to Glu); G
SA103[g]	TCAAGGAATATTTCTACCATCAG**C**A**C**AAATCAGAAAGACTCAACTATA	91	*COX15* (TAA to Ser); G
SA104[g]	TCAAGGAATATTTCTACCATCAG**T**A**T**AAATCAGAAAGACTCAACTATA	91	*COX15* (TAA to Leu); G
SA111	CCTATAACCTGTTTCATCCAC	+242	*SUP8*; E

[a] Plus or minus signed numbers indicate the number of nucleotides that the 5′ end of the primer is from the start (−) or stop (+) codons of ORFs or the beginning (−) or end (+) of tRNA coding sequences. Primers without signs are within ORFs or tRNA-TYR genes, and the number indicates the number of nucleotides that the 5′ end of the primer is from the start codon of ORFs or from the beginning of the tRNA.

[b] A, primers used to verify homologous integration of cassette at designated gene(s); B, primers used to amplify MX cassettes for homologous integration at the designated gene; C, primers used to amplify specified gene; D, primers used to verify homologous integration of the *cox15-421* gene for introduction into the S288c background; E, primers used for MASA PCR; F, primers used for sequencing; G, primers used for mutagenesis. *SUP2* = tY(GUA)D; *SUP3* = tY(GUA)O; *SUP4* = tY(GUA)J2; *SUP5* = tY(GUA)M1; *SUP6* = tY(GUA)F2; *SUP7* = tY(GUA)J1; *SUP8* = tY(GUA)M2; *SUP11* = tY(GUA)F1.

[c] The number of nucleotides that the 5′ end of the primer is from the stop codon of any of the dominant drug resistance ORFs.

[d] Underlined sequences are homologous to a portion of the multicloning site of the MX cassette.

[e] The nucleotide(s) shown in boldface type indicate the specific sequence for the anticodon, which corresponds to wild-type anticodon or ochre, amber, or opal mutant anticodons.

[f] Underlined sequences are additional nucleotides to introduce the *Hin*dIII recognition site.

[g] Oligonucleotides cotransformed into a *cox15-421* strain to determine which amino acids might result in a functional Cox15p. The nucleotide sequence change in ochre mutation (underlined) is indicated in boldface type.

derivatives of the haploid *cox15-421* strains V1-1− and S183 had to meet two criteria. First, in conjunction with locus-specific primers, a *Hpy*CH4III-resistant tRNA-Tyr locus had to fail to produce MASA-PCR products with primers SA86 (3′ end homologous to the wild-type tRNA-Tyr anticodon), SA88 (3′ end homologous to tRNA-Tyr with an amber suppressor anticodon), and SA89 (3′ end homologous to tRNA-Tyr with an opal suppressor anticodon). Second, in conjunction with locus-specific primers, a *Hpy*CH4III-resistant tRNA-Tyr locus had to produce MASA-PCR products with both primer SA90 (which, at its 3′ end, encompasses the two bases of the *Hpy*CH-4III site outside of the anticodon) and primer SA87 (3′ end homologous to tRNA-Tyr with an ochre suppressor anticodon).

YJM421 (*SUP7*-**o**/+) and Pet$^+$ derivatives of the *cox15-421* diploid strain YSA3 contained tRNA-Tyr loci with both *Hpy*CH-4III-sensitive and -resistant PCR products. Therefore, the criteria for identifying an ochre suppressor in these *SUP*-**o**/+ strains differed slightly from the criteria used for haploid strains in that a MASA-PCR product was expected with both primer SA86 and primer SA87.

Introduction of *SUP2*-o and *SUP8*-o mutations into the S288c background: Spontaneous *SUP2*-**o** and *SUP8*-**o** mutants were not isolated in the S288c genetic background. To introduce *SUP2*-**o** and *SUP8*-**o** into the S288c genetic background, *SUP2*-**o** and *SUP8*-**o** were first amplified from *SUP*-**o**-containing, V1-1-derived strains using primers SA1 and SA2 (*SUP2*-**o**) and primers SA13 and SA14 (*SUP8*-**o**). The resulting *SUP2*-**o**- and *SUP8*-**o**-containing PCR products were then transformed, separately, into the *ura3*Δ *cox15-421* strain S183, together with the kanMX4-containing *CEN* plasmid pSA15. G418^r transformants were selected; screening for Pet$^+$ identified putative *SUP*-**o**-containing cotransformants. Replacement of *sup*$^+$ with *SUP*-**o** at the *SUP2* or *SUP8* locus in G418^r Pet$^+$ transformants was examined by the destruction of the *Hpy*CH4III recognition sequence and then confirmed by MASA-PCR, as described above. Finally, derivatives of the *SUP2*-**o**- and *SUP8*-**o**-containing strains that had lost pSA15 were selected by demanding growth on 5-fluoroorotic acid-containing medium. The plasmid-less strains S1260 and S1269 (*SUP2*-**o**) and S1258 and S1259 (*SUP8*-**o**) were used for further analysis.

Determination of suppressor efficiency: Ochre suppressor efficiency was determined by quantification of the suppression of the ochre mutation in the plasmid-borne β-galactosidase gene of pUKC817 (*URA3 lacZ*-ochre) relative to the control plasmid pUKC815 (*URA3 lacZ*$^+$), as described previously (Firoozan *et al.* 1991; Stansfield *et al.* 1995). For each *SUP*-**o** locus, except for *SUP3*-**o**, two or three independently isolated *SUP*-**o**-containing strains were assayed for β-galactosidase. For each experimental measurement, three independent transformants of each *SUP*-**o**-containing Pet$^+$ strain were assayed for β-galactosidase. Assays from each culture were performed in duplicate.

Oligonucleotide-mediated transformation of the *cox15-421* ochre mutation to sense: Oligonucleotide-mediated transformation was performed using a modification of a previously described (Moerschell *et al.* 1991) procedure. Seven single base-pair substitution mutations can convert an ochre codon to one of seven sense codons, representing six amino acid substitutions. To determine which of these six amino acid substitutions might result in a functional Cox15p, strain S183 was cotransformed with (i) plasmid pSA11 (*CEN* natMX4) and (ii) one of seven oligonucleotides (SA98–SA104; using 10 μl of 500 μm solution of each oligonucleotide), which could convert the ochre codon of *cox15-421* to one of the seven sense codons. After 2 days growth at 30°, nourseothricin-resistant transformants were screened for Pet$^+$ by replica plating to YEPEG. The nourseothricin-resistant Pet$^+$ transformants were

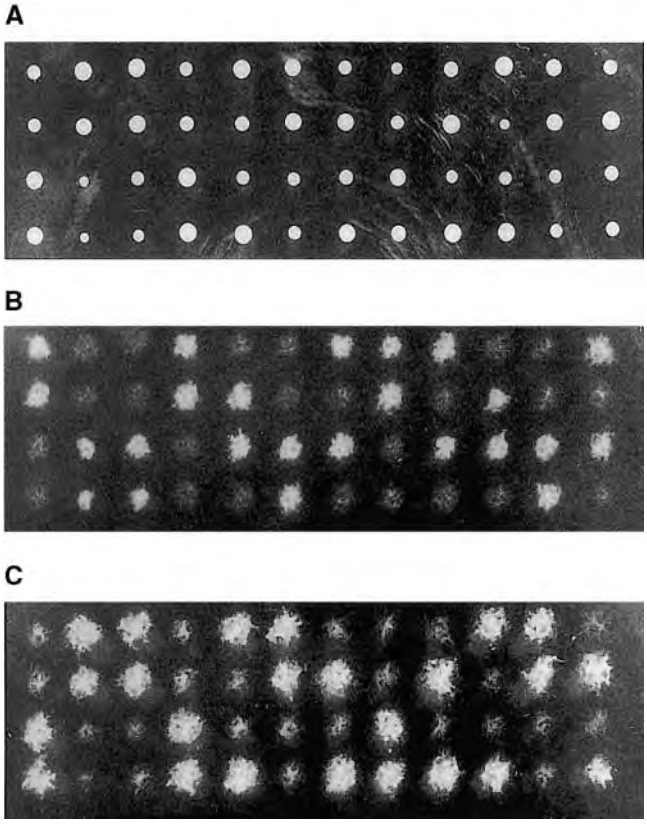

FIGURE 1.—Tetrads of YJM421 showing (A) colony size on YEPD, (B) growth on YEPEG, and (C) growth on YEPD + 1.5 M KCl.

tested for osmotic sensitivity to exclude all of the tyrosine-inserting *SUP-***o** except *SUP11-***o**. The *SUP11* genotype of Pet^+ Osm^+ transformants was determined as described above. For *sup11*$^+$ Pet^+ Osm^+ transformants, the region around codon 39 of *COX15* was amplified (using primers PH132 and PH158), cloned into pCR2.1-TOPO (Invitrogen, San Diego), and sequenced. For each *sup11*$^+$ Pet^+ Osm^+ transformant, two independent plasmid clones were sequenced on both strands using M13 and reverse primers. To exclude the presence of [*PSI*$^+$], four *sup11*$^+$ Pet^+ Osm^+ *cox15-421* isolates were grown under [*psi*$^-$]-inducing conditions (LUND and COX 1981; TUITE *et al.* 1981); that is, they were streaked for single colonies on YEPD + 5 mM guanidine hydrochloride followed by replica plating twice to YEPD + 5 mM guanidine hydrochloride. After growth under these [*psi*$^-$]-inducing conditions, the Pet^+ phenotypes of the strains were retested.

