Nonsense Mutations in Essential Genes of *Saccharomyces cerevisiae*

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ABSTRACT

A new method for isolating nonsense mutations in essential yeast genes has been used to develop a collection of 115 ochre mutations that define 94 complementation groups. The mutants are isolated in a genetic background that includes an ochre suppressor on a metastable plasmid and a suppressible colony-color marker on a chromosome. When the parental strain is plated on a rich medium, the colonies display a pattern of red, plasmid-free sectors on a white background. Mutants containing an ochre mutation in any essential yeast gene give rise to nonsectoring, white colonies, since cell growth is dependent on the presence of the plasmid-borne suppressor. Analysis of the data suggests that mutations are being recovered from a pool of approximately 250 genes.

STUDIES of essential genes in yeast have relied almost entirely on the isolation of temperature-conditional lethal mutations or on the creation of disrupted genes by DNA transformation. While these two methods have allowed genetic access to a large number of essential functions in yeast, there remains a need for alternative methods to discover new genes that are required for cell growth. Several lines of evidence suggest that many essential genes are not represented in existing collections of temperature-conditional lethals. For example, when MOIR *et al.* (1982) analyzed a series of cold-sensitive lethals containing cell division cycle (*CDC*) defects, they found few genes in common with the set of over 50 *CDC* genes that had previously been detected using temperature-sensitive (*ts*) lethals (PRINGLE and HARTWELL 1981). On the other hand, recent searches for new *ts* lethals have yielded few new genes. For example, all 32 members of a set of *ts* lethals recovered on chromosome *I* by KABACK *et al.* (1984) fell into three previously known complementation groups; no mutations were recovered in at least two other genes on chromosome *I* that are known to be essential.

In principle, the search for new essential genes could rely on recovery of unconditional recessive lethals in diploids, rather than on further collection of haploid temperature-conditional mutants. Because of its potential for associating a gratuitous genetic and physical marker with the lethal mutation, gene disruption—induced by the homology-directed integration of random DNA sequences cloned into a suitable transformation vector—is an attractive method of creating recessive-lethal mutations. This approach was illustrated in a recent study by GOEBL and PETES (1986), which demonstrated that about 10% of random integration events generate recessive-lethal mutations. Gene disruption is presently most useful, however, for testing whether or not a particular cloned gene is essential for growth (SHORTLE, HABER and BOTSTEIN 1982). In a major search for new genes, much of the power of haploid yeast genetics would be sacrificed in this method because of the need to propagate the mutants as diploids.

A third strategy in the search for new essential genes would be to rely on nonsense mutations. Nonsense mutations can arise in any protein-coding gene at many sites, and they usually cause tight null phenotypes. Despite these attractive properties, nonsense mutations have only been widely used to define essential genes in bacteriophage (for example, see STUDIER 1969, 1972). In bacteriophage systems, the lethality associated with a nonsense mutation in an essential gene can be expressed or suppressed simply by changing hosts. There is no straightforward counterpart to this device when manipulating nonsense mutations in essential cellular genes, although a variety of alternative strategies have been explored. In yeast, for example, RAI and CARTER (1981) isolated 33 *CDC* amber mutants by recovering *ts* lethals in a genetic background that contained a *ts* amber suppressor and then by screening these mutants for cases in which the *ts* lethality co-segregated with the suppressor. REED (1980) isolated amber mutations in two yeast *CDC* genes, but the method employed depended on the prior availability of a *ts* mutation in the target gene.

In this report, we describe a general technique for isolating nonsense mutations in essential yeast genes. We have established the technique's practicality by isolating 115 mutants, which define 94 complementation groups. The mutants are isolated starting with a strain that contains an ochre suppressor on a CEN plasmid (CLARKE and CARBON 1980). CEN plasmids are lost during colony formation at a frequency of

approximately 3% per generation in typical host strains (MAINE, SINHA and TYE 1984). Plasmid loss is readily monitored by colony color in hosts containing the *ade2-1* ochre mutation: in the presence of a strong ochre suppressor, colonies are white (wild type), while in the absence of a suppressor, they are red. Colonies grown from a cell containing an ochre suppressor on a CEN plasmid in an *ade2-1* ochre background display a striking pattern of red and white sectors (SHAW and OLSON 1984; HIETER *et al.* 1985). When a lethal ochre mutation is present in a chromosomal gene, cells can grow only if they retain the suppressor-bearing plasmid; consequently, the mutant cell clones give rise to white nonsectoring colonies. OLIVER and BECKWITH (1982) have described a related method, which also depends on a genetically unstable nonsense suppressor, for recovering lethal nonsense mutations in *E. coli*.

