Evolution in *Saccharomyces cerevisiae*: Identification of Mutations Increasing Fitness in Laboratory Populations

Victoria M. Blanc[1] and Julian Adams[2]

Department of Biology, University of Michigan, Ann Arbor, Michigan 48109-1048

Manuscript received June 16, 2003
Accepted for publication June 25, 2003

ABSTRACT

Since the publication of the complete sequence of the genome of *Saccharomyces cerevisiae*, a number of comprehensive investigations have been initiated to gain insight into cellular function. The focus of these studies has been to identify genes essential for survival in specific environments or those that when mutated cause gross phenotypic defects in growth. Here we describe Ty1-based mutational approaches designed to identify genes, which when mutated generate evolutionarily significant phenotypes causing small but positive increments on fitness. As expected, Ty1 mutations with a positive fitness effect were in the minority. However, mutations in two loci, one inactivating *FAR3* and one upstream of *CYR1*, identified in evolving populations, were shown to have small but significantly positive fitness effects.

THE number of complete genome sequences that has been published is now large and includes a diverse array of organisms. The genome of *Saccharomyces cerevisiae*, ~12 Mb in size and containing ~6000 protein-coding genes (Goffeau *et al.* 1996), was one of the first to be sequenced completely. Much information on these loci has been gained by comparison of sequences between organisms. However, there is a limit to the amount of biologically important information that can be revealed by such comparisons. Determining the cellular role of each gene in a genome is the logical next step in understanding the functioning of the organism as a whole.

Recently, a number of experimental approaches have been utilized to gain insight into cellular function in *S. cerevisiae*. One strategy that has been utilized has been to analyze the phenotypes resulting from a series of systematically constructed deletions (Oliver 1996; Winzeler *et al.* 1999). Other workers have analyzed the phenotypes of strains bearing gene disruptions systematically constructed by using either Ty1 (Smith *et al.* 1995, 1996) or a mini-Tn*3* transposon (Ross-MacDonald *et al.* 1997, 1999; Thatcher *et al.* 1998; Kumar *et al.* 2000). Many of the strains carrying deleted or disrupted loci did not show any readily observable phenotypic changes (*cf.* Goebl and Petes 1986). Others, however, were either inviable or conditional lethals or exhibited a variety of other deleterious phenotypes. These results allowed predictions to be made concerning the cellular function of a given gene, which could then be tested by further experimentation.

As informative as these studies have been, they were designed to identify mutations with a neutral or deleterious effect on fitness; and they will not necessarily identify evolutionarily significant mutations with small but positive effects on fitness. In this article, we describe and utilize two alternative experimental approaches to identify mutations enhancing fitness. Both approaches use Ty1 transposon tagging to identify adaptive mutations. The first approach relies on spontaneous Ty1 transpositions occurring in an initially genetically homogeneous population during long-term culture (~1000 generations), whereas the second approach relies on the generation of large amounts of genetic variation due to Ty1 transposition, followed by short-term culture (~100 generations) to allow the most fit clone to predominate. These two approaches may be considered to be complementary and possess their own advantages and disadvantages. Non-Ty1-associated adaptive mutations may complicate the analysis of the populations obtained from the first rather than the second approach. However, multiple Ty1 insertions, some with a nonzero fitness effect, will complicate the analysis of the populations obtained from the second approach, but will rarely be a factor in the first.

Using both approaches mutations were identified, one inactivating the *FAR3* locus and one upstream of *CYR1*, which encodes adenylate cyclase. Reconstruction experiments confirmed the adaptive advantage of both mutations.

MATERIALS AND METHODS

Media, growth, and sampling: Batch cultures were grown in 10 ml 1% yeast extract, 2% peptone, and 2% glucose (YPD), at 30°, in a gyratory shaker at 150–200 gyrations/min. Solid YPD medium contained 1.4% agar. When indicated, G418

[1]*Present address:* Asterand Inc., Detroit, MI 48201.

[2]*Corresponding author:* Departments of Molecular, Cellular, and Developmental Biology and Ecology and Evolutionary Biology, 830 N. University, University of Michigan, Ann Arbor, MI 48109-1048. E-mail: julian@umich.edu

TABLE 1

Strains used in this work

Strain	Description	Source
337	*MAT*α *ura3 gal3* zero Ty1 elements	P. Philippsen and M. Ciriacy
CMW101	*MAT*α *ura3 gal3* one Ty1 element, defined in this report as clone A	Wilke and Adams (1992)
X2180-1B	*MAT*α *SUC2 mal mel gal2 CUP1*	Yeast Genetic Stock Center
VB1X2180-1B	*MAT*α *SUC2 mal mel gal2 CUP1 far3*Δ::*Kan*MX	This study
VB2X2180-1B	*MAT*α *SUC2 mal mel gal2 CUP1 leu2*Δ::*Kan*MX	This study

(200 μg/ml) was added after autoclaving. Synthetic complete medium lacking uracil (SC − ura) or leucine (SC − leu) was made as described (Sherman *et al.* 1986), except when galactose was substituted for glucose as the carbon source for induction of pGTyH3 (see below). Cell densities were measured using an electronic particle counter (Coulter, Hialeah, FL). Samples from liquid culture were stored in 40% glycerol at −70°.

Strains: Strains used in this study are listed in Table 1. Clones derived from CMW101 and isolated during the experiment are listed in Table 2. Disruptions of the *LEU2* and *FAR3* loci were generated by transformation with purified amplification products containing the *Kan*MX cassette (Wach *et al.* 1994), with the appropriate ends for homologous recombination. Cells were transformed using the lithium acetate procedure as previously described (Geitz *et al.* 1995), and transformants were selected on YPD plates plus G418, followed by growth in liquid YPD + G418 media. Disruptions of *FAR3* and *LEU2* were confirmed by Southern blotting of *Eco*RI-digested genomic DNA and probed with either the *FAR3* or the *LEU2* open reading frame (ORF). *LEU2* replacements were also tested for their ability to grow on synthetic media with or without leucine. *FAR3* gene disruptions were further tested by Southern blotting of pulsed-field gels and probing with PCR-amplified *Kan*MX cassette DNA to confirm integration in the correct chromosome.

