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An Examination of Adaptive Reversion in *Saccharomyces cerevisiae*

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ABSTRACT

Reversion to Lys^+ prototrophy in a haploid yeast strain containing a defined *lys2* frameshift mutation has been examined. When cells were plated on synthetic complete medium lacking only lysine, the numbers of Lys^+ revertant colonies accumulated in a time-dependent manner in the absence of any detectable increase in cell number. An examination of the distribution of the numbers of early appearing Lys^+ colonies from independent cultures suggests that the mutations to prototrophy occurred randomly during nonselective growth. In contrast, an examination of the distribution of late appearing Lys^+ colonies indicates that the underlying reversion events occurred after selective plating. No accumulation of Lys^+ revertants occurred when cells were starved for tryptophan, leucine or both lysine and tryptophan prior to plating selectively for Lys^+ revertants. These results indicate that mutations accumulate more frequently when they confer a selective advantage, and are thus consistent with the occurrence of adaptive mutations in yeast.

A central tenet of evolutionary biology is that genetic mutations occur in a random manner which is not dependent upon exposure to selective agents. This point was first demonstrated by LURIA and DELBRUCK (1943) through combined experimental and statistical analyses of mutations to phage T1 resistance in bacterial cells. In these classic experiments, independent cultures of wild-type, T1-sensitive *Escherichia coli* were grown nonselectively and then were plated in the presence of phage T1. LURIA and DELBRUCK found that the numbers of mutant, T1-resistant bacterial cells obtained from the independent cultures varied far more than would be expected if the mutations conferring phage resistance occurred upon exposure to the phage. This indicated that the mutations conferring T1 resistance were present at the time of plating and hence occurred during nonselective growth without regard to immediate utility. Subsequent, more direct demonstrations by LEDERBERG and LEDERBERG (1952) and others have further ensconced the notion that mutations occur randomly and continuously in growing cells prior to exposure to the selective agent. Although the bacterial experiments could not exclude the occurrence of additional mutations in direct response to a selective agent (the selective agent killed wild-type cells in these experiments), geneticists and evolutionary biologists have interpreted these classic experiments to mean that *all* mutations arise in a random manner. Natural selection thus is assumed to work on preexisting spontaneous mutations without directly influencing the frequency with which such mutations occur.

Recently, this central dogma concerning the spontaneous origin of mutations has been challenged (for a review see DRAKE 1991; FOSTER 1992). CAIRNS, OVERBAUGH and MILLER (1988), CAIRNS and FOSTER (1991) and HALL (1990, 1991) have reported results which suggest that nongrowing *E. coli* cells can acquire additional advantageous mutations when a selective pressure is imposed. CAIRNS, OVERBAUGH and MILLER (1988), working with *lacZ* amber mutations, observed that the number of Lac^+ revertants arising in independent cultures deviated significantly from the expected theoretical distributions derived assuming the occurrence of random mutations only during the preselective growth of the cultures (LEA and COULSON 1949). The direction of the deviation led to the suggestion that additional, adaptive mutations can arise in response to the selective agent. In support of the view that such reversions are adaptive, it was further reported that colonies (presumably resulting from new mutations) continued to appear on the selective medium for several days post-plating, while the number of mutations in an unselected gene failed to increase during the same time period. CAIRNS and FOSTER (1991) have obtained similar results using a constitutively expressed *lacZ* frameshift mutation and have demonstrated that some function of RecA influences the rates of adaptive mutation.

HALL (1990) has provided evidence that reversions of *trp* operon missense mutations in *E. coli* continue to occur for many days following plating on selective medium. As in the CAIRNS, OVERBAUGH and MILLER (1988) experiments, the production of the extra Trp^+ revertants appears to be dependent upon exposure to the selective medium. Hall further demonstrated that the appearance of "late" revertants was not due to cryptic growth of some cells and that the growth rates

of the revertants were comparable regardless of the time of appearance. In addition, reconstruction experiments defined the window of time during which the revertants should appear if they were present at the time of plating. Taken together, these results seem to eliminate any simple, physiological explanation of the late appearing revertants. In more recent work, HALL (1991) has provided evidence that adaptive mutants also arise when two separate mutations are required for prototrophy.