MATERIALS AND METHODS

Strains: Two main genetic backgrounds were used in this study (Table 1). Despite their superficial similarity, ochre suppressors behave quite differently in the two backgrounds, probably because of the action of ψ or some closely related cytoplasmic modifier of suppressor phenotypes. AB1610 and AB1620 are meiotic progeny of W87 (ROTHSTEIN, ESPOSITO and ESPOSITO 1977), which has been used in a number of studies of nonsense suppressors derived from the *tRNA*^{Tyr} genes (ROTHSTEIN 1977, 1979; KURJAN and HALL 1982). AB1375 and AB1380 are sister spore strains constructed for this study in a series of 20 crosses that introduced a variety of genetic backgrounds. AB1377 was derived from AB1375 by transformation with the *SUP4-o* CEN3 *TRP1* plasmid, pC689$_{UAA}$ (SHAW and OLSON 1984). AB1400 is related to AB1375 and AB1380. Experiments were carried out on standard media (SHERMAN, FINK and HICKS 1986) at 30°.

The W87-derived strains sporulate quickly and abundantly, tolerate the presence of ochre suppressors well and are easily transformed with pC689$_{UAA}$. However, a relatively high proportion of plasmid-bearing colonies do not exhibit red sectors, which gives rise to a background of false positives in mutant screens. In contrast, the diploid formed by mating AB1375 and AB1380 sporulates rather poorly. Plasmid-bearing colonies nearly always produce multiple red sectors in these strains, giving rise to few false positives when screening for mutants. Several efforts to transform AB1375 with pC689$_{UAA}$ produced a single haploid transformant, AB1377, while all attempts to transform AB1380 with this plasmid failed. Control transformations of both strains with the UGA version of the plasmid proceeded normally.

The most likely explanation of the behavior of AB1375 and AB1380 is that these strains are ψ$^+$, where ψ is a cytoplasmically inherited determinant of enhanced ochre suppression (COX 1965; SHERMAN 1982). Consistent with this hypothesis was the response of AB1375 and AB1380 to guanidine hydrochloride (GUHCl), an agent that efficiently converts ψ$^+$ strains to ψ$^-$ (TUITE, MUNDY and COX 1981). The strains were spread onto synthetic complete plates containing 5 mM GUHCl, incubated for 1 week, and then restreaked onto YPD plates. GUHCl-treated AB1375 and AB1380 were easily transformed with pC689$_{UAA}$.

Mutant isolation: Cells from AB1377 were grown on synthetic medium lacking lysine and tryptophan to select for suppression and for the plasmid. Single colonies were diluted in water, spread onto YPD or onto complete synthetic medium containing 10 mg/liter of adenine sulfate at 100–200 cells/plate and lightly mutagenized as follows: the lids were removed and the plates, inverted on a Spectroline Transilluminator Model TR-302 (302 nm ultraviolet) light box, were irradiated for 15 sec and then incubated for 4 days at 30°. The level of induced lethality was low (5–10%). Can^r mutants were induced in AB1400 under this treatment at a rate of 2.7 per 10^5 cells, an induction of 40 times the spontaneous rate. Plates were removed from 30° to room temperature after 4 days and then left for an additional 7 days to allow for optimal color development.