RESULTS

The unusual genetic behavior of YJM421: In spite of being *HO* and self-diploidized, tetrads of the clinically derived Spo^+ strain YJM421 (MCCUSKER *et al.* 1994) dissected onto YEPD showed Mendelian (2:2) segregation for colony size, suggesting heterozygosity at one locus. Small colony size is frequently indicative of a respiration defect. Therefore, segregants were tested for their ability to respire by replica plating to YEPEG; 2 Pet^+:2 Pet^- segregation was observed with, unexpectedly, all of the large colonies being Pet^- and all of the small colonies being Pet^+ (Figure 1, A and B). There was no difference in cell size between the isogenic Pet^+ and Pet^- segregants, which suggested that the difference in colony size was due to a difference in growth rate. As expected for an *HO* strain, all segregants were nonmating but, unexpectedly, all segregants were sporulation deficient (Spo^-).

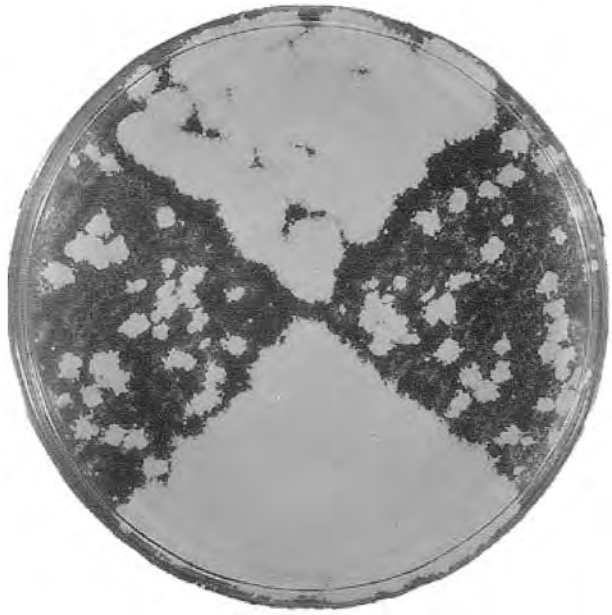

FIGURE 2.—Tetrad of YJM421 on YEPEG—frequent Pet^+ papilli from Pet^- segregants.

The Pet^- phenotype of YJM421 segregants was unstable. All Pet^- segregants produced Pet^+ papilli after prolonged incubation on YEPD and all Pet^- segregants produced abundant Pet^+ papilli when replica plated from YEPD to YEPEG medium (Figure 2). The $Pet^- \rightarrow Pet^+$ variants were Spo^+ and the original segregation pattern of YJM421 was recapitulated in these $Pet^- \rightarrow Pet^+$ variants; that is, after sporulation and dissection, all $Pet^- \rightarrow Pet^+$ variants produced tetrads with 2 Spo^- Pet^- large colony:2 Spo^- Pet^+ small colony segregation.

The results were consistent with a formal model where the unusual behavior and phenotypic variability in the YJM421 genetic background was due to a *ph*enotypic *v*ariability locus (*PHV1*), with two alleles (*PHV1-1* and *PHV1-2*) that had pleiotropic effects on respiration and sporulation. Formally, the *PHV1-1* allele would be necessary for respiration and dominant to *PHV1-2* with respect to the Pet^+ phenotype. However, since heterozygosity was required for sporulation, *PHV1-1* and *PHV1-2* would be either codominant or dosage dependent.

Genetic behavior of the Pet^- phenotype in crosses with S288c background strains: To aid in the design of a cloning strategy for the hypothetical *PHV1* locus, haploid Pet^- *ho*Δ YJM421 background strains were crossed with laboratory S288c background strains to further analyze the Pet^- phenotype. YJM421 background

*ho*Δ Pet$^-$ strains crossed with S288c background strains yielded Pet$^+$ diploids. In addition, tetrads from these crosses showed 2 Pet$^+$:2 Pet$^-$ segregation with the Pet$^-$ segregants being capable of producing Pet$^+$ papilli (data not shown). Because the Pet$^-$ phenotype was complemented in crosses with laboratory strains and showed single gene segregation in meiosis, it seemed likely that we would be able to complement the Pet$^-$ phenotype with a clone from a laboratory strain library.

Complementation of the Pet$^-$ phenotype: To complement the unstable Pet$^-$ phenotype of YJM421-derived strains, a YCp50-based (*CEN URA3*) *S. cerevisiae* library (Rose *et al.* 1987) was transformed into the *ura3* Pet$^-$ strain V1-13−. Over 26,000 Ura$^+$ colonies were screened and 9 Pet$^+$ colonies were isolated. The Pet$^+$ phenotype of three of these isolates was determined to be plasmid dependent; each of these plasmids had overlapping restriction fragment sizes after *Eco*RI and *Hin*dIII digestion. The insert ends of these three plasmids were sequenced, revealing that the Pet$^+$-complementing region spanned the 450,009–462,741 region of chromosome 5.

The presumed sequence for one clone (7A), derived from the Saccharomyces Genome Database, was used to identify available restriction sites and thereby design and construct a series of deletions within the insert. These insert-deletion-containing plasmids were transformed into the Pet$^-$ strain V1-13−. Analysis of these plasmids demonstrated that the *COX15* (YER141w) region was required to complement the Pet$^-$ phenotype in YJM421-derived strains. *COX15* encodes a protein required for cytochrome oxidase assembly and *cox15* mutations have been shown to result in a Pet$^-$ phenotype (Glerum *et al.* 1997). Crosses of *COX15* and *cox15*Δ S288c background strains with Pet$^-$ YJM421-derived strains showed that *COX15* was required to complement the Pet$^-$ phenotype. Therefore, alteration in *COX15* expression or Cox15p function was solely responsible for the variable Pet$^-$ phenotype of YJM421 background strains.

Sequence analysis of *COX15* from Pet$^-$ and Pet$^+$ YJM421 background strains: Two alternative hypotheses for the variable Pet$^-$ phenotype would be that *COX15* expression or Cox15p function differed between the Pet$^-$ and Pet$^+$ variants because of a sequence change (i) at *COX15* or (ii) at a locus unlinked to *COX15*. To distinguish between these two hypotheses, we cloned *COX15* by gap repair (Rothstein 1991) from both Pet$^+$ and Pet$^-$ YJM421 background strains. A *Pvu*II deletion of clone 7A (7A-p), which removed the *COX15* ORF as well as the flanking sequence 2000 bp upstream and 800 bp downstream, was used to gap repair clone *COX15* and surrounding sequences from the Pet$^-$ strain V1-13− and its Pet$^+$ variant V1-13+. Gap repaired plasmids from both Pet$^+$ and Pet$^-$ variants were rescued and sequenced. Sequence analysis of the YJM421-derived *COX15* from both Pet$^+$ and Pet$^-$ strains showed a C → T transition at nucleotide 115 of the 1461-bp open reading frame, which changed the CAA (encoding glutamine) of codon 39 to a TAA (ochre) codon; sequence analysis of the corresponding region of the S288c-derived *COX15* showed the expected C at position 115. Since there was no sequence variation at *cox15* in Pet$^+$ and Pet$^-$ variants, the basis for Pet$^+$ variants must lie elsewhere, presumably in the formation of ochre suppressors.

Identification of the ochre suppressor in YJM421: Libraries containing YJM421-derived DNA that were propagated and amplified in an *E. coli* host failed to yield yeast transformants with a plasmid-dependent Pet$^+$ phenotype (data not shown). This result suggested that a sequence near the putative ochre suppressor was unstable in or deleterious to *E. coli*. A library not propagated in *E. coli*, but instead directly transformed into yeast, would presumably contain sequences that would be unstable in or deleterious to *E. coli*. Therefore, two libraries containing genomic DNA from YJM421 were constructed and directly transformed into a *cox15-421*-containing *S. cerevisiae* strain to clone the presumed ochre suppressor.