While there is substantial evidence supporting the notion of what appears to be a nonrandom source of mutations in bacterial cells, no comparable data have been reported for any eukaryotic organism. In the experiments described here, we have investigated this phenomenon in the budding yeast, *Saccharomyces cerevisiae*. The reversion of a defined frameshift mutation at the *LYS2* locus has been examined by following the time of appearance of prototrophic colonies after plating cultures on selective medium. As in the bacterial experiments, we find that Lys$^+$ revertants continue to arise after exposure to the selective medium in the absence of any detectable cell growth. Statistical analyses, as well as reconstruction and transformation experiments, indicate that the late appearing colonies result from genetic events occurring after selective plating. Since revertants do not accumulate under nonselective starvation conditions, they may be considered to be adaptive rather than resulting from a generally higher rate of mutation under starvation conditions.

MATERIALS AND METHODS

Yeast strains and plasmids: Strain SJR159 was used for measuring reversion frequencies. This strain contains a defined *lys2* frameshift mutation (P_{CYC1}-*lys2ΔBgl*), an undefined *trp1* amber mutation and a nonreverting *leu2* allele. The *lys2ΔBgl* allele was constructed *in vitro* by filling in a unique *Bgl*II restriction site near the 5′ end of the *LYS2* coding sequence with the Klenow fragment of DNA polymerase, thus creating a *Cla*I site (plasmid pSR125). Standard yeast transformation procedures were used to replace the wild-type information at the *LYS2* locus in strain BC3 [*MATα leu2 ura3::Ty trp1$_{am}$ ade2* P_{CYC1}-*LYS2*; see FLEIG, PRIDMORE and PHILIPPSEN (1986) for construction of the P_{CYC1}-*LYS2* fusion] with the *lys2ΔBgl* frameshift allele, thus generating strain SJR159. This strain was originally constructed for recombination work, and fusion of *LYS2* sequences to the heterologous *CYC1* promoter was essential for the analysis of recombinants. For the reversion studies, it was hoped that use of the glucose-repressible fusion gene would allow the effect of transcription on reversion to be examined. The transcription experiments were inconclusive, however, and are not reported here.

The presence of amber-suppressing tRNAs in SJR159 Trp$^+$ revertants was ascertained by mating revertants with strain YP45 (*MAT***a** *ura3-52 lys2-801$_{am}$ ade2-101$_{am}$ trp1Δ1*; obtained from P. HIETER) and looking for suppression of the *lys2* amber mutation. Strains SJR122 (*MATα trp1Δ1*) and DA2100 (*MATα leu2-3,112 ura3-52 his4 suc2Δ9 lys2Δ1::URA3*; see BARNES and THORNER 1986) were used as nongrowing backgrounds in reconstruction experiments with the Trp$^+$ and Lys$^+$ revertants, respectively. Plasmids YCp631 (BARNES and THORNER 1986) and YEp24 (BOTSTEIN *et al.* 1979) contain the selectable *LYS2* and *URA3* genes, respectively, and were used in the yeast transformation experiments.

Media and growth conditions: Yeast strains were grown nonselectively in YPD medium (1% yeast extract, 2% Bactopeptone, 2% dextrose; 2.5% agar for plates). Strains were grown selectively on minimal SC drop-out media (SHERMAN 1991) containing all amino acids and nucleotides except the one corresponding to the auxotrophy being analyzed. SC-Lys plates, for example, contain all amino acids except lysine. Canavanine medium (SC-Arg+Can) was made by adding 45 µg/ml L-canavanine sulfate to SC-Arg medium. All experiments were conducted at 30°.