Population construction: Strain 337, containing no Ty1 elements, was transformed using the lithium acetate procedure (Geitz *et al.* 1995), with pGTyH3 (Boeke *et al.* 1985). This plasmid contains a transposition-competent Ty1 element fused to the inducible *GAL1* promoter and the *URA3* selectable marker. Transformants were selected on the basis of their ability to grow on SC − ura. Fifty milliliters of SC − ura medium with galactose as the carbon source was inoculated with ~5×10^5 transformed cells. Cells were incubated for 2–3 days before sampling every 6–12 hr. A portion of each sample was plated to YPD, and the remainder was stored in 40% glycerol at −70°. Analysis of the samples taken at each time point indicated that the sample from the fifth time point contained cells with a small range in the number of Ty1 insertions (0–8). To generate populations used in selection experiments, cells from the frozen stock of time point number five were added directly to 10 ml YPD medium.

TABLE 2

Identification of Ty1 insertions in clones isolated from the long-term serial dilution population

Name of clone	No. of Ty1 insertions	Description of insertions	Observed in generations
A	1	tRNA-Lys	0
B	2	As A and unidentified	197
C	2	As A and LTR cluster	346–546
D	3	As C and σ	346
E	3	As C and unidentified	346–546
F	3	As C and *FAR3* promoter	546–911
G	4	As F and δ	546
H	3	As C and unidentified	546
I	4	As H and unidentified	546
J	4	As F and unidentified	911

Serial dilution cultures and sampling: Competition experiments between *FAR3*$^+$ and *far3* strains were initiated with frequencies of *far3* of ~0.1 or ~0.5. The *far3* strain was isolated from generation 546 of the long-term population described by Wilke and Adams (1992). *FAR3*$^+$ cells were obtained by pooling 19 *FAR3*$^+$ clones isolated from the same generation. Cultures were begun with 1×10^3 cells/ml, grown to ~1×10^8 cells/ml, and transferred to fresh YPD at ~1×10^3 cells/ml. *far3*::Ty1 allele frequency was monitored by PCR amplification of the disrupted *FAR3* locus, using primers to the 3′ ends of Ty1 and the *FAR3* ORF, and/or by Southern blotting, using the *FAR3* ORF as a probe. In experiments where *FAR3* was disrupted by *Kan*MX, changes in the frequency of G418 resistance were estimated by plating samples of appropriate dilutions onto YPD plates and then picking colonies onto YPD plus G418. Relative fitnesses were calculated as described previously (Modi and Adams 1991; Wilke and Adams 1992).

DNA manipulations: Yeast nuclear DNA was isolated using methods previously described (Sherman *et al.* 1986).

PCR: For inverse PCR (Ochman *et al.* 1988), 1 μg genomic DNA was digested with 10 units of either *Nla*III or *Rsa*I (New England Biolabs, Beverly, MA) at 37° for 4–6 hr, with 5 μg RNase A in a 50-μl reaction. The reaction was then terminated by incubation at 65° for 20 min. Digested DNA was incubated under conditions that favored intramolecular ligation to produce circular DNA: 500 ng/ml DNA was incubated with ligation buffer (Promega, Madison, WI), 1 mM ATP, and 1 μl ligase (Promega HC, 20 units/μl), in 800 μl. Ligations were performed overnight at either 16° or room temperature for DNA digested with *Nla*III or *Rsa*I. To facilitate efficient DNA precipitation, completed ligation reactions were split into two tubes, each containing 400 μl of the ligation mixture, 44 μl of 3 M sodium acetate, and 1 ml ethanol. DNA was resuspended in dH_2O at 10 ng/μl.

For flank PCR (Siebert *et al.* 1995), 1 μg genomic DNA was digested with any or all of the following in individual reactions: *Eco*RV, *Nru*I, *Hpa*I, *Dra*I, and *Sca*I. Sequencing adapters were ligated to restriction fragments in a 20-μl reaction including 0.5 μg digested DNA, 100 pmol flanking sequence adapter, 1.5 units T_4 DNA ligase, and 1.5 mM ATP. The se-

quences of the flanking sequence adapter and the primers complementary to it have been described elsewhere (Siebert *et al.* 1995). The Ty1-specific primer 5′-GGAGTGCTCAGAGG CGTTCCAACTGAT GAT-3′ and an adapter-specific primer were used for a first-round PCR amplification. A second round of amplification was performed on a 1:100 dilution of the products in the first, using nested primers complementary to the Ty1 and the adapter. Ty1 nested flank primer has the sequence 5′-GTAAAATGACCAACCAGATGGATTGGCTTGG-3′ and is located 89 bp upstream of the first Ty1 primer. Nested products were analyzed on and excised from a 1% agarose gel and purified using the QIAGEN (Santa Clara, CA) gel extraction kit. In some cases products were cloned into the pCR-XL-TOPO vector available from Invitrogen (Carlsbad, CA).

Amplification was carried out under the following conditions: 100 ng digested and ligated genomic DNA, 50 pmol of primer "VB1" 5′-GATCGTTGATCTACTATCAGTAAG-3′ and 50 pmol primer "VB2" 5′-GAGACTTAGAGATGAAGTATC-3′, 200 μM dNTPs, 1× Taq Polymerase buffer, 2.5 mM $MgCl_2$, and 1 unit Taq Polymerase (Promega) in a 100-μl reaction. Samples were heated in an MJ Research (Waltham, MA) thermal cycler at 94° for 3 min and then cycled 30 times through the following temperature profile: 1 min 94°, 45 sec 42°, 2 min 72°, followed by a final 5-min extension at 72°. PCR products were separated on a 1.5% low-melting-point agarose gel, and bands >630 bp in size were cut out of the gel. Two volumes of water were added to the gel slice, which was then melted at 65° for 20 min, and 5–10 μl was used for a nested PCR. Nested PCR was performed under the same conditions as above, using VB1 and nested primer VB3 5′-AGAACTTCTAG TATATTCTG-3′, which anneals to the Ty1 template 402 bp downstream of VB2. Nested products were gel extracted using either Schleicher and Schuell (Keene, NH) NA45 DEAE cellulose paper or the QIAquick gel extraction kit (QIAGEN). These DNA fragments were subsequently sequenced (see below). The *FAR3* and *ACT1* ORF sequences were amplified from strain 337 using "GenePairs" primers for YMR052W and YFL039C, respectively, available from Research Genetics (Huntsville, AL). PCR was performed under the conditions recommended by the company. YMR052W-reverse primer and primer VB4 5′-GATCTATTACATTATGGGTG-3′, which anneals to a region in the 3′ end of Ty1, were used in experiments to monitor the frequency of the Ty1::*FAR3* allele in reconstruction experiments. Cycling conditions were as described for inverse PCR.