Twenty-seven Pet$^+$ colonies from the *Hin*dIII library had a plasmid-dependent Pet$^+$ phenotype. Four out of the first eight of these plasmids recovered from the yeast transformants were able to propagate in *E. coli* and these were amplified for further analysis. All four of these plasmids complemented (or suppressed) the *cox15-421* mutation. Although all four plasmids contained multiple *Hin*dIII fragments, all had a 10-kb *Hin*dIII fragment in common. Six of the Pet$^+$ transformants from the *Xba*I library had a plasmid-dependent Pet$^+$ phenotype, only one of which could be propagated in *E. coli*. This plasmid isolate, named X2, contained a single insert of ~15 kb, which, like the plasmids from the *Hin*dIII library, contained an internal *Hin*dIII fragment of 10 kb. We sequenced the ends of the X2 insert; the resulting sequences corresponded to chromosome 10 positions 342,491–357,278 (Saccharomyces Genome Database). This 14,787-bp fragment contained three tRNA sequences, one of which, tY(GUA)J1, had previously been determined to be *SUP7* (Hawthorne and Mortimer 1968; Olson *et al.* 1977, 1979).

The cloned tRNAs could be separated into distinct *Eco*RI fragments, one containing the tyrosine-inserting tRNA tY(GUA)J1 (*SUP7*-**o**) and the other fragment containing the other two tRNAs. These fragments were subcloned into the *TRP1*-containing plasmid pRS314 (Sikorski and Hieter 1989) and individually transformed into strain V1-22−. Only the plasmid containing the tY(GUA)J1 region conferred Trp$^+$ Pet$^+$ growth on V1-22−. Sequence analysis of this smaller clone showed that tY(GUA)J1 contained a single G → T mutation in the anticodon (ACT**G**TAA → ACT**T**TAA), which converted it into the ochre suppressor *SUP7*-**o**.

Genotyping of tY(GUA)J1/*SUP7*-o in YJM421: The genetic (2 Pet$^+$ small colony:2 Pet$^-$ large colony segrega-

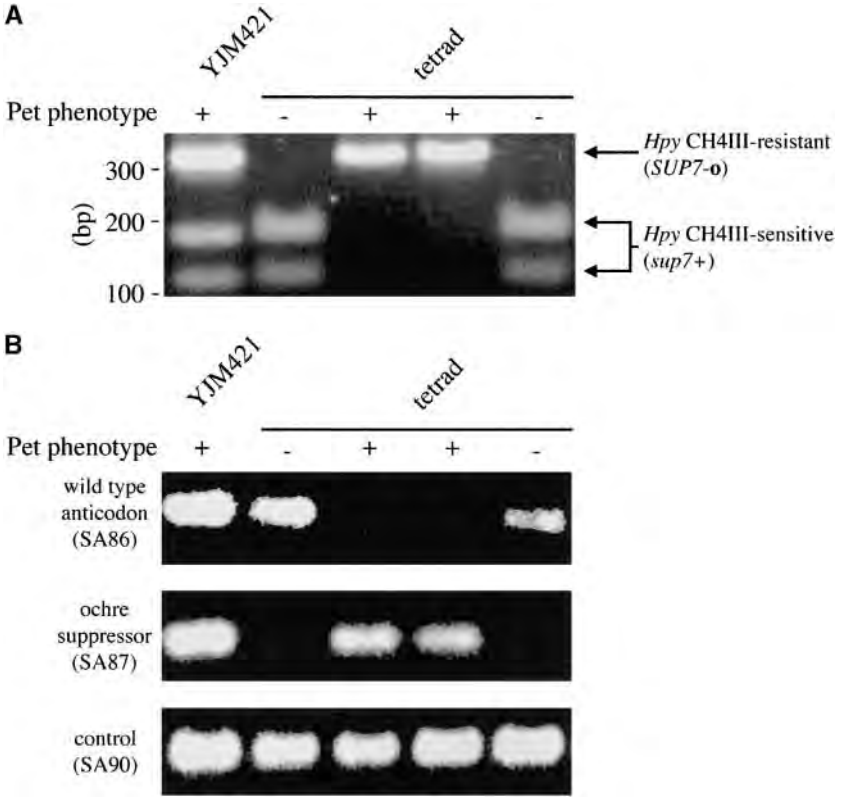

FIGURE 3.—Genotyping of YJM421 and one tetrad from YJM421 by (A) *Hpy*CH4III digestion and (B) MASA PCR.

tion) and molecular analysis (the *cox15-421* ochre mutation and *SUP7*-**o** ochre suppressor) of YJM421 was consistent with the hypothesis that the genotype of YJM421 was *cox15-421*/*cox15-421 SUP7*-**o**/+. To test the hypothesis that the YJM421 genotype was *SUP7*-**o**/+, we took advantage of the fact that wild-type tRNA-Tyr loci contain an *Hpy*CH4III site (ACNGT), which overlaps the anticodon (ACTGTA) and is destroyed when the anticodon is mutated to form an ochre suppressor (ACT TTA).

The eight tRNA-Tyr loci of YJM421 and the control S288c background strain S1 (Southern analysis showed that both genetic backgrounds had eight tRNA-Tyr loci; data not shown) were amplified, separately, to yield 200- to 300-bp PCR products. Agarose gel electrophoresis analysis of the undigested PCR products of all tRNA-Tyr loci from both S1 and YJM421 showed the expected band sizes. The *Hpy*CH4III-digested PCR products of all tRNA-Tyr loci from S1 and, with the exception of tY(GUA)J1/*SUP7*, all tRNA-Tyr loci from YJM421 showed two bands of the expected sizes; that is, none of these loci were ochre suppressors. In contrast, the *Hpy*CH4III-digested PCR product of tY(GUA)J1/*SUP7* from YJM421 showed three bands, one corresponding to the undigested or *Hpy*CH4III-resistant PCR product and two bands corresponding to the *Hpy*CH4III-sensitive PCR product (Figure 3A). The results were consistent with the hypothesis that the genotype of YJM421 was *SUP7*-**o**/+.

Ochre suppressors are known to confer osmotic sensitivity (SINGH 1977). Consistent with the ochre suppressor hypothesis, tetrads from YJM421 showed 2 Osm^s Pet^+:2 Osm^+ Pet^- segregation (Figure 1, B and C). As a final test of the hypothesis that the genotype of YJM421 was *SUP7*-**o**/+ and that *SUP7*-**o** was the sole suppressor in YJM421, 14 YJM421 tetrads were phenotyped for Pet and their tY(GUA)J1/*SUP7*-**o** PCR products were genotyped using *Hpy*CH4III. Consistent with the *SUP7*-**o**/+ hypothesis, the 14 tetrads showed 2 *Hpy*CH4III-sensitive (2 bands) Pet^-:2 *Hpy*CH4III-resistant (one band) Pet^+ segregation (Figure 3A) and 2:2 segregation in the MASA genotyping assay (Figure 3B).

Mutation frequencies and rates in the YJM421 and S288c genetic backgrounds: Mutation frequencies and rates were determined for the *CAN1* and *LYP1* loci and for $Pet^- \rightarrow Pet^+$ in the *cox15-421* strains V1-1− (isogenic with YJM421) and S183 (isogenic with S288c). To avoid selecting or enriching for Pet^+, cultures were grown in YEPD containing 5% dextrose (instead of the usual 2% dextrose) and were harvested in log phase. As shown in Table 3, the *CAN1*, *LYP1*, and $Pet^- \rightarrow Pet^+$ mutation rates and frequencies in the YJM421 and S288c genetic backgrounds were similar, differing at most by approximately twofold.

Genotypes of spontaneous Pet^+ mutants: Spontaneous Pet^+ mutants from three strains (YSA3, V1-1−, and S183) were genotyped (the tRNA-Tyr loci by *Hpy*CH4III digestion of PCR products and MASA and, in cases where all the tRNA-Tyr loci were wild type, *COX15* by sequencing) to determine which loci had mutated. Of 129 spontaneous, independently isolated Pet^+ mutants,

TABLE 3

Spontaneous mutation frequencies and rates

Strain	*CAN1* → *can1*		*LYP1* → *lyp1*		Pet$^-$ → Pet$^+$	
	Frequency ($\times 10^6$)	Rate ($\times 10^8$)	Frequency ($\times 10^6$)	Rate ($\times 10^7$)	Frequency ($\times 10^8$)	Rate ($\times 10^9$)
V1-1	1.0 ± 0.6[a]	7.2 ± 3.9	2.7 ± 0.9	1.9 ± 0.6	7.1 ± 5.6	4.7 ± 3.6
S183	2.1 ± 1.4	14 ± 0.9	9.4 ± 2.3	6.1 ± 1.7	4.1 ± 2.8	2.7 ± 1.9

[a] Standard deviation.