TABLE 1

Genotypes of strains used in this study

Strain		Genotype
AB1610	α	*ade2-1o lys2-1o can1-100o trp5-2o met 4-1o leu1-12 ura3*
AB1620	a	*ade2-1o lys 2-1o can1-100o trp5-2o met4-1o leu1-12 ura1*
AB1375	α	Ψ$^+$ *ade2-1o lys2-1o can1-100o leu2*$_{UGA}$ *trp1 his5*$_{UGA}$
AB1377		AB1375[pC689$_{UAA}$]
AB1380	a	Ψ$^+$ *ade2-1o lys2-1o can1-100o ura3 trp1 his5*$_{UGA}$
AB1400	α	*ade2-1o lys1o met4-1o leu2*$_{UGA}$ *trp1 ile1*

Mutants were collected in nine separate experiments that employed 40 independent colonies of the parental strain. Approximately 50,000 colonies were screened for the nonsectoring phenotype. Candidate mutants were streaked to YPD, and four nonsectoring subclones of each mutant that passed this secondary test were picked to YPD grids. After incubation for one day at 30°, these plates were replica-plated to YP-glycerol plates and also to synthetic plates that contained L-canavanine sulfate (80 μg/ml), lacked arginine, and contained all other required nutrients. Mutants that failed to grow on the YP-glycerol plates were not analyzed further, since *ade2* strains with gross respiratory defects do not synthesize the red pigment on which the sectoring colony assay depends. The level of growth on the canavanine plates, on the other hand, provided a useful secondary phenotype for mutants containing ochre mutations in essential genes (see RESULTS).

Complementation testing: The mutations were obtained in the opposite mating type by crossing the original mutants in the AB1377 background to AB1380. Before sporulation, these diploids were routinely treated with GUHCl (see above), which increased the fraction of meiotic progeny containing the plasmid from 0% before treatment to 30% after treatment. Relatively poor sporulation characterized the 1377 × 1380 diploid with or without the presence of the plasmid, an essential gene mutation, or ψ. Consequently, strains carrying mutations in a *MAT***a** background were isolated by random spore analysis rather than by tetrad dissection. Approximately 10^6–10^7 sporulated cells were incubated in 100 μl of 0.1 M dithiothreitol at room temperature for 30 min. After further incubation for 3.5 hr in a 1:10 dilution of glusulase in water, 500–1000 cells were spread onto synthetic plates lacking adenine and leucine in order to select for the plasmid and against *leu2* spores. Fifty to 100 colonies were picked to YPD grids and, after growth at 30° for 1 day, the plates were replica-plated to canavanine plates and to complete plates lacking uracil. Strains that were unable to grow on either medium were checked for mating type. One *MAT***a** *ura3* isolate was chosen for each mutation.

*MAT***a** *ura3* strains carrying an ochre mutation in an essential gene were crossed on YPD grids with the original set of mutant strains, which had been isolated in a *MAT*α *leu2* background. Mating grids were replica-plated to complete medium lacking uracil and leucine. In many cases, the complementation results could be read directly from the diploids growing at grid intersections on the -leu -ura plates. However, strains known to complement one another did not always produce red sectors in the dense growth at grid intersections. For this reason, diploids from the complementation crosses were picked to grids on canavanine plates, which were scored after one week. On these plates, most of the noncomplementing diploids produced no growth, or at most sparse white patches. Complementing diploids always produced dense red growth.

Segregation patterns were examined by crossing mutant strains with AB1610 or AB1620 and dissecting eight tetrads from diploids that lacked the plasmid. Sporulation was normal in these crosses.

RESULTS

Mutant phenotypes: Ochre mutations in essential genes were selected as white non-sectoring colonies in an *ade2-1o* strain that carries a *SUP4-o* CEN plasmid. Normally, because of plasmid loss at a frequency of a few percent per generation, such strains give rise to red and white sectored colonies (SHAW and OLSON 1984; HIETER *et al.* 1985). Cells with lethal ochre mutations cannot survive loss of the plasmid and, therefore, form white nonsectoring colonies (Figure 1a).