To generate deletion cassettes for *FAR3* and *LEU2*, oligonucleotides were synthesized such that their 5′ ends would generate 60 bp of DNA homologous to the gene being replaced, and the 3′ ends were complementary to the *Kan*MX gene, located on the plasmid pFA6-*Kan*MX4, and used as the template for PCR. The 5′ ends were designed such that the entire genomic ORF would be replaced with *Kan*MX. The primer used for homologous recombination at the 5′ end of *FAR3* has the sequence 5′-CCGGCTAGCATTCGGCGATTAATGAA GAAAGTAAAACCGTGATTTATTACTTCTTGCTCGCAGCT GAAGCTTCGTACGC-3′ and the primer for 3′ end recombination is 5′-AATGCATTCAAGGTTTGCTATTTCACGTCTG CTTACACTTTTGTTCGATCCATCGTAGGGCATAGGCCAC TAGTGGATCTG-3′. To replace *LEU2*, the following primer pair was used: 5′-TTCTAACTTTTCTTACCTTTTACATTTCA GCAATATATATATATATATTTCAAGGATATACAGCTGAA GCTTCGTACGC-3′ and 5′-ACCCTATGAACATATT CCATTT TGTAATTTCGTGTCGTTTCTATTATGAATTTCATTTAGC ATAGGCCACTAGTGGATCTG-3′, which recombined with the 5′ and 3′ ends of the gene, respectively. The underlined sequences anneal to *Kan*MX. PCR conditions were as described (Wach *et al.* 1994).

Cycle sequencing: We used the Perkin Elmer (Norwalk, CT) fluorescent-dye termination kit, following manufacturer's instructions, except that all reactions using 2.5 pmol VB3 were halved. Cycling conditions were: 2 min at 96°, followed by 25 cycles of 96° for 30 sec, 42° for 20 sec, and 60° for 4 min. Products were sequenced using an ABI prism 310 or 377 automated sequencer. Sequenced products were compared to GenBank (http://www.ncbi.nlm.nih.gov/) and/or the *Saccharomyces cerevisiae* Genome Database (SGD; http://genome-www.stanford.edu/Saccharomyces/) using the BLAST algorithm (Altschul *et al.* 1997).

Southern blotting and hybridization conditions: Genomic yeast DNA was isolated as described (Sherman *et al.* 1986) from 5 ml YPD cultures grown overnight. For standard gel electrophoresis, 1–5 μg DNA was digested with 10 units *Eco*RI (Promega, Madison, WI), according to manufacturer's specifications. Restriction fragments were separated on 0.7% agarose gels in 0.5× TBE containing 10 μg/ml ethidium bromide. DNA was transferred to Hybond N+ nylon membrane (Amersham, Piscataway, NJ), using the alkaline transfer method as per the manufacturer's instructions. Prehybridization and hybridization were carried out at 65° in 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7% sodium dodecyl sulfate (SDS; Church and Gilbert 1984). After hybridization, membranes were washed at 65°, first in 5× SSC, 0.1% SDS, two times; followed by two washes each in 2× SSC, 0.1% SDS and 0.5× SSC, 0.1% SDS. When necessary, blots were stripped by washing in boiling 0.5% SDS and allowed to cool to room temperature before subsequent use. Autoradiographs were exposed overnight at −70°, with intensifying screens. A 3.5-kbp fragment of Ty1 ("probe 2"; Wilke and Adams 1992) was used for detection of Ty1 sequences; *FAR3* sequences were detected using a probe consisting of the *FAR3* ORF amplified from strain 337, using the appropriate "gene pair" primers. All probes were labeled with [α-^{32}P]dATP, by random priming, using a kit by Promega.

Northern analysis: Yeast cells were grown to midlog phase and total RNA was isolated as previously described (Maddock *et al.* 1996) or by using a kit by Gentra Systems (Minneapolis, MN). Poly(A)$^+$ RNA was isolated using a kit from Promega. All solutions were made with diethylpyrocarbonate-treated water to protect against nucleases (Sambrook *et al.* 1989). Twelve micrograms total RNA or 1.5 μg poly(A)$^+$ RNA was loaded onto a 1% agarose gel containing 3.3% formaldehyde and 20 mM sodium phosphate and run at 90–100 V for 1–2 hr. The gel running buffer was 10 mM sodium phosphate, 2.8% formaldehyde. RNA was transferred to Hybond N+ (Amersham) via capillary action using 25 mM sodium phosphate as the transfer buffer. The blot was then baked at 80° for 1–2 hr and then exposed to UV light for 1–3 min. Prehybridization, hybridization, and washes were the same for Northerns as for Southerns, except that the blot was prehybridized for 2–4 hr. Blots were probed using the *FAR3, CYR1*, and *ACT1* ORFs, generated as described above.

RESULTS

Two different approaches were used to search for mutations that increased fitness under our conditions.