126 (42 in each of the three strains) had mutated one of the tRNA-Tyr loci (in the case of Pet$^+$ derivatives of the diploid strain YSA3, one of the two copies of a given locus) to form *SUP*-**o** and only three had wild-type tRNA-Tyr at all eight loci. Of the three Pet$^+$ mutants with all wild-type tRNA-Tyr loci, the one in S183 had mutated the TAA codon of *cox15-421* to CAA, the wild-type glutamine; the other two Pet$^+$ mutants, both in V1-1−, had mutated the TAA codon of *cox15-421* to TAC (tyrosine) codons, the same amino acid inserted by the tRNA-Tyr *SUP*-**o**. Therefore, in spite of the fact that seven base-pair changes could result in a functional Cox15p (TAA → CAA, AAA, GAA, TCA, TTA, TAC, or TAT; see below), the *cox15-421* ochre codon had a mutation frequency per base pair substantially lower than that of the average tRNA-Tyr locus anticodon mutating to form an ochre suppressor. In addition, only tRNA-Tyr loci mutated to suppress *cox15-421*.

Mutation frequencies to *SUP*-o within the tRNA-Tyr gene family: Aside from a single base-pair polymorphism within the intron, all members of the tRNA-Tyr gene family have identical sequences. Given their identical sequences, one hypothesis would be that the eight tRNA-Tyr loci would be equally likely to mutate to ochre suppressors. The alternative hypothesis would be that members of the dispersed tRNA-Tyr gene family would differ in their ability to mutate to ochre suppressors.

To test these two hypotheses, the data in Table 4 were analyzed by a χ^2 test (Sokal and Rohlf 2000) for 7 d.f. This analysis showed that the biased distribution of *SUP*-**o** mutations at the tRNA-Tyr loci was significant for YSA3 ($\chi^2 = 15.52$ at $P = 0.05$) and highly significant for V1-1− ($\chi^2 = 20.86$ at $P = 0.005$) and S183 ($\chi^2 = 44.86$ at $P = 0.001$). A G-statistic analysis (Sokal and Rohlf 2000) showed that there were no significant differences ($P = 0.05$) in the distribution of mutants between the three strains. Therefore, ploidy, mating type (YSA3 *vs.* V1-1−), and genetic background (V1-1− *vs.* S183) had no significant effect on the relative mutation frequencies of members of the tRNA-Tyr gene family to ochre suppressors. Since there were no significant differences in the distributions of *SUP*-**o** mutants between the three strains, the *SUP*-**o** data for all three strains were combined. Analysis of the combined *SUP*-**o** mutant data by a χ^2 test showed a highly significant deviation from the expected distribution ($\chi^2 = 66.25$ at $P = 0.001$), again indicating that the members of the dispersed tRNA-Tyr gene family differed significantly in their frequency of mutation to ochre suppressors.

To determine which of the tRNA-Tyr loci mutated to ochre suppressors at frequencies higher or lower than expected, the combined *SUP*-**o** mutant data for the three strains were modeled (Sokal and Rohlf 2000); that is, the three tRNA-Tyr loci with the highest and lowest observed mutation frequencies (*sup6*$^+$, *sup2*$^+$, and *sup8*$^+$) that contributed the most to the highly significant χ^2 value were lowered (*SUP6*-**o**) and raised (*SUP2*-**o** and *SUP8*-**o**) to the mean value of the entire set of the eight loci (mean = 15.75). When the modeled data were analyzed by a χ^2 test, the χ^2 statistic was reduced to a nonsignificant value ($P = 0.05$), indicating that *sup6*$^+$ mutated at a frequency higher than expected while *sup2*$^+$ and *sup8*$^+$ mutated at frequencies lower than expected.

Examination of *SUP*-o suppression efficiencies for possible correlation with mutation frequencies in the tRNA-Tyr gene family: Because a correlation between suppressor efficiencies and locus-specific mutation fre-

TABLE 4

Number of independently isolated *SUP*-o mutants

Strain	*SUP2*-**o**	*SUP3*-**o**	*SUP4*-**o**	*SUP5*-**o**	*SUP6*-**o**	*SUP7*-**o**	*SUP8*-**o**	*SUP11*-**o**
YSA3	1	4	7	4	11	7	1	7
V1-1−	1	2	3	8	11	7	1	9
S183	0	1	10	5	17	5	0	4
Total	2	7	20	17	39	19	2	20

TABLE 5

***lacZ* expression and *lacZ*-ochre suppression**

Strain[a]	*COX15* genotype	*SUP* genotype	β-Galactosidase activity in strain containing: Wild-type *lacZ*[b]	*lacZ*-ochre[c]	% suppression[d]
S183	*cox15-421*	+	335.6 ± 28.1	ND[e]	NA[f]
S183 (+AA)[g]	*cox15-421*	+	338.5 ± 21.0	ND	NA
S1219	*COX15*	+	303.4 ± 11.4	ND	NA
S1219 (+AA)	*COX15*	+	317.3 ± 8.7	ND	NA
S1238	*COX15*	+	268.1 ± 20.2	ND	NA
S1238 (+AA)	*COX15*	+	313.2 ± 21.8	ND	NA
S1260, S1269	*cox15-421*	*SUP2*-**o**	150.0 ± 25.2	23.1 ± 2.4	15.4
S1233[h]	*cox15-421*	*SUP3*-**o**	92.5 ± 16.7	19.4 ± 3.3	21.0
S1201, S1256	*cox15-421*	*SUP4*-**o**	128.9 ± 26.7	29.6 ± 5.2	23.0
S1202, S1257	*cox15-421*	*SUP5*-**o**	170.2 ± 28.7	28.2 ± 6.1	16.6
S1203, S1254[i]	*cox15-421*	*SUP6*-**o**	144.6 ± 17.0	28.7 ± 4.5	19.8
S1204, S1255	*cox15-421*	*SUP7*-**o**	143.9 ± 6.7	28.7 ± 6.2	20.0
S1258, S1259	*cox15-421*	*SUP8*-**o**	174.9 ± 5.5	29.9 ± 1.7	17.1
S1205, S1252, S1253	*cox15-421*	*SUP11*-**o**	205.4 ± 11.7	26.5 ± 5.2	12.9
S1205/S1228[j]	*cox15-421*/*cox15-421*	*SUP11*-**o**/+	97.9 ± 8.0	4.4 ± 0.1	4.5
S1205/S1228 (+KCl)[k]	*cox15-421*/*cox15-421*	*SUP11*-**o**/+	71.7 ± 5.3	4.1 ± 0.4	5.7

[a] All strains were isogenic with S288c. With the exception of *SUP2*-**o** and *SUP8*-**o**, all *SUP*-**o** were spontaneous Pet$^+$ mutants from S183. S1219 was a segregant from a cross between S183 and the isogenic strain S95. S1238 was isolated by transforming S183 with an oligonucleotide (Moerschell *et al.* 1991) that converted the TAA ochre mutation to the wild-type CAA.

[b] β-Galactosidase activity in strains containing pUKC815 (*lacZ* reporter).

[c] β-Galactosidase activity in strains containing pUKC817 (*lacZ* reporter with ochre mutation).

[d] (*lacZ*-ochre units/*lacZ* units) × 100.

[e] ND, not detected. Both in the presence and absence of the *lacZ*-ochre reporter, the *sup*$^+$ control strains S183, S1219, and S1238 showed no detectable β-galactosidase activity within the time frame used to assay β-galactosidase activity in *SUP*-**o** strains.

[f] NA, not applicable.

[g] (+AA), grown in the presence of 1 μg/ml of the respiration inhibitor antimycin A.

[h] The same *SUP3*-**o** mutant (the only one isolated from S183) was assayed in three independent experiments.

[i] The same *SUP6*-**o** mutant was assayed in two independent experiments.

[j] S1205 was crossed with S1228 that had been transformed, separately, with pUKC815 or pUKC817; diploids were selected by growth on synthetic minimal medium containing ethanol and glycerol as the sole carbon sources.

[k] (+KCl), grown in SDC-URA containing 1.5 M KCl.

quencies might offer insight into the mechanism responsible for the locus-specific mutation frequencies, the suppressor efficiencies of S183-derived *SUP*-**o** mutants were characterized. The correlation coefficient (Sokal and Rohlf 2000) between mutation frequencies for S183 (Table 4) and suppressor efficiencies for S183-derived *SUP*-**o** mutants (Table 5) was found to be not significant ($r = 0.38$ at $P = 0.05$).

Locus- and dosage-dependent *SUP*-o mutant phenotypes: Because such phenotypes would be relevant to phenotypic variation, the *SUP*-**o** mutants were examined to determine if there were locus- and/or dosage-specific phenotypes. In addition to their suppression of nonsense mutations, some *SUP*-**o** mutations have an osmotic sensitivity phenotype (Osm^s; Singh 1977). With the exception of the *SUP11*-**o** mutants, all of the *SUP*-**o** mutants isolated in the haploid strains V1-1− and S183 were Osm^s. All of the 42 *SUP*-**o**/+ mutants isolated in the diploid strain YSA3 were Osm$^+$; similarly, when 19 *SUP*-**o** mutants isolated in the haploid strain S183 were crossed with an isogenic *sup*$^+$ strain, the resulting diploids were Osm$^+$. Therefore, osmotic sensitivity was recessive, or dosage dependent, for the *SUP*-**o** mutants. The suppression efficiency of S1205/S1228 (*SUP11*-**o**/+ *cox15-421*/*cox15-421*) was substantially lower than that of haploid *SUP11*-**o** strains (Table 5), consistent with suppression efficiency being dosage dependent.