Isolation of revertants: Colonies were excised from YPD plates and grown overnight in 5 ml liquid YPD. Cells were harvested from the 5-ml cultures by centrifugation, washed once with 5 ml sterile H_2O and resuspended in 1 ml sterile H_2O. Aliquots of 100 µl containing approximately 10^8 cells were plated on selective media to select revertants; this concentration of cells is within the linear range for measuring prototroph frequencies (data not shown). Appropriate dilutions also were plated nonselectively on YPD to determine the total number of viable cells in each culture. For most UV experiments, 300-µl aliquots were transferred to Petri plates and exposed to UV in a Stratalinker 2400 (Stratagene, La Jolla, CA) set at 7.5 mJ. Although reversion was stimulated at this dosage of UV, there was no detectable reduction in cell viability. Aliquots of 100 µl of the UV-irradiated cells were immediately plated on the appropriate selective medium to determine prototroph frequencies. In addition, a 100-µl aliquot was diluted 10-fold into liquid YPD and allowed to grow for 8 hr before plating. In separate experiments designed to assay adaptive reversion in UV-treated cells, the cells were plated on the appropriate media and then were irradiated with UV in a Stratalinker set at 5.0 mJ. Irradiated plates were wrapped with aluminum foil to prevent photoreactivation of UV-induced DNA damage.

Reconstruction experiments: Prototrophic revertants were grown overnight in YPD liquid medium, washed with sterile H_2O and diluted into washed cultures of an appropriate nonreverting, auxotrophic strain such that approximately 100 prototrophs were present for every 10^8 nonreverting cells. Cells were plated selectively at densities comparable to those used to identify revertants.

Mathematical methods: Statistical comparisons of independent cultures were done by one-way analysis of variance (ANOVA); each data point was transformed by adding 0.5 and calculating the square root in order to conform to the assumptions of the method. Analysis of the effects of nonspecific starvation on the accumulation of Lys$^+$ revertants was by two-way ANOVA for the paired case (SOKAL and ROHLF 1981). The theoretical LEA and COULSON (1949) and Poisson distributions were derived using our median and mean experimental values, respectively. The Lys$^+$ distributions in Figure 1 were calculated as according to Equation 11 of LEA and COULSON (1949) using a computer program provided by F. STEWART (STEWART, GORDON and LEVIN 1990). The Trp$^+$ distributions in Figure 3 were calculated using an approximate closed solution to the same equation (D. F. STEELE, unpublished).

The determination of the LEA and COULSON component of the distribution of Lys$^+$ revertants was done following subtraction of an estimated Poisson component from the number of colonies appearing on days 3 and 4 post-plating. Colonies appearing after day 4 were Poissonally distributed

and thus represented events that occurred after selective plating. As a first estimate of the Poisson component, it was assumed that the numbers of Poissonally distributed colonies on days 3 and 4 were the same as those observed on later days (100 per day using the data in Table 1). The mean expected Poisson contribution per culture was then subtracted from the numbers of colonies appearing on days 3 and 4 from each culture. The median of the "corrected" numbers of revertants was determined and the theoretical LEA and COULSON distribution was calculated and plotted against the corrected data. Goodness of fit was estimated by inspection. Values for hypothesized Poisson components both greater and lesser than the first estimate were similarly tested. Use of the first Poisson contribution estimate to correct the data yielded a curve virtually coincident with the theoretical curve for the corresponding median. Other Poisson estimates yielded progressively poorer fits to corresponding theoretical curves as they deviated from the initial Poisson estimate.

RESULTS

Reversion of a *lys2* frameshift mutation: The reversion behavior of a *lys2* frameshift allele (*lys2ΔBgl*) was studied in strain SJR159 in order to determine whether reversion events in yeast occur strictly in a random manner before selective plating, or whether they can occur as an adaptive response after a selective pressure has been imposed. The *lys2ΔBgl* allele contains a 4-bp duplication and was constructed by filling in a *Bgl*II restriction site (see MATERIALS AND METHODS). In genetic and physical analyses of approximately 100 independent Lys^+ revertants, we have found that approximately 90% contained an additional, compensatory frameshift in the *LYS2* coding sequence. The remainder were true revertants that had regained the *Bgl*II restriction site; none was the result of extragenic suppression (M. E. MORRIS and J. HOLBROOK, unpublished observations). The numbers of Lys^+ revertants in nonselectively grown, independent cultures of strain SJR159 were determined by plating aliquots on minimal medium deficient in lysine (SC-Lys). The numbers of prototrophic colonies appearing daily were counted beginning at 3 days after selective plating. Although colonies continued to appear indefinitely (we have followed cultures as long as 26 days post-plating; data not shown), we have chosen to limit the data reported to the first 8 days post-plating. Our rationale for this limitation is that cell viability decreased rapidly after day 5 (see below for viability measurements), thus making growth of the remaining cells very difficult to monitor.