Analysis of a population maintained for ~1000 generations: In work described by Wilke *et al.* (1992), a population of *S. cerevisiae* inoculated with a clone of strain CMW101 containing a single active genomic Ty1 element was maintained in serial dilution in rich medium for ~1000 generations. Samples were taken for analysis every 200 generations, and the changes in Ty1 number were monitored by hybridization of a Ty1-specific probe

TABLE 3

Relative fitnesses estimated from reconstruction experiments

Strain	No. of replicates	Fitness ± SE relative to wild type (unity)[a]
Clone F (FAR3⁻)	5	1.0149 ± 0.0074
*far3*Δ::*Kan*MX	11	1.0204 ± 0.0021
*leu2*Δ::*Kan*MX	8	0.9972 ± 0.0034

[a] See text for details.

to yeast genomic DNA, and the differences were compared between the clones. Over the course of the experiment a small but significant increase in Ty1 number/genome was observed. The loci of insertions of the Ty1 elements were determined by Ty1-specific inverse PCR (Ochman *et al.* 1988) or by flank PCR (Siebert *et al.* 1995), followed by direct sequencing of the PCR products, and subsequent comparison of the sequence information obtained to the SGD. Many of the Ty1 insertions were present in the population for only a short period of time and were insertions into σ, δ, or τ elements (Blanc 2000). Consequently, there was little *a priori* evidence that they conferred an adaptive advantage to the cells harboring them. However, one clone, designated clone F, first identified at generation 546 persisted in the population until the termination of the experiment at generation 911. Additionally, all other clones present at generation 546 had disappeared from the population by generation 746. This evidence taken together suggested that this clone was selectively favored in comparison to other clones present in the population during that period. Accordingly, the clone containing this insertion was isolated for further study.

To determine if this clone possessed a selective advantage relative to other clones coexisting in the population at the time it was identified, competition experiments were initiated to reconstruct the changes occurring in the population. Clone F was inoculated in 10 ml YPD at frequencies of either 0.10 or 0.50. The remainder of the population was composed of 19 other clones in equal frequencies, isolated from the population at generation 546, the generation sample at which clone F was first identified. In all replicates, the frequency of clone F increased significantly. The average fitness of clone F was 1.0149 ± 0.0074 (relative to 1 for the other 19 clones) and was independent of the initial frequency of clone F (Table 3). Thus, clone F possesses a small, but significant growth advantage over other clones isolated from the sample taken at generation 546. Furthermore, there is no evidence that this selective advantage is dependent on the frequency of clone F in the population.

Clone F possessed three Ty1 elements whose order of insertion can be traced from the population history. The first insertion was identified near tRNA-Lys, and the second is located within a cluster of LTRs, while the third is found upstream of the *FAR3* locus, in the 5′ noncoding region of *FAR3*, at −35 relative to the translation start site and in the same transcriptional orientation (Figure 1). Upstream from *FAR3* is a tRNA-Trp gene and several solo δ elements indicative of past Ty insertions. Considering the location of the three insertions, and that clones containing only the tRNA-Lys and composite LTR insertions were lost from the population, the results suggest that the most likely cause of the selective advantage observed is the third insertion—upstream of *FAR3*.

FAR3 is expressed constitutively in *a*- and α-haploid and diploid cells and is required for pheromone-mediated cell cycle arrest (Horecka and Sprague 1996). The position and orientation of the Ty1 insertion upstream from *FAR3* suggests that the expression of *FAR3* is attenuated or even eliminated (see Figure 1). Analysis of the phenotype of clone F revealed that it is insensitive to pheromone-mediated cell cycle arrest, consistent with the loss or reduction of FAR3 expression. We therefore assayed the expression of *FAR3* by Northern analysis. As expected, there was no detectable expression of *FAR3* in clone F, whereas 337, the strain used to inoculate the population, showed normal *FAR3* expression levels (data not shown).

Production of genetic variation for Ty1 transposition events: Strain 337, transformed with pGTyH3 (see materials and methods), was grown in liquid SC − ura media with galactose as the carbon source, to induce transposition. After ∼2.5 generations of growth, the average number of Ty1 insertions was 2.0 ± 2.3 including clones containing zero Ty elements (∼25%) and those containing up to eight insertions. The short duration of growth in galactose medium ensured that the overwhelming majority of the genetic variation was generated by Ty1 insertion.

Each clone possessing one or more Ty elements exhibited a different Ty banding pattern, indicative of a high level of variability for Ty1 insertion sites. Three populations were then inoculated with ∼140 cells in 10 ml YPD and grown overnight. The following day, each population was split evenly into two flasks and allowed to reach stationary phase, when the cells were diluted to fresh medium at ∼1000 cells/ml in 10 ml. This process of serial dilution was repeated until 120–130 generations of growth had elapsed. Colonies were then sampled and assayed for Ty1 pattern by Southern blotting. In all three populations, the same clone type predominated in both replicates, indicating the presence of an adaptively favored clone at the start of the experiment.

To confirm that these clones possessed an increased fitness compared to the progenitor strain 337, reconstruction competition experiments were initiated with 337 and clones containing the same Ty1 insertions (as determined by hybridization profiles) isolated from the earliest samples available. The use of such clones minimized the confounding of any Ty1-associated selective effect with that of any potential spontaneous non-Ty1-

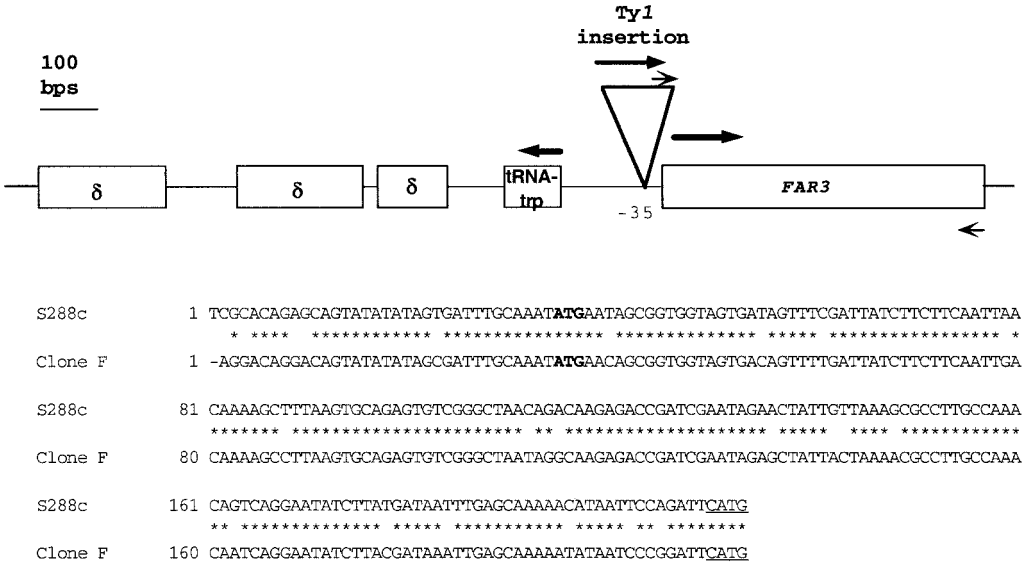


FIGURE 1.— Ty1 insertion upstream of *FAR3*, in clone F isolated from the long-term serial dilution population. Structure of chromosome VIII in S288c in the *FAR3* region and Ty1 insertion in clone F. Ty1 insertion is not to scale. Arrows indicate direction of transcription. The nucleotide sequence of the *FAR3* fragment generated from inverse PCR is compared to the same region in S288c. Translation start sites in the two sequences are in boldface type, and the *Nla*III sites are underlined. Thick arrows indicate direction of transcription and thin arrows show primer binding sites for PCR amplification of the Ty1::*far3* allele. δ, δ elements.