Of all the *SUP*-**o** mutants, only the *SUP11*-**o** mutants were Osm$^+$. One hypothesis for the Osm$^+$ phenotype of *SUP11*-**o** would be that *SUP11*-**o** expression was reduced or abolished under high-osmolarity conditions. However, all *SUP11*-**o** *cox15-421* strains grew on YEPEG containing 1.5 M KCl. In addition, the suppression efficiency of S1205/S1228 (*SUP11*-**o**/+ *cox15-421*/*cox15-*

421) was not reduced in medium containing 1.5 M KCl (Table 5). Therefore, similar to the Osm$^+$ phenotype of *SUP*-**o**/+ strains, the Osm$^+$ phenotype of *SUP11*-**o** was probably due to the suppression efficiency of *SUP11*-**o**, the least efficient of the tRNA-Tyr *SUP*-**o** mutants, being below a critical threshold for conferring the Osm^s phenotype.

Measuring the suppressor efficiencies of *SUP*-**o** mutants required the use of the control plasmid pUKC815, which contains wild-type *lacZ*$^+$ under the control of the *PGK1* promoter (Firoozan *et al.* 1991; Stansfield *et al.* 1995), in both *sup*$^+$- and *SUP*-**o**-containing strains. For pUKC815-containing strains grown in the absence of antimycin A, the average β-galactosidase level of *sup*$^+$ *COX15* strains (285.7 Miller units) was considerably higher than that for *SUP*-**o** *cox5-421* (Pet$^+$) strains (Table 5). Therefore, the *SUP*-**o** mutants as a class had substantially reduced *PGK1* promoter activity.

The effect of the *SUP*-**o** mutations as a class on *PGK1* promoter activity suggested a correlation between suppressor efficiency, which was *SUP*-**o** locus specific, and *PGK1* promoter activity. The correlation coefficient (Sokal and Rohlf 2000) between *PGK1* promoter activity and suppressor efficiency (Table 5) was highly significant ($r = -0.86$ at $P = 0.01$): that is, the more efficient the suppressor, the lower the *PGK1* promoter activity.

The low frequency of intragenic *COX15* revertants and restricted spectrum of *SUP*-o mutations: The low frequency of intragenic *cox15-421* revertants could be partially dependent upon the amino acid substitutions at position 39 of Cox15p, which would result in a functional gene product. Similarly, the restricted spectrum of extragenic suppressors of *cox15-421* could be dependent upon the amino acid substitutions at position 39 of Cox15p, which would result in a functional gene product, and/or upon the low suppression efficiency of non-tRNA-Tyr *SUP*-**o**. Alternatively, relative to the average mutation frequency of the tRNA-Tyr loci, intragenic *cox15-421* revertants and ochre suppressors at other tRNA loci might be formed at very low frequencies.

We used oligonucleotide cotransformation (Moerschell *et al.* 1991) to address the question of permissible amino acid substitutions at position 39 of Cox15p. Seven single base-pair substitution mutations can convert a TAA ochre codon to one of seven sense codons: CAA (Gln), TAC (Tyr), TAT (Tyr), AAA (Lys), GAA (Glu), TCA (Ser), and TTA (Leu). Therefore, we transformed the *cox15-421* strain S183 with (i) a plasmid containing a natMX4 selectable marker and (ii) oligonucleotides that would convert the ochre codon of *cox15-421* to one of the seven sense codons. Nourseothricin-resistant (plasmid-containing) transformants were selected and then screened for Pet$^+$. After excluding tRNA-Tyr *SUP*-**o**-containing Pet$^+$ strains, the region around codon 39 of *COX15* of the remaining plasmid-containing Pet$^+$ transformants was sequenced.

As expected, wild-type glutamine (oligonucleotide SA98) and tyrosine (inserted by tRNA-Tyr *SUP*-**o**; oligonucleotides SA99 and SA100) amino acid substitutions at amino acid 39 of Cox15p were recovered (2, 1, and 3 cotransformants, respectively). In addition, lysine (oligonucleotide SA101), glutamate (oligonucleotide SA102), serine (oligonucleotide SA103), and leucine (oligonucleotide SA104) substitutions were recovered (3, 1, 1, and 1 cotransformants, respectively). Since all of these amino acids resulted in a functional Cox15p, all possible seven intragenic single base-pair substitution mutations of *cox15-421* occurred at low frequencies relative to the average tRNA-Tyr locus mutating to *SUP*-**o**. In addition, the results suggested that the failure to isolate non-tRNA-Tyr tRNA *SUP*-**o** mutants was not due to the inserted amino acid at position 39 resulting in a nonfunctional Cox15p.

Four Pet$^+$ Osm$^+$ *sup11*$^+$ *cox15-421* mutants were also isolated. In principle, the Pet$^+$ phenotype of these mutants could have been due to [*PSI*$^+$] or to non-tRNA-Tyr *SUP*-**o** mutations. However, after being grown in [*psi*$^-$]-inducing conditions, these Osm$^+$ *sup11*$^+$ *cox15-421* mutants retained their Pet$^+$ phenotypes; that is, the Pet$^+$ phenotypes of these Osm$^+$ *sup11*$^+$ *cox15-421* mutants were not due to [*PSI*$^+$]. These results suggested that, at a low frequency relative to the average tRNA-Tyr locus, some tRNA genes other than the tRNA-Tyr loci could apparently mutate to *SUP*-**o** and suppress *cox15-421*.

DISCUSSION

The high-frequency formation of tRNA-Tyr *SUP*-**o** mutants, along with the presence of the *cox15-421* reporter mutation, is responsible for the phenotypic variation described in this work. In addition, the analysis of YJM421 and other *cox15-421*-containing strains explains the more subtle nuances of this intriguing case of phenotypic variation. First, although dominant in terms of their suppressor function, ochre suppressors confer a recessive sporulation defect (Rothstein *et al.* 1977), which explains why the Pet$^+$ Spo$^+$ strain YJM421 (*HO*/*HO MAT***a**/*MAT*α *cox15-421*/*cox15-421 SUP7*-**o**/+) produces Pet$^+$ Spo$^-$ segregants (*HO*/*HO MAT***a**/*MAT*α *cox15-421*/*cox15-421 SUP7*-**o**/*SUP7*-**o**). Second, ochre suppressors have been described as being detrimental to cell growth (Sherman 1982), which explains why Pet$^+$ (*SUP7*-**o**) segregants of YJM421 form colonies smaller than those of Pet$^-$ (*sup7*$^+$) segregants. Finally, the high frequency of tyrosine-inserting ochre suppressor formation (relative to *cox15-421* → *COX15*) explains the phenotypic instability of the Pet$^-$ segregants and the recapitulation by the resulting Pet$^+$ Spo$^+$ *SUP*-**o**/+ variants of the original behavior of YJM421.

Diversity in *SUP*-o mutant phenotypes—implications for phenotypic variation: Although phenotypically indistinguishable on the basis of their Pet$^+$ phenotypes, the

SUP-**o** mutants in this study differ clearly in their suppression efficiencies. In turn, the different suppression efficiencies of the *SUP*-**o** mutants result in clear differences in other phenotypes. For example, the severity of the sporulation defect of *SUP*-**o** mutants correlates with suppression efficiency (Rothstein *et al.* 1977). All of the tRNA-Tyr *SUP*-**o** mutants in this study are osmotic sensitive, except for *SUP11*-**o**, which is the least efficient of the tyrosine-inserting ochre suppressors. Finally, the different tRNA-Tyr *SUP*-**o** mutations have substantial effects on *PGK1* promoter activity that correlates with suppressor efficiency. Since the suppression efficiency of *SUP* mutants is dosage sensitive and *SUP* mutants exhibit locus-specific suppression efficiencies, *SUP* mutant formation is a modulatable phenotypic variation mechanism. Clearly, phenotypic variants that are phenotypically indistinguishable at the gross level may arise from mutational events at different loci; it is only upon closer examination that these variants may have different phenotypes. This *SUP*-**o** locus-specific and -dependent diversity and the resulting phenotypic diversity have implications for the study of other phenotypic variations in *S. cerevisiae* and for the study of phenotypic variation in other yeasts and fungi.

Genetic diversity and heterogeneity—implications for phenotypic variation: Naturally occurring nonsense mutations have been described in different *S. cerevisiae* genetic backgrounds, such as *suc*^{0} (Gonzalbo and Hohmann 1989, 1990), *flo8* (Liu *et al.* 1996), and a "delayed homothallism" *ho* mutation (Tani *et al.* 1994; Ekino *et al.* 1999). The *cox15-421* mutation is another example of a naturally occurring nonsense mutation. In the context of this work, naturally occurring nonsense mutations are genetic-background-specific nonsense suppressor and phenotypic variation reporters. Viewed from the perspective of *SUP*-**o**-mediated variation, phenotypic variants in different genetic backgrounds may arise from mutational events in the same gene but will have profoundly different phenotypes due to the different nonsense mutations in each genetic background; a similar argument has been made with respect to [*PSI*$^+$]-mediated phenotypic variation (True and Lindquist 2000). Therefore, what may appear to be different phenotypic variation systems in unrelated members of the same species could in fact be due to mutational or switching events in the same gene.