A useful secondary phenotype of mutations in essential genes is the inability to grow on a medium that selects against the plasmid. The particular system employed depended on the presence of the *can1-100o* mutation in all strains used for mutant isolation and analysis. The *can1-100o* mutation confers canavanine resistance unless suppressed. Since colonies of the parental strain, AB1377, are normally mixtures of suppressor-containing and suppressor-free cells, they give dense growth when replica-plated to canavanine medium. In contrast, mutants with ochre mutations in essential genes generally give little or no growth on canavanine medium since neither the suppressor-containing nor the suppressor-free cells can grow.

Although the primary nonsectoring phenotype was absolute in all cases analyzed, the amount of growth obtained after replica-plating to canavanine medium allowed considerable phenotypic discrimination amongst the mutants. Of the 144 mutants that were analyzed, 99 gave little or no growth on canavanine plates and were scored as having tight phenotypes. A second group of 21 mutants, which produced significant growth, but much less than the parental strain, was scored as having leaky phenotypes. Finally, 24 mutants, which were difficult to distinguish from the parental strain on canavanine plates, were not analyzed further.

A total of 50,000 colonies was screened to obtain the 120 mutants with distinct nonsectoring and canavanine-sensitive phenotypes. This frequency of one mutant per 420 cells plated, emphasizes the ease of obtaining nonsense mutations in essential genes by this method. All the mutations were recessive: crosses to an *ade2-1o can 1-100o* tester strain invariably produced sectoring colonies that gave dense growth when replica-plated to canavanine plates.

Complementation patterns: In preparation for complementation testing, the lethal nonsense mutations from 116 out of the 120 mutants described above were recovered in the opposite mating type. Unequivocal complementation data were obtained for 114 of these 116 cases. All distinct pairwise combinations of the mutations were examined in a total of 6555 complementation tests. As discussed in MATERIALS AND METHODS, the testing was carried out by replica-plating mating grids to a medium that selected for diploids, which were then picked to canavanine medium. On the latter plates, diploids containing complementing pairs of mutations gave dense red growth, while those containing noncomplementing pairs gave little or no growth and no pigment development (Figure 1b). All cases of noncomplementation were re-checked using a reciprocal cross (e.g., if the first test had employed *MAT***a** *mut1* × *MAT*α *mut2*, the *MAT*α *mut1* × *MAT***a** *mut2* diploid was used for the second test). Only one inconsistency was encountered during the complementation testing; it was traced to a double mutant in the original collection, which segregated mutations in two different complementation groups after out-crossing.

This double mutant was clearly an exceptional case, since segregation data confirmed that the overwhelming majority of the mutants contained a single lethal mutation. The segregation patterns of the lethal mutations were directly examined for 67 of the mutants by crossing them to AB1620, sporulating a diploid that had spontaneously lost the plasmid, and then dissecting tetrads. The diploids segregated an excess of dead spores in only 5 of the 67 cases. Of these 5 cases, random-spore analysis indicated that only the double mutant discussed above segregated mutations in two different complementation groups.

Taking into account the two mutations in the single identified double mutant, complementation data were obtained on 115 mutations, as summarized in Table 2. The mutations fell into 94 complementation groups, confirming the expectation that mutations would be recovered in a broad sampling of essential genes. Consideration of the clonal history of the different strains and the results of the complementation tests indicates that at least 114 of the 115 mutations had independent origins. As discussed below (see DISCUSSION), statistical analysis of the data in Table 2 suggests that the pool of accessible genes