associated adaptive mutations, which may have occurred during the 120−130 generations of growth. As expected, in every replicate, the frequency of the clone containing the Ty1 insertions increased over time. Thus, the data indicate that the clones predominating in the populations were selectively favored compared to their progenitor 337. In the case of population II, the predominating clone possessed only one Ty1 insertion.

Table 4 shows the loci of insertions of the Ty1 elements in the three populations. The predominant clone in population I possessed seven Ty1 elements. Of these, five were sequenced, and three were located near tRNA genes. One insertion matched nothing in the database. The last insertion identified was located at +180 bp from the ATG codon of the *FAR3* gene (Figure 2). The fitness of this clone (relative to a fitness of 1 for strain 337) was estimated, from reconstruction experiments, to be 1.0123 ± 0.0019.

The predominant clone in population II possessed only one element located 512 bp upstream of *CYR1*, which encodes adenylate cyclase (CASPERSON *et al.* 1985; KATAOKA *et al.* 1985). The fitness of this clone (relative to a fitness of 1 for strain 337) was estimated, from reconstruction experiments, to be 1.0340 ± 0.0040.

The predominant clone in population III possessed three Ty1 elements, of which one was also located upstream of the *CYR1* locus. However, Southern analysis indicated that the location of the insertion in population II upstream of *CYR1* was different from that seen in population III and was located closer to the *CYR1* start codon. The fitness of this clone (relative to a fitness of 1 for strain 337) was estimated, from reconstruction experiments, to be 1.0355 ± 0.0173.

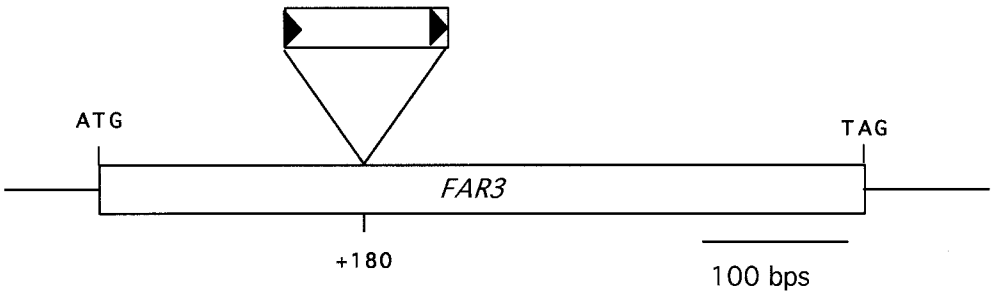
Molecular analysis of *FAR3* and *CYR1*: Given that the *FAR3* locus in population I is disrupted by a Ty1 insertion, it seemed probable that its expression was lost. Analysis of the expression of *FAR3*-specific mRNA in population I by Northern blotting showed the expression of a shorter mRNA, as compared to that in 337 (data not shown). This lower molecular weight mRNA can be attributed to expression from the intact *FAR3* promoter through to the Ty1. Translation of this mRNA would produce a protein consisting of the first 60 amino acids of Far3p, followed by 11 amino acids encoded fortuitously by the LTR of Ty, until a stop codon is reached. Truncations of Far3p after codon 122 have been shown to be inactive (HORECKA and SPRAGUE 1996). Therefore, any protein translated from this smaller mRNA is unlikely to be functional.

TABLE 4

Locus of Ty1 insertions in predominating clones after selection

Population	No. of Ty1 insertions	Identified mutations
I	7[a]	+180 bp of *FAR3* δ-tRNA-Arg, CHX δ-tRNA-Leu, CHIII δ-tRNA-Gly, chromosome unknown No match to database
II	1	−512 bp *CYR1* and +130 bp upstream of tRNA-Leu
III	3	tRNA-Thr, CHVII σ 5′-*CYR1*

[a] Only five of the seven insertions were identified.




```
1    ATG AAC AGC GGT GGT AGT GAC AGT TTT GAT TAT CTT CAA CAA TTG ACA
      M   N   S   G   G   S   D   S   F   D   Y   L   Q   Q   L   T

49   AAA GCC TTA AGT GCA GAG TGT CGG GCT AAT AGG CAA GAG ACC GAT CGA
      K   A   L   S   A   E   C   R   A   N   R   Q   E   T   D   R

98   ATA GAG CTA TTA CTA AAA CGC CTT GCC AAC AAT CAG GAA TAT GCT TAC
      I   E   L   L   L   K   R   L   A   N   N   Q   E   Y   A   Y

146  GAT CAA TTG AGC AAA AAT ATA ATC CCG GAT TCA TGG TGT TGG AAT AGA
      D   Q   L   S   K   N   I   I   P   D   S   W   C   W   N   R

193  AAT CAA CTA TCA TCT ACT AAC TAG TAT TTA CAT TAC TAG TAT ATT ATC
      N   Q   L   S   S   T   N   *   Y   L   H   Y   *   Y   I   I

242  ATA TAC GGT GTT AGA AGA TGA CGC AAA TGA TGA
      I   Y   G   V   R   R   *   R   K   *   *
```

FIGURE 2.—Ty1 insertion into *FAR3* in the clone isolated from population I, genetically variable for Ty1 insertions (see text for details). Insertion occurs after nucleotide 180. The amino acid sequence of *FAR3* translated from the DNA sequence is shown below the diagram. The sequence includes the site of Ty1 insertion, as well as part of the Ty1 sequence, shown in boldface type. Asterisks represent stop codons.