We have examined *lys2ΔBgl* reversion in more than 100 independent cultures; data from one experiment in which the entire contents of 22 cultures were plated are presented in Table 1. As is evident from the table, the numbers of Lys^+ revertants continued to increase after the initial appearance of prototrophic colonies on day 3 so that by day 8, the average increase in the number of revertants per culture was 3.7-fold. It should be noted that the numbers of revertants appearing after day 3 ("late appearing" revertants) are similar in all cultures, in spite of the fact that some cultures contained jackpots of "early appearing" Lys^+ colonies on day 3. The general observation that the number of late appearing revertants is independent of the number of early appearing revertants was verified by statistically comparing the numbers of early *vs.* late appearing revertants derived from the individual cultures. A correlation coefficient of 0.04 was obtained, thus eliminating crossfeeding by early appearing Lys^+ colonies as a possible explanation for the late appearing revertants.

TABLE 1

Time-dependent appearance of SJR159 Lys^+ revertants

Cells plated ($\times 10^7$)	No. of newly arising colonies on day:						-Fold increase
	3	4	5	6	7	8	
105	24	9	6	5	6	0	2.08
84	8	16	10	7	5	2	6.00
93	7	10	5	2	4	7	5.00
80	7	11	5	5	4	4	5.14
79	12	18	6	3	5	6	4.17
108	13	9	6	1	4	7	3.08
80	7	23	6	5	3	2	6.57
81	2	5	2	3	4	1	8.50
90	10	16	8	3	4	2	4.30
82	27	14	5	2	3	4	2.04
80	81	14	2	4	13	6	1.48
95	105	8	6	5	4	4	1.26
99	10	11	2	3	4	4	3.40
78	38	13	3	2	4	2	1.63
86	91	14	7	7	5	3	1.40
86	6	17	6	7	7	2	7.50
78	37	23	3	6	3	2	2.00
79	17	21	10	4	3	3	3.41
94	34	23	7	8	4	0	2.24
95	8	5	2	4	7	4	3.75
83	8	8	1	3	2	5	3.38
93	10	13	3	4	4	1	3.50
Mean							3.72

Data obtained from 22 independent cultures are given. For each culture, a single colony was inoculated into 5 ml YPD and grown overnight at 30°. Each overnight culture was washed with H_2O and resuspended in a final colume of 1 ml H_2O. The entire culture was then plated in 0.1-ml aliquots on 10 selective plates; there were thus approximately 10^8 cells per plate. Each number given represents the sum of the colonies on all 10 plates for a given day post-plating. The -fold increase for each culture is the ratio of the cumulative number of colonies through day 8 to the number of colonies counted on day 3.

In addition to examining the time-dependent appearance of Lys^+ revertants, we also examined the distribution of the total numbers of revertants among independent cultures using the data presented in Table 1. Figure 1 presents theoretical distributions for random mutations occurring in the absence of selection [calculated according to LEA and COULSON (1949) and hereafter referred to as LEA and COULSON distributions]; theoretical distributions (Poisson distributions) for adaptive mutations occurring in response

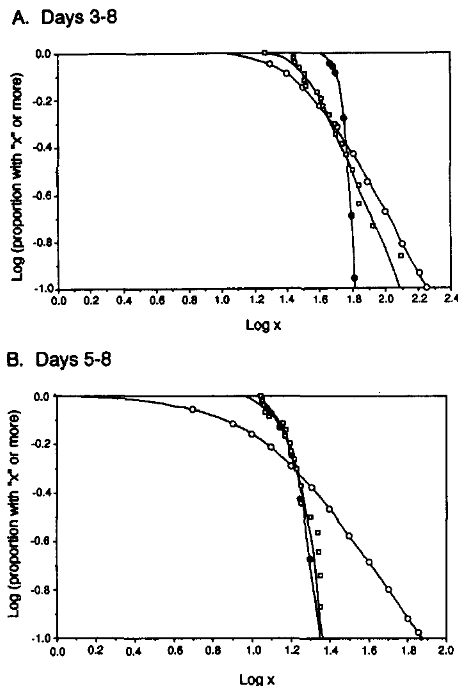


FIGURE 1.—Distributions of the numbers of Lys$^+$ revertants. The theoretical and experimental distributions of the numbers of Lys$^+$ revertants were obtained by plotting log (x) *vs.* log (proportion of cultures with x or more mutants), where "x" is the number of mutants per culture (see CAIRNS, OVERBAUGH and MILLER 1988). The squares represent experimental values calculated using the data in Table 1, the open circles are values for the LEA and COULSON distribution and the closed circles are values for the Poisson distribution.