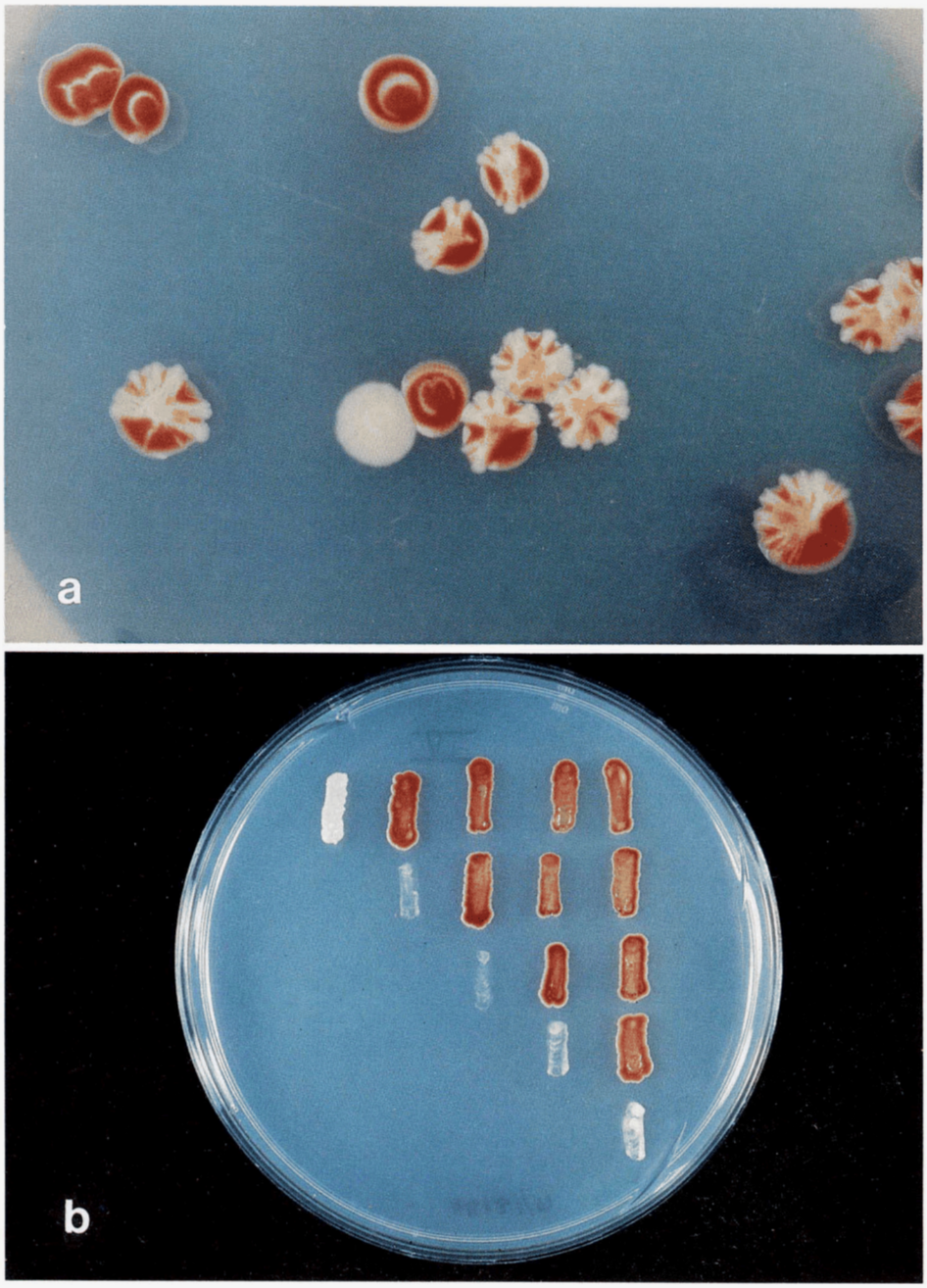

FIGURE 1.—Phenotypes of typical mutants during primary screening and complementation testing. a, Primary screen for nonsectoring mutants showing red colonies that have lost the CEN plasmid, red and white sectored colonies, and a single white mutant colony (the white circles in the red, plasmid-free colonies are caused by reflected light); b, complementation grid on a canavanine plate, showing dense red growth of complementing diploids compared with generally sparse, nonpigmented growth of noncomplementing diploids.

was sampled with little bias and that the mutants with tight and leaky phenotypes do not arise by the sampling of distinct sub-pools.