Since loss of *CYR1* function has been shown to be lethal (*e.g.*, MORISHITA *et al.* 1993), loss of expression similar to that seen for *FAR3* was not expected. However, Northern analysis of exponential-phase cultures did not reveal any gross differences in *CYR1* mRNA levels between 337 and the mutant strains (data not shown). This could be due to changes in the amount of *CYR1* mRNA, of a magnitude too small to be detected under our experimental conditions. In addition, the strains possessing the Ty1 insertions upstream of *CYR1* did not exhibit phenotypes characteristic of strains with altered levels of adenylate cyclase, namely altered thermotolerance and altered growth characteristics on a nonfermentable carbon source such as glycerol.

Selective effect of loss of *FAR3* expression: To confirm that the observed increase in fitness was due to the loss of *FAR3* expression, rather than to a different spontaneous mutation, competition experiments were performed with a pair of strains isogenic except for a deletion of the *FAR3* locus. A deletion of *FAR3* was constructed in the standard laboratory strain X2180 using the *Kan*MX deletion cassette (WACH *et al.* 1994). Deletion of the *FAR3* locus in G418-resistant transformants was confirmed by genomic DNA digestion followed by Southern blotting, as well as by Southern blotting of whole-chromosome preparations separated in a pulsed-field gel. In both cases, no hybridization was seen using a *FAR3*-specific probe (data not shown).

As before, competition experiments were initiated with two initial frequencies of the *FAR3* deletion strain, 0.10 and 0.50. In all replicates, an increase in the relative frequency of cells resistant to G418 was observed. The relative fitness of the *far3*Δ strain was 1.0204 ± 0.0021 (Table 3). The increment in fitness observed was not significantly different from that observed for clone F (Table 3).

To confirm that that the selective difference observed was due to the loss of *FAR3* and not to the presence of the *Kan*MX cassette, similar competition experiments were performed using a strain in which the *LEU2* gene had been replaced with the *Kan*MX cassette. Previous experiments had indicated that *leu2* auxotrophs had no selective advantage in rich medium. Deletion of the *LEU2* locus was confirmed by Southern blotting of genomic DNA digests using a *LEU2*-specific probe. No hybridization was observed in G418-resistant transformants. In addition, these cells were unable to grow on medium lacking leucine, but were capable of growth when leucine was supplied. As expected, the fitness of the *leu2*Δ::*Kan*MX strain was not significantly different from that of the *LEU2*$^+$ parent strain (X2180; Table 3).

DISCUSSION

Ty1 is the most abundant of the yeast transposable elements, present in >30 full-length copies in laboratory strains. The effects of Ty1 transposition can be deleterious, neutral, or beneficial. Arguments for the neutrality

of Ty1 elements come from the observation of their abundance in yeast; laboratory strains show no apparent harmful effects of having such high numbers, although in wild strains the average Ty1 number is somewhat lower (Wilke *et al.* 1992). Nevertheless, in studies where Ty1 numbers were doubled in laboratory strains, or greatly increased in strain 337, growth rates and stationary-phase cell densities were reduced (Boeke *et al.* 1991; Wilke and Adams 1992), indicative of an overall negative effect of insertions in those strains.

A number of studies have systematically analyzed the genome of *S. cerevisiae* to identify genes that when mutated or deleted have a deleterious or lethal effect. The focus of this communication was to identify genes that when mutated enhance fitness. Our results show that the experimental design allows us to identify mutations that have a quite small, but significant beneficial effect—on the order of 2%. It is also clear from the results that such mutations constitute a small proportion of the total mutational spectrum, as the selective effects of a large number of Ty1 insertions were assayed as the populations evolved. Ty1 transpositions occur at a rate of 10^{-5}–10^{-7}/element/cell division (Curcio and Garfinkel 1991). The population maintained in serial dilution for ~1000 generations ranged in cell number from 10^4 immediately after transfer to fresh medium to 10^9 at stationary phase. Therefore, at a minimum, there was less than one new transposition event per generation, and at a maximum, there were 10,000 for each element. Consequently, between 1000 and 1×10^7 Ty1-induced mutations occurred during this time. Nevertheless, beneficial mutations were seen at only two loci. Moreover, mutations at the same two loci were seen in independent populations, providing further evidence that there are a limited number of loci at which beneficial Ty1-induced mutations may occur.

Our study does not permit us to identify Ty1 mutations that have a deleterious or lethal effect on fitness. However, our results indicate that the majority of the Ty1-induced mutations identified in this work (Blanc 2000) have no selective effect. A number of studies have shown that Ty1 displays a target site preference inserting preferentially into regions of duplicated DNA. Thus, genomic analysis of Saccharomyces and the positions of all Ty elements showed that 90% of Ty1 elements are found within 750 bases of tRNA genes or other genes transcribed by RNA polymerase III (Kim *et al.* 1998). Experimental analysis of Ty1 insertions on chromosome III showed that most Ty insertions occur in regions containing tRNA-coding regions and/or LTRs of preexisting retrotransposons (Ji *et al.* 1993). In the studies reported here, a large proportion of the identified insertions were found in or near LTRs or tRNA genes (80%). The neutrality of Ty1-mediated mutations in such regions will be the subject of a separate communication (Blanc and Adams 2003).

One insertion was identified in the 5′ region of the *FAR3* gene. This is consistent with the observation that Ty1 target site preference is not exclusive to tRNAs and LTRs. In fact, when Ty1 is observed in or near protein-encoding genes, it shows another level of targeting to the 5′ regions (Eibel and Philippsen 1984; Natsoulis *et al.* 1989; Wilke *et al.* 1989).