Mutation frequencies and rates: A high frequency of mutators has been reported in specific ecotypes (Denamur *et al.* 2002) and in clinical isolates of pathogenic bacteria and these mutators have been suggested to be important for adaptation to a pathogenic life style (LeClerc *et al.* 1996). Given its clinical origin, the possibility that a mutator might play a role in the phenotypic variability seen in YJM421 was intriguing. However, the mutation frequencies and rates in the S288c and YJM421 genetic backgrounds are similar, which argues strongly against the mutator hypothesis.

Base-pair substitution frequencies—the average tRNA-Tyr locus anticodon *vs.* the *cox15-421* ochre codon: One base-pair substitution mutation in the anticodon (GTA to TTA) can convert any of the eight tRNA-Tyr loci to ochre suppressors. In contrast, seven single base-pair substitution mutations can convert the TAA ochre codon of *cox15-421* to one of seven sense codons resulting in a functional Cox15p. Given the gene copy numbers and the number of permissible base substitution mutations per gene, one might expect comparable numbers of tRNA-Tyr *SUP*-**o** mutants and intragenic (nonsense to sense) *COX15* mutants. However, in our sample size of 129 spontaneous Pet$^+$ mutants, we find 126 tyrosine-inserting *SUP*-**o** mutants but only 3 intragenic *COX15* revertants. Therefore, relative to the ochre codon of *cox15-421* mutating to sense, the average tRNA-Tyr locus mutates to *SUP*-**o** at a very high frequency.

As argued for [*PSI*$^+$] (True and Lindquist 2000), translational misreading allows the potential value of nonsense mutations to be assessed. Viewed from the perspective of phenotypic variation, the high mutation frequency of the average tRNA-Tyr locus from wild type to *SUP*-**o**, combined with the tRNA-Tyr gene copy number, would facilitate both the retention of naturally occurring nonsense mutations, such as *cox15-421*, and the testing of these nonsense mutations for potentially beneficial effects.

The position effect on tRNA-Tyr mutation frequency: One reasonable hypothesis would be that the eight tRNA-Tyr loci, which aside from a 1-bp intron polymorphism are identical in sequence, would mutate to form ochre suppressors at equal frequencies. However, as shown in this work, members of the tRNA-Tyr gene family mutate to form ochre suppressors at significantly different frequencies. To the best of our knowledge, no previous studies have examined the effect of gene position in the genome on mutation frequency. Now that a very substantial position effect on mutation frequency has been found, the challenge is to deduce a mechanism. Toward this end, we examined the association between tRNA-Tyr locus-specific mutation frequencies and multiple factors.

The eight tRNA-Tyr genes have a very short, simple structure: exon 1 (base pairs 1–39), an intron (base pairs 40–53), and exon 2 (base pairs 54–89). Although flanking sequences can affect transcription efficiency, the tRNA-Tyr promoter is intragenic (reviewed in Geiduschek and Tocchini-Valentini 1988; Paule and White 2000). The sequences of exon 1 and exon 2 are identical in all eight members of the tRNA-Tyr gene family. However, there is a 1-bp sequence polymorphism in the intron. At the polymorphic intron position (base 44) of the two lowest frequency tRNA-Tyr loci, there is a T at *sup2*$^+$ and a C at *sup8*$^+$. Similarly, *sup5*$^+$, *sup7*$^+$, *sup11*$^+$ (three of the four average frequency loci), and *sup6*$^+$ (the highest frequency locus) all have a C at

position 44. Therefore, there is no association between the one polymorphism in the tRNA-Tyr loci and their mutation frequencies.

Is there any correlation between tRNA-Tyr gene-centromere or -telomere distances and locus-specific mutation frequencies? The distances (in ascending order) between the tRNA-Tyr genes and their centromeres are *sup11* (19 kb), *sup3* (39 kb), *sup6* (63 kb), *sup7* (82 kb), *sup5* (99 kb), *sup4* (107 kb), *sup2* (500 kb), and *sup8* (570 kb). Similarly, the distances (in ascending order) between the tRNA-Tyr genes and their telomeres are *sup6* (59 kb), *sup8* (86 kb), *sup11* (103 kb), *sup5* (169 kb), *sup4* (203 kb), *sup3* (288 kb), *sup7* (354 kb), and *sup2* (586 kb). There is no significant correlation between tRNA-Tyr mutation frequencies and distances to centromeres ($r = -0.56$ at $P = 0.05$) or telomeres ($r = -0.41$ at $P = 0.05$).

Using the Saccharomyces Genome Database, the regions flanking the tRNA-Tyr genes were examined for transposon-related or transcriptional characteristics that might associate with mutation frequency. No Ty1 or Ty3 elements are in the regions immediately flanking any of the tRNA-Tyr genes. A sigma element is near (17 bp) *sup2* but no sigma elements are near any of the other tRNA-Tyr genes. The number and location of delta elements relative to the tRNA-Tyr genes are *sup2* [one delta element (639 bp) in a 6.5-kb region], *sup3* (no delta elements in a 9.8-kb region), *sup4* [five delta elements (the nearest being 404 bp) in a 7.3-kb region], *sup5* [one delta element (114 bp) in a 6.4-kb region], *sup6* [one delta element (3.5 kb) in a 9.4-kb region], *sup7* [two delta elements (the nearer being 206 bp) in a 10-kb region], *sup8* [one delta element (164 bp) in a 8.8-kb region], and *sup11* [one delta element (5.3 kb) in a 10.4-kb region]. Finally, all of the RNA polymerase II transcribed genes near the tRNA-Tyr genes are transcribed at low levels (for most genes, less than one transcript per cell). Therefore, there is no obvious association between Ty elements, delta/sigma elements, or RNA polymerase II transcription levels and tRNA-Tyr mutation frequencies.

One model for the highly skewed mutation frequencies would be that recovery of mutants in specific tRNA-Tyr genes is biased by suppressor efficiency. For example, there might be reduced recovery of more efficient ochre suppressors when their high suppression efficiency is extremely deleterious to the cell. Alternatively, there might be reduced recovery of inefficient ochre suppressors when their low suppression efficiency is incapable of producing sufficient Cox15p. However, there is no significant correlation between suppressor efficiency and mutation frequency. In addition, the spectrum of tRNA-Tyr mutants in isogenic haploid and diploid strains (all *SUP*-**o**/+ heterozygotes, which, due to dosage, have reduced suppression efficiency relative to *SUP*-**o**-containing haploids) is indistinguishable. These results argue strongly against the skewed mutation frequencies being due to differences in suppressor efficiency.

Another model for the highly skewed mutation frequencies in the eight tRNA-Tyr genes would be that the level of transcription of these genes influences the mutation frequencies. Although RNA polymerase III-transcribed tRNA genes have not been examined, there is evidence in *S. cerevisiae* for a correlation between transcription of RNA polymerase II-transcribed genes and mutation frequency (Datta and Jinks-Robertson 1995; Morey *et al.* 2000). However, the lack of correlation between suppressor efficiency, which is presumably a measure of transcription efficiency of these eight identical tRNA-Tyr genes, and mutation frequency argues strongly against the transcription hypothesis.

One aspect of DNA replication that might be relevant to the position effect on mutation frequency is the timing of replication during S phase, which has recently been determined for the entire *S. cerevisiae* genome. The times of replication of the tRNA-Tyr loci are *sup2* (35 min), *sup3* (19 min), *sup4* (18 min), *sup5* (20 min), *sup6* (17 min), *sup7* (29 min), *sup8* (27 min), and *sup11* (18 min; data derived from Raghuraman *et al.* 2001). However, there is no significant correlation between the times of replication of the tRNA-Tyr loci and their frequencies of mutation to *SUP*-**o** ($r = -0.35$ at $P = 0.05$).

Another aspect of DNA replication that might be relevant to the position effect on mutation frequency is the rate of fork movement during replication of the tRNA-Tyr loci, which might influence replication fidelity or DNA repair. The rates of fork movement (in ascending order) of the members of the tRNA-Tyr gene family are *sup2* (1.3 kb/min), *sup6* (1.4 kb/min), *sup7* (1.7 kb/min), *sup5* (1.8 kb/min), *sup4* (2.1 kb/min), *sup3* (2.3 kb/min), *sup11* (2.4 kb/min), and *sup8* (2.7 kb/min; data derived from Raghuraman *et al.* 2001). One caveat with respect to these fork rate movements is that they were determined for relatively large (compared to the size of tRNA-Tyr loci) regions and local fork rate movements may differ. Indeed, DNA replication fork pause sites have been localized to tRNA loci that are transcribed in opposition to replication forks (Deshpande and Newlon 1996). However, with this caveat in mind, there is no significant correlation between the rate of replication and locus-specific mutation frequencies ($r = -0.14$ at $P = 0.05$).