Terminal phenotypes: A crude indication of the number of cell divisions that could be completed by germinating spores that contained an unsuppressed lethal mutation was obtained by measuring the sizes of 10 to 16 abortive colonies that these spores formed. A measurement of 10 microns corresponds to two to six cells in wild-type strains. Cells in microcolonies formed by a number of the mutant strains were abnormally large and sometimes irregular in shape (Figure 2). Not surprisingly, strains with tight mutations tended to form smaller abortive colonies than strains with leaky ones. Nearly 75% of the tight mutants formed colonies with diameters of less than or equal to 20 microns, while approximately 70% of the leaky mutants produced colony diameters of more than 20 μm. The latter group generally had greater variation in colony size. In most cases, different alleles

TABLE 2

Results of complementation testing

No. of mutants defining complementation group	No. of complementation groups		
	Mutants with tight phenotypes	Mutants with leaky phenotypes	All mutants
1	67	17	78
2	10	1	12
3	3	0	3
4	0	0	1
Total	80	18	94

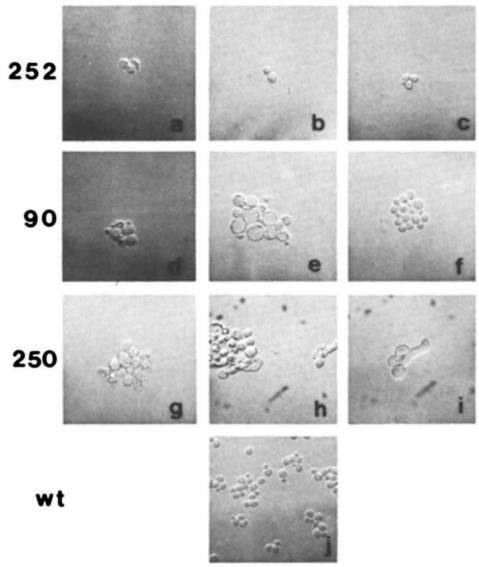

FIGURE 2.—Terminal phenotypes of three mutants that contain ochre mutations in essential genes. a–c, Three colonies of mutant 252 (1–5 cells); d–f, three colonies of mutant 90 (20–40 μm); g–h, two colonies of mutant 250 (20–50 μm); i, elongate cell, mutant 250; j, wild-type cells, photographed 1 hr after spreading a small colony.

of the same complementation group had similar terminal colony sizes. Terminal colony phenotypes of three essential-gene mutants are illustrated in Figure 2.

The sizes of abortive mitotic colonies were also determined by inspection of the colonies that formed from individual haploid cells that had spontaneously lost the plasmid. In a control experiment, 100 haploid cells from the parental strain, AB1377, formed normal colonies after the cells had been arranged in an orderly grid by micromanipulation. Twelve of these colonies were plasmid free. For the six mutant strains tested in the same way, a similar proportion of the cells formed abortive, presumably plasmid-free colonies. The sizes of abortive mitotic colonies were generally similar to those of terminal meiotic colonies.

Growth rates in YPD broth for six mutant strains that had a range of abortive colony sizes were determined (data not shown). There appears to be no correlation between growth rate of suppressed mutant strains and terminal-colony size in the absence of the suppressor.

DISCUSSION

We describe a method for isolating nonsense mutations in yeast genes that are required for growth on a rich medium. The lethality associated with these mutations is suppressed by a nonsense suppressor on a metastable plasmid. Individual colonies comprise a mixture of suppressor-containing and suppressor-free cell clones, which can be phenotypically distinguished because of the presence of a suppressible colony-color marker in the genetic background. Colonies that arise from cells that have suffered a lethal nonsense mutation are recognizable, because the suppressor-free sectors are unable to grow.

Because of the large number of sites that can give rise to nonsense mutations in all protein-coding genes, it is practical to isolate mutants from lightly mutagenized cell populations. At levels of UV mutagenesis that induce few double mutants, one in several hundred colonies have the nonsectoring phenotype expected for nonsense mutants. Furthermore, when each of the 114 haploid nonsectoring mutants was crossed to an appropriate tester strain, the resultant diploids had sectoring colony phenotypes, and the suppressor-free sectors harbored recessive lethal mutations. Although there are various other types of mutants whose colonies would also fail to display visible sectoring, the frequency of occurrence of lethal nonsense mutations is so high that the background due to all other classes of events was negligible.