Mutations in *FAR3* enhance fitness: Our results show unambiguously that mutations inactivating *FAR3* are selected in laboratory culture. *FAR3* was previously identified as a gene required for pheromone-mediated G1 arrest (Horecka and Sprague 1996). G1 arrest is essential for mating cells to ensure that they are synchronized in their cell cycles upon fusion to form a diploid cell. Activation of Fus3p and Kss1p, two MAP kinases, is coupled to G1 arrest at "start," the commitment phase of the cell cycle (Sprague and Thorner 1993). Fus3p and Kss1p also promote other responses required for mating, including activation of the Ste12p transcription factor, "shmoo" formation, and signal attenuation (Elion *et al.* 1991; Ma *et al.* 1995; Gartner *et al.* 1998; Cherkasova *et al.* 1999; Farley *et al.* 1999). Together these two proteins have been shown to inhibit G1 cyclin expression and promote recovery from G1 arrest via mechanisms distinct for each protein (Cherkasova *et al.* 1999). These authors also suggest that in addition to repressing G1 cyclin gene expression, Fus3p and Kss1p may have additional targets of negative control, including perhaps Far3p. It is also conceivable that Far3p functions as a "fine-tuning" molecule in the G1 to S-phase transition, together with Pho85p-Pcl1/2p (Horecka and Sprague 1996), or another protein/protein complex. In this regard it is noteworthy that in two large-scale two-hybrid screens, interactions of FAR3 with three other proteins, YKE2, YFR008W, and YDR200C, were seen (Uetz *et al.* 2000) and have been reported to be associated in a complex with 12 other proteins (Gavin *et al.* 2002).

Our results show that *FAR3* may operate in a more general cell arrest pathway, particularly since it is expressed constitutively through the cell cycle in both *a* and α cells (Horecka and Sprague 1996). As cells enter stationary phase or perceive low nutrient concentrations, cell division is arrested. In serial dilution or in continuous (chemostat) culture, FAR^+ cells would effectively arrest at G1, whereas cells mutant for *FAR3* would not. Thus, $Far3^-$ cells would possess a selective advantage, and would increase in frequency in the population.

Mutations 5′ to CYR1 enhance fitness: Three lines of evidence point to a selective advantage of mutations upstream of *CYR1*.

i. Clones containing two different mutations upstream of *CYR1* were selected in two independent populations constructed so that the overwhelming majority of variation was due to Ty1 insertion.
ii. One clone possessed only one Ty1 insertion—upstream of *CYR1*. Consequently, the only alternative explanation for the predominance of this clone would be a

non-Ty1-based adaptive mutation—which can be considered to be unlikely given the experimental design.

iii. The fitness increments associated with the two clones possessing Ty1 insertions upstream of *CYR1* possessed the same fitnesses, relative to the parent strain 337: 0.040 (population II) and 0.055 (population III). The clone selected in population III contained, in addition to a Ty1 insertion upstream of *CYR1*, two other insertions, one near a tRNA locus and one within a σ element. However, the results from independent experiments have shown that such insertions have no significant selective effect and thus can be considered neutral (Blanc 2000).

Our results provide no indication that the Ty1 insertions upstream of *CYR1* alter the expression of this locus. Indeed, the distance between the locus of one of the insertions and the *CYR1* start codon, 512 bp, renders this possibility quite unlikely. Nevertheless, it is tantalizing to speculate that such insertions have effects on *CYR1* expression that are too subtle to be detected by our assays, as previous work by Iida (1988) showed that Ty1 insertions into the promoter region of *CYR1* confer multistress resistance (Thevelein and De Winde 1999)—providing the cells with a survival advantage under certain conditions. An alternative explanation is that the insertions upstream of *CYR1* have targeted an ORF upstream, which has not yet been identified. In this regard, minitransposon insertions ~130 bp upstream of the *CYR1* start codon indicate the existence of a previously unidentified ORF in this region (A. Kumar, personal communication).

In conclusion, the work presented here demonstrates how Ty1 transposon tagging may be used to identify fitness-enhancing mutations. Two such mutations have been identified: in one case, identifying a novel phenotype associated with a previously characterized mutation; and in the second, a possible new ORF, which has not been previously characterized. In this article we employed populations maintained in large volumes (10–150 ml). However, the same experimental procedures could conceivably be carried out using much smaller volumes, in microtiter plates, thus allowing for a much larger-scale screening for adaptive mutations.

We thank M. Blot, J. Maddock, L. Olsen, M. Savageau, and C. Wilke for helpful discussion. This work was supported in part by National Institutes of Health (NIH) grant AI55756. V.M.B. acknowledges support of NIH training grants NIH 5 T32 GM07544 and NIH 5-T32 HG00040.

LITERATURE CITED

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang *et al.*, 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389–3402.

Blanc, V. M., 2000 Effects of retrotransposon Ty1 on fitness in *Saccharomyces cerevisiae*. Ph.D. Thesis, University of Michigan, Ann Arbor, MI.

Blanc, V. M., and J. Adams, 2003 Ty1 insertions in intergenic regions of the genome of *Saccharomyces cerevisiae* transcribed by RNA polymerase III have no detectable selective effect. FEMS Yeast Res. (in press).

Boeke, J. D., D. J. Garfinkel, C. A. Styles and G. R. Fink, 1985 Ty elements transpose through an RNA intermediate. Cell **40:** 491–500.

Boeke, J. D., D. J. Eichinger and G. Natsoulis, 1991 Doubling Ty1 element copy number in *Saccharomyces cerevisiae*: host genome stability and phenotypic effects. Genetics **129:** 1043–1052.

Casperson, G. F., N. Walker and H. R. Bourne, 1985 Isolation of the gene encoding adenylate cyclase in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **82:** 5060–5063.

Cherkasova, V., D. M. Lyons and E. A. Elion, 1999 Fus3p and Kss1p control G1 arrest in *Saccharomyces cerevisiae* through a balance of distinct arrest and proliferative functions that operate in parallel with Far1p. Genetics **151:** 989–1004.

Church, G. M., and W. Gilbert, 1984 Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:** 1991–1995.

Curcio, M. J., and D. J. Garfinkel, 1991 Single-step selection for Ty1 element retrotransposition. Proc. Natl. Acad. Sci. USA **88:** 936–940.

Eibel, H., and P. Philippsen, 1984 Preferential integration of yeast transposable element Ty into a promoter region. Nature **307:** 386–388.

Elion, E. A., J. A. Brill and G. R. Fink, 1991 FUS3 represses *CLN1* and *CLN2* and in concert with *KSS1* promotes signal transduction. Proc. Natl. Acad. Sci. USA **88:** 9392–9396.