The final aspect of DNA replication that might be relevant to the position effect on mutation frequency is gene orientation relative to the nearest origin(s) of replication. Gene origin of replication orientation effects on mutation that are attributable to differences in fidelity in leading- *vs.* lagging-strand DNA synthesis have been described in *E. coli* (Fijalkowska *et al.* 1998; Maliszewska-Tkaczyk *et al.* 2000). In *S. cerevisiae*, the effect

of gene orientation relative to the origin of replication on the distribution and frequency of mutations has been examined using a plasmid-borne *SUP4*-**o** forward mutation detection system where there is only a single origin of replication. While the spontaneous *sup4*$^{-}$ mutation frequency is orientation independent, the distribution of *sup4*$^{-}$ mutations is influenced by gene orientation (Karthikeyan *et al.* 2000).

The gene origin of replication orientation hypothesis has specific predictions: (1) bidirectionally replicated tRNA-Tyr loci (*e.g.*, roughly equidistant between two efficient origins) should have average mutation frequencies and (2) unidirectionally replicated tRNA-Tyr loci (*e.g.*, physically close to one efficient origin), depending upon the gene orientation, should have either high or low mutation frequencies. The locations of origins of replication, as well as probable replication termini, have recently been determined across the entire *S. cerevisiae* genome (Raghuraman *et al.* 2001). An examination of origin locations and efficiencies, as well as likely replication termini relative to tRNA-Tyr gene locations, suggests that all of the tRNA-Tyr genes are replicated unidirectionally but the predicted bimodal (high or low, depending upon gene orientation) distribution in mutation frequencies is not observed. However, it is interesting to note that *sup2*, *sup3*, and *sup8*, the three loci with the lowest mutation frequencies, are all transcribed in the same direction as the most likely replication forks. In contrast, *sup4*, *sup5*, *sup7*, and *sup11*, the four loci with average mutation frequencies, as well as *sup6*, the locus with the highest mutation frequency, are all transcribed in the opposite direction to the most likely replication forks. This suggests that gene orientation relative to the direction of replication may be a factor in locus-specific mutation frequencies. However, if gene origin of replication orientation is a factor, the distribution of mutation frequencies suggests that one or more additional factors contribute to locus-specific mutation frequencies.

In addition to the analysis of position effects on mutation frequency in this study, the tRNA-Tyr gene family has been used to examine the effect of genomic position on gene conversion. Gene conversion frequencies have been determined for all eight tRNA-Tyr loci in *SUP*/+ diploids: that is, the same base pair, in the same immediate sequence context, in the same dispersed gene family as this study. There are clear differences between the position effects on gene conversion frequencies and the position effects on mutation frequencies in this study; specifically, gene conversion frequencies for seven of the eight tRNA-Tyr loci did not differ significantly, ranging from 1.5 to 5.3%. However, it is interesting to note that *SUP6*, which had the highest mutation frequency in this study, had a significantly higher gene conversion frequency of 21% (Mortimer and McKey 1969). While it is not clear whether there is any relationship between the position effects on gene conversion and mutation, this gene conversion study provides a clear precedent for significantly different behavior of the same sequence, in this case members of the dispersed tRNA-Tyr gene family, in different genomic locations.

Conclusion: Like [*PSI*$^{+}$], tRNA-Tyr suppressors are a mechanism for generating phenotypic variation. There are clear differences between [*PSI*$^{+}$] and tRNA-Tyr suppressors, such as the amino acid(s) inserted at nonsense codons (apparently unknown for [*PSI*$^{+}$] but specific for tRNA *SUP*), suppression efficiency (low for [*PSI*$^{+}$], high for tRNA-Tyr *SUP*), and specificity of suppression (all three types of nonsense codons for [*PSI*$^{+}$] but specific to a single type of nonsense codon for tRNA *SUP*). However, there are many similarities between [*PSI*$^{+}$] and tRNA-Tyr suppressors, such as suppression of nonsense mutations, modulatable suppression efficiency, and the frequency of formation and loss.

The finding in this study that members of the tRNA-Tyr gene family are agents of phenotypic variation has multiple implications. First, phenotypic variants can arise by multiple mechanisms; this may involve mutating different genes, such as different tRNA-Tyr loci, as well as mechanisms of loss of *SUP*-**o**. Second, variants that are indistinguishable at the gross phenotypic level may be due to mutations at different loci that, upon closer examination, differ phenotypically. Finally, in different backgrounds the same switch or mutational event can produce quite different phenotypes. All of these results have implications for the study of other phenotypic variations in *S. cerevisiae* and the study of phenotypic variation in other yeasts and fungi. Models for phenotypic variation must take into account not only the phenotypic diversity but also the genetic diversity, such as strain-to-strain differences that could be due to both strain-specific reporter(s) as well as different phenotypic variation mechanisms/systems.

For the first time to our knowledge, this work demonstrates that there is a strong position effect on mutation frequency. A position effect on mutation frequency has a variety of implications in the field of genetics. For example, different regions of the genome may vary in how quickly they diverge and in the amount of sequence diversity they exhibit and, in experimental systems, mutant recovery in different screens and selections may be influenced by genomic location.

With respect to mechanisms for the position effect on mutation frequency, most of the usual suspects appear to be excluded, at least as sole factors, from consideration; it is likely that multiple factors interact to produce the position effects on mutation frequency. Although the tRNA-Tyr gene family offers a powerful, naturally occurring system to examine the effect of genomic position on mutation frequency, the tRNA-Tyr system has two limitations: one can assay only a single base pair for one substitution mutation and one must assay eight loci

in every mutant strain. Other mutation reporter systems will be considerably more flexible with respect to the selections for mutations that can be applied, the number and different types of mutations that can be assayed, the assay method(s), and the experimental placement of reporter gene(s) in different genomic locations. In the future, we will use other systems to more explicitly examine the effects of reporter gene position within the genome, including the effects of gene orientation and transcription, on both mutation frequency and mutational spectrum.

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LITERATURE CITED

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith *et al.* (Editors), 2002 *Current Protocols in Molecular Biology*. Wiley Interscience, New York.

Datta, A., and S. Jinks-Robertson, 1995 Association of increased spontaneous mutation rates with high levels of transcription in yeast. Science **268:** 1616–1619.

Denamur, E., S. Bonacorsi, A. Giraud, P. Duriez, F. Hilali *et al.*, 2002 High frequency of mutator strains among human uropathogenic *Escherichia coli* isolates. J. Bacteriol. **184:** 605–609.

Deshpande, A. M., and C. S. Newlon, 1996 DNA replication fork pause sites dependent on transcription. Science **272:** 1030–1033.

Dower, W. J., J. F. Miller and C. W. Ragsdale, 1988 High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:** 6127–6145.

Drake, J. W., 1991 A constant rate of spontaneous mutation in DNA-based microbes. Proc. Natl. Acad. Sci. USA **88:** 7160–7164.

Ekino, K., I. Kwon, M. Goto, S. Yoshino and K. Furukawa, 1999 Functional analysis of *HO* gene in delayed homothallism in *Saccharomyces cerevisiae* wy2. Yeast **15:** 451–458.

Erbs, P., F. Exinger and R. Jund, 1997 Characterization of the *Saccharomyces cerevisiae* FCY1 gene encoding cytosine deaminase and its homologue FCA1 of *Candida albicans*. Curr. Genet. **31:** 1–6.

Fijalkowska, I. J., P. Jonczyk, M. M. Tkaczyk, M. Bialoskorska and R. M. Schaaper, 1998 Unequal fidelity of leading strand and lagging strand DNA replication on the *Escherichia coli* chromosome. Proc. Natl. Acad. Sci. USA **95:** 10020–10025.

Firoozan, M., C. M. Grant, J. A. Duarte and M. F. Tuite, 1991 Quantitation of readthrough of termination codons in yeast using a novel gene fusion assay. Yeast **7:** 173–183.

Geiduschek, E. P., and G. P. Tocchini-Valentini, 1988 Transcription by RNA polymerase III. Annu. Rev. Biochem. **57:** 873–914.

Gietz, R. D., R. H. Schiestl, A. R. Willems and R. A. Woods, 1995 Studies on the transformation of intact yeast cells by the LiAc/SS- DNA/PEG procedure. Yeast **11:** 355–360.

Glerum, D. M., I. Muroff, C. Jin and A. Tzagoloff, 1997 *COX15* codes for a mitochondrial protein essential for the assembly of yeast cytochrome oxidase. J. Biol. Chem. **272:** 19088–19094.