Complementation testing of the mutants is straightforward. When two mutants containing mutations in different complementation groups are crossed, the diploids have sectoring phenotypes that can either be assessed directly using the suppressible colony-color marker or indirectly by virtue of the ability of the diploids to grow on a medium that selects for suppressor-free cells. Crosses between mutants containing mutations in the same complementation groups yield nonsectoring diploids that cannot grow on a medium that selects against the suppressor.

The 115 mutants that we analyzed defined 94 complementation groups (Table 2). A striking feature of these data is the extent to which they conform to a random-sampling model. In contrast to the situation commonly encountered when collecting temperature-conditional mutants, the results in Table 2 are consistent with the hypothesis that there is an equal probability of recovering a mutation in each gene

that can yield a mutant with the required phenotype. This conclusion follows from comparing the predicted and observed numbers of complementation groups defined by 1, 2, 3, and 4 independently isolated mutants, while regarding the number of accessible genes as an adjustable parameter. Using the pooled data for mutants with both tight and leaky phenotypes, the best fit to a random-sampling model is obtained by assuming that the mutations are being sampled from a pool of 250 genes. When the fit is carried out using 3 degrees of freedom, pooling the data for the 4 complementation groups that were defined by either 3 or 4 mutants, the χ^2 statistic is 3.6, which compares favorably with the most probable value of 3 that is predicted for random sampling.

There are no statistical grounds for treating the data for the tight and leaky mutants separately. The amount of overlap between the two sets of complementation groups defined by these two classes of mutants is close to that expected if they both arose by random sampling from the same set of genes (*i.e.*, 5 of the 18 complementation groups defined by the 19 leaky mutants are also represented amongst the 80 complementation groups defined by the 96 tight mutants, whereas the expected number of common complementation groups is 6 if the tightness of the phenotype exerted no sampling bias).

Given the good fit between the observed data and the predictions of a random-sampling model, the value of 250 provides a rough estimate of the number of genes that could be defined by this method if exhaustive sampling were carried out. Extrapolating from their detailed study of essential genes on chromosome *I*, KABACK *et al.* (1984) estimated that a similar number of genes could be defined by exhaustive use of established methods of recovering *ts* lethals. Although mutations can undoubtedly be recovered in many genes by either method, the two approaches are also likely to have substantial complementarity. The most obvious advantage of nonsense mutations lies in the large cross section for the occurrence of such mutations in typical coding regions. For example, in a study of mutations in the nonessential yeast gene *LYS2*, CHATTOO *et al.* (1979) found that 30% of all loss-of-function mutations were nonsense mutations. Conversely, the most obvious limitation on the use of nonsense mutations to define essential genes is that nonsense suppression may not restore enough activity to allow recovery of nonsense mutations in some genes, particularly those that code for structural proteins (FLOOR 1970). In yeast, typical strong ochre suppressors, such as the one used in the current study, restore only 5–10% of wild-type protein levels (GILMORE, STEWART and SHERMAN 1971). It should be noted, however, that low activity under permissive conditions is also a common problem with temperature-conditional mutations.

Given that nonsense mutations in essential genes are readily recovered in the presence of a plasmid-borne suppressor, the next challenge will be to develop methods of screening these mutants for specific classes of defects. It should be possible to screen the mutants for some biochemical and morphological defects simply by examining the mosaic cell populations that are generated during steady-state growth. The low efficiency of nonsense suppression may allow some biochemical defects to be detected by bulk assays on the entire cell populations. For phenotypes that can be recognized at the cellular level, a more powerful approach would be to screen the mixture of suppressor-containing and suppressor-free cells using an assay that can detect those individual cells whose growth has been arrested following loss of the suppressor. Finally, the ability to generate lethal nonsense mutations in a convenient way may stimulate renewed interest in the development of conditionally expressed nonsense suppressors. RASSE-MESSENGUY and FINK (1973) described a *ts* allele of *SUP4* many years ago, and other methods for making nonsense suppression conditional have also been proposed (SHAW and OLSON 1984). A conditional source of nonsense suppression has the potential of allowing biochemical assays to be carried out on whole populations of cells that are expressing the defect associated with the lethal nonsense mutation.

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