Farley, F. W., B. Satterberg, E. J. Goldsmith and E. A. Elion, 1999 Relative dependence of different outputs of the *Saccharomyces cerevisiae* pheromone response pathway on the MAP kinase Fus3p. Genetics **151:** 1425–1444.

Gartner, A., A. Jovanovic, D. I. Jeoung, S. Bourlat, F. R. Cross *et al.*, 1998 Pheromone-dependent G1 cell cycle arrest requires Far1 phosphorylation, but may not involve inhibition of Cdc28-Cln2 kinase, *in vivo*. Mol. Cell. Biol. **18:** 3681–3691.

Gavin, A.-C., M. Bösche, R. Krause, P. Grandi, M. Marzioch *et al.*, 2002 Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature **415:** 141–147.

Geitz, 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.

Goebl, M. G., and T. D. Petes, 1986 Most of the yeast genomic sequences are not essential for cell growth and division. Cell **46:** 983–992.

Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon *et al.*, 1996 Life with 6000 genes. Science **274:** 546, 563–567.

Horecka, J., and G. F. Sprague, Jr., 1996 Identification and characterization of *FAR3*, a gene required for pheromone-mediated G1 arrest in *Saccharomyces cerevisiae*. Genetics **144:** 905–921.

Iida, H., 1988 Multistress resistance of *Saccharomyces cerevisiae* is generated by insertion of retrotransposon Ty into the 5′ coding region of the adenylate cyclase gene. Mol. Cell. Biol. **8:** 5555–5560.

Ji, H., D. P. Moore, M. A. Blomberg, L. T. Braiterman, D. F. Voytas *et al.*, 1993 Hotspots for unselected Ty1 transposition events on yeast chromosome III are near tRNA genes and LTR sequences. Cell **73:** 1007–1018.

Kataoka, T., D. Broek and M. Wigler, 1985 DNA sequence and characterization of the *S. cerevisiae* gene encoding adenylate cyclase. Cell **43:** 493–505.

Kim, J. M., S. Vanguri, J. D. Boeke, A. Gabriel and D. F. Voytas, 1998 Transposable elements and genome organization: a comprehensive survey of retrotransposons revealed by the complete *Saccharomyces cerevisiae* genome sequence. Genome Res. **8:** 464–478.

Kumar, A., K. H. Cheung, P. Ross-Macdonald, P. S. Coelho, P. Miller *et al.*, 2000 TRIPLES: a database of gene function in *Saccharomyces cerevisiae*. Nucleic Acids Res. **28:** 81–84.

Ma, D., J. G. Cook and J. Thorner, 1995 Phosphorylation and localization of Kss1, a MAP kinase of the *Saccharomyces cerevisiae* pheromone response pathway. Mol. Biol. Cell **6:** 889–909.

Maddock, J. R., J. Roy and J. L. Woolford, Jr., 1996 Six novel genes necessary for pre-mRNA splicing in *Saccharomyces cerevisiae*. Nucleic Acids Res. **24:** 1037–1044.

Modi, R. I., and J. Adams, 1991 Coevolution in bacterial-plasmid populations. Evolution **45:** 656–667.

Morishita, T., A. Matsuura and I. Uno, 1993 Characterization of the *cyr1–2* UGA mutation in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **237:** 463–466.

Natsoulis, G., W. Thomas, M. C. Roghmann, F. Winston and J. D. Boeke, 1989 Ty1 transposition in *Saccharomyces cerevisiae* is nonrandom. Genetics **123:** 269–279.

Ochman, H., A. S. Gerber and D. L. Hartl, 1988 Genetic applications of an inverse polymerase chain reaction. Genetics **120:** 621–623.

Oliver, S., 1996 A network approach to the systematic analysis of yeast gene function. Trends Genet. **12:** 241–242.

Ross-Macdonald, P., A. Sheehan, G. S. Roeder and M. Snyder, 1997 A multipurpose transposon system for analyzing protein production, localization, and function in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **94:** 190–195.

Ross-MacDonald, P., P. S. R. Coelho, T. Roemer, S. Agrawal, A. Kumar *et al.*, 1999 Large-scale analysis of the yeast genome by transposon tagging and gene disruption. Nature **402:** 413–418.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sherman, F., G. R. Fink and J. B. Hicks, 1986 *Laboratory Course Manual for Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Siebert, P. D., A. Chenchik, D. E. Kellogg, K. A. Lukyanov and S. A. Lukyanov, 1995 An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Res. **23:** 1087–1088.

Smith, V., D. Botstein and P. Brown, 1995 Genetic footprinting: a genomic strategy for determining a gene's function given its sequence. Proc. Natl. Acad. Sci. USA **92:** 6479–6483.

Smith, V., K. Chou, D. Lashkari, D. Botstein and P. Brown, 1996 Functional analysis of the genes of yeast chromosome V by genetic footprinting. Science **274:** 2069–2074.

Sprague, Jr., G. F., and J. W. Thorner, 1993 Pheromone response and signal transduction during the mating process of *Saccharomyces cerevisiae*, pp. 657–744 in *The Molecular and Cellular Biology of the Yeast Saccharomyces*, edited by E. Jones, J. Pringle and J. Broach. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Thatcher, J. W., J. M. Shaw and W. J. Dickinson, 1998 Marginal fitness contributions of nonessential genes in yeast. Proc. Natl. Acad. Sci. USA **95:** 253–257.

Thevelein, J. M., and J. H. de Winde, 1999 Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. **33:** 904–918.

Uetz, P., L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson *et al.*, 2000 A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature **403:** 623–627.

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.

Wilke, C. M., and J. Adams, 1992 Fitness effects of Ty transpositions in *Saccharomyces cerevisiae*. Genetics **131:** 31–42.

Wilke, C. M., S. H. Heidler, N. Brown and S. W. Liebman, 1989 Analysis of yeast retrotransposon Ty insertions at the *CAN1* locus. Genetics **123:** 655–665.

Wilke, C. M., E. Maimer and J. Adams, 1992 The population biology and evolutionary significance of Ty elements in *Saccharomyces cerevisiae*. Genetica **86:** 155–173.

Winzeler, E. A., D. D. Shoemaker, A. Astromoff, H. Liang, K. Anderson *et al.*, 1999 Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science **285:** 901–906.

Communicating editor: H. Ochman