Goldstein, A. L., and J. H. McCusker, 1999 Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast **15:** 1541–1553.

Goldstein, A. L., and J. H. McCusker, 2001 Development of *Saccharomyces cerevisiae* as a model pathogen: a system for the genetic identification of gene products required for survival in the mammalian host environment. Genetics **159:** 499–513.

Goldstein, A. L., X. Pan and J. H. McCusker, 1999 Heterologous URA3MX cassettes for gene replacement in *Saccharomyces cerevisiae*. Yeast **15:** 507–511.

Gonzalbo, D., and S. Hohmann, 1989 The naturally occurring silent invertase structural gene *suc2*^{0} contains an amber stop codon that is occasionally read through. Mol. Gen. Genet. **216:** 511–516.

Gonzalbo, D., and S. Hohmann, 1990 Nonsense suppressors partially revert the decrease of the mRNA level of a nonsense mutant allele in yeast. Curr. Genet. **17:** 77–79.

Gottschling, D. E., O. M. Aparicio, B. L. Billington and V. A. Zakian, 1990 Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. Cell **63:** 751–762.

Hasegawa, Y., S. Takeda, S. Ichii, K. Koizumi, M. Maruyama *et al.*, 1995 Detection of K-ras mutations in DNAs isolated from feces of patients with colorectal tumors by mutant-allele-specific amplification (MASA). Oncogene **10:** 1441–1445.

Hawthorne, D. C., and R. K. Mortimer, 1968 Genetic mapping of nonsense suppressors in yeast. Genetics **60:** 735–742.

Karthikeyan, R., E. J. Vonarx, A. F. Straffon, M. Simon, G. Faye *et al.*, 2000 Evidence from mutational specificity studies that yeast DNA polymerases delta and epsilon replicate different DNA strands at an intracellular replication fork. J. Mol. Biol. **299:** 405–419.

LeClerc, J. E., B. Li, W. L. Payne and T. A. Cebula, 1996 High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. Science **274:** 1208–1211.

Lindegren, C. C., 1956 Mutation and other variations in microorganisms. Compt. Rend. Trav. Lab. Carlsberg Ser. Physiol. **26:** 253–272.

Liu, H., C. A. Styles and G. R. Fink, 1996 *Saccharomyces cerevisiae* S288C has a mutation in *FLO8*, a gene required for filamentous growth. Genetics **144:** 967–978.

Lund, P. M., and B. S. Cox, 1981 Reversion analysis of [*psi-*] mutations in *Saccharomyces cerevisiae*. Genet. Res. **37:** 173–182.

Maliszewska-Tkaczyk, M., P. Jonczyk, M. Bialoskorska, R. M. Schaaper and I. J. Fijalkowska, 2000 SOS mutator activity: unequal mutagenesis on leading and lagging strands. Proc. Natl. Acad. Sci. USA **97:** 12678–12683.

McCusker, J. H., and R. W. Davis, 1991 A novel cell type switching system, high temperature growth and protease secretion in a clinical *S. cerevisiae* isolate. Genetics Society of America Yeast Genetics and Molecular Biology Meeting, San Francisco, p. 78.

McCusker, J. H., K. V. Clemons, D. A. Stevens and R. W. Davis, 1994 Genetic characterization of pathogenic *Saccharomyces cerevisiae* isolates. Genetics **136:** 1261–1269.

Moerschell, R. P., G. Das and F. Sherman, 1991 Transformation of yeast directly with synthetic oligonucleotides, pp. 362–369 in *Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology*, edited by C. Guthrie and G. R. Fink. Academic Press, San Diego.

Morey, N. J., C. N. Greene and S. Jinks-Robertson, 2000 Genetic analysis of transcription-associated mutation in *Saccharomyces cerevisiae*. Genetics **154:** 109–120.

Mortimer, R. K., and T. J. McKey, 1969 Gene conversion of nonsense suppressors in *Saccharomyces*. Antonie Van Leeuwenhoek **35** (Suppl.): C23–24.

Moxon, E. R., P. B. Rainey, M. A. Nowak and R. E. Lenski, 1994 Adaptive evolution of highly mutable loci in pathogenic bacteria. Curr. Biol. **4:** 24–33.

Niedenthal, R. K., L. Riles, M. Johnston and J. H. Hegemann, 1996 Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. Yeast **12:** 773–786.

Olson, M. V., D. L. Montgomery, A. K. Hopper, G. S. Page, F. Horodyski *et al.*, 1977 Molecular characterisation of the tyrosine tRNA genes of yeast. Nature **267:** 639–641.

Olson, M. V., K. Loughney and B. D. Hall, 1979 Identification of the yeast DNA sequences that correspond to specific tyrosine-inserting nonsense suppressor loci. J. Mol. Biol. **132:** 387–410.

Paule, M. R., and R. J. White, 2000 Survey and summary: transcription by RNA polymerases I and III. Nucleic Acids Res. **28:** 1283–1298.

Pillus, L., and J. Rine, 1989 Epigenetic inheritance of transcriptional states in *S. cerevisiae*. Cell **59:** 637–647.

Raghuraman, M. K., E. A. Winzeler, D. Collingwood, S. Hunt, L. Wodicka *et al.*, 2001 Replication dynamics of the yeast genome. Science **294:** 115–121.

Rainey, P. B., E. R. Moxon and I. P. Thompson, 1993 Intraclonal polymorphism in bacteria. Adv. Microbiol. Ecol. **13:** 263–300.

Robertson, B. D., and T. F. Meyer, 1992 Genetic variation in pathogenic bacteria. Trends Genet. **8:** 422–427.

Rose, M. D., P. Novick, J. H. Thomas, D. Botstein and G. R. Fink, 1987 A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. Gene **60:** 237–243.

Rose, M. D., F. Winston and P. Hieter, 1990 *Methods in Yeast Genetics: A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Rothstein, R., 1991 Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol. **194:** 281–301.

Rothstein, R. J., R. E. Esposito and M. S. Esposito, 1977 The effect of ochre suppression on meiosis and ascospore formation in Saccharomyces. Genetics **85:** 35–54.

Scheda, R., and D. Yarrow, 1966 The instability of physiological properties used as criteria in the taxonomy of yeasts. Arch. Mikrobiol. **55:** 209–225.

Scheda, R., and D. Yarrow, 1968 Variation in the fermentative pattern of some *Saccharomyces* species. Arch. Mikrobiol. **61:** 310–316.

Sherman, F., 1982 Suppression in the yeast *Saccharomyces cerevisiae*, pp. 463–486 in *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression*, edited by J. N. Strathern, E. W. Jones and J. R. Broach. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sherman, F., G. R. Fink and C. W. Lawrence, 1974 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Singh, A., 1977 Nonsense suppressors of yeast cause osmotic-sensitive growth. Proc. Natl. Acad. Sci. USA **74:** 305–309.

Skovsted, A., 1956 Some unstable mutations in yeast. Compt. Rend. Trav. Lab. Carlsberg Ser. Physiol. **26:** 335–347.

Sokal, R. R., and F. J. Rohlf, 2000 Analysis of frequencies, pp. 685–793 in *Biometry: The Principles and Practice of Statistics in Biological Research*. W. H. Freeman, New York.

Soll, D. R., 1992 High-frequency switching in *Candida albicans*. Clin. Microbiol. Rev. **5:** 182–203.

Soll, D. R., 1997 Gene regulation during high-frequency switching in *Candida albicans*. Microbiology **143** (Pt. 2): 279–288.

Stansfield, I., Akhmaloka and M. F. Tuite, 1995 A mutant allele of the *SUP45* (*SAL4*) gene of *Saccharomyces cerevisiae* shows temperature-dependent allosuppressor and omnipotent suppressor phenotypes. Curr. Genet. **27:** 417–426.

Takeda, S., S. Ichii and Y. Nakamura, 1993 Detection of K-ras mutation in sputum by mutant-allele-specific amplification (MASA). Hum. Mutat. **2:** 112–117.

Tani, Y., T. Kurokui, C. Masaki, M. Hayakawa, K. Ekino *et al.*, 1994 A novel type of life cycle "delayed homothallism" in *Saccharomyces cerevisiae* wy2 showed slow interconversion of mating-type. Biosci. Biotechnol. Biochem. **58:** 2228–2231.

True, H. L., and S. L. Lindquist, 2000 A yeast prion provides a mechanism for genetic variation and phenotypic diversity. Nature **407:** 477–483.

Tuite, M. F., C. R. Mundy and B. S. Cox, 1981 Agents that cause a high frequency of genetic change from [*psi*+] to [*psi*−] in *Saccharomyces cerevisiae*. Genetics **98:** 691–711.

Wach, A., A. Brachat, R. Pohlmann and P. Philippsen, 1994 New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10:** 1793–1808.

Winge, O., 1944 On segregation and mutation in yeast. Compt. Rend. Trav. Lab. Carlsberg Ser. Physiol. **24:** 79–96.

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