to selection; and the actual distributions of the numbers of Lys$^+$ colonies in our experiments. The notable difference in the shapes of the two types of theoretical distributions results largely from differences in the predicted variances. The LEA and COULSON distributions are broad due to the large fluctuation in the number of revertants per independent culture. In contrast, the Poisson distributions expected if mutations occur strictly in response to the selective pressure are much narrower due to the smaller variance expected between independent cultures. The distribution of Lys$^+$ revertants obtained using the summed data for days 3–8 is intermediate between the predicted LEA and COULSON and Poisson distributions (Figure 1A). One interpretation of this composite distribution is that some mutations occurred during nonselective pregrowth of the cultures (those giving rise to the early appearing revertants) while other mutations occurred after selective plating (those giving rise to the late appearing revertants). Consistent with this interpretation is the observation that the distribution of the numbers of late appearing revertants (days 5–8; Figure 1B) very closely approximates the theoretical Poisson distribution.

Below, we address several phenomena other than adaptive mutation (*e.g.*, phenotypic lag or continued cell growth after selective plating) that could account for the late appearing revertants. In addition, we present a strong statistical argument supporting the interpretation that the late appearing revertants reflect mutations occurring after selective plating. Finally, we demonstrate that the late appearing Lys$^+$ revertants arise only when they confer a direct selective advantage to the cells.

Reconstruction and transformation experiments: A trivial explanation for the late appearance of some Lys$^+$ revertants is that the time of appearance of a Lys$^+$ revertant is directly proportional to the growth rate of the revertant. An early appearing colony would thus correspond to a fast growing revertant while a late appearing colony would have a slow growth rate. This hypothesis was tested by doing reconstruction experiments similar to those done by HALL (1990) with approximately 100 revertants which made their appearance at defined times (day 3, day 4, etc.) during reversion assays. In these experiments, approximately 100 cells of each revertant were plated selectively in the presence of 10^8 nonreverting Lys$^-$ cells (strain DA2100) in order to achieve cell densities comparable to those used in the reversion experiments. The time frames in which colonies appeared on the selective plates were then determined. The growth rates of 99% of the Lys$^+$ revertants analyzed in the reconstruction assay were such that they appeared within 3 days after plating, regardless of the time the individual revertants made their debuts in the original reversion assay (data not shown). Thus, the hypothesis that the time of appearance of a Lys$^+$ prototrophic colony in the reversion assay is directly related to the growth rate of that revertant is not valid.

In the reconstruction assays described above, all of the Lys$^+$ revertants examined had been expressing the *LYS2* gene product for many generations. In fact, many Lys$^+$ colonies consistently appeared by day 2 in these assays, 1 day earlier than revertants appeared in the reversion assays. In addition, many of the Lys$^+$ colonies in "jackpot" cultures (cultures containing large numbers of Lys$^+$ prototrophs derived from a reversion event occurring relatively early during the nonselective growth of the culture) appeared by day 2, 1 day earlier than the day 3 debut of Lys$^+$ colonies in most cultures. These observations suggest that revertants arising late during the growth of a culture, immediately prior to plating, may not contain sufficient *LYS2* product to begin immediate growth. Conceivably, it could take several days under the starvation conditions of the SC-Lys plates to produce sufficient gene product so that cellular division could begin. Given this flaw in the reconstruction experiments, we designed an experiment which should more accurately measure the time it takes a cell to produce a colony after it has acquired a *LYS2* gene. Yeast transformation using a lithium acetate procedure

TABLE 2

Time-dependent appearance of SJR159 Lys$^+$ transformants

Tube	DNase (μg)	No. of newly arising transformants on day: 3	4	5	6	7	Total
1	1	314 (0.93)	17	2	3	0	336
2	10	236 (0.86)	23	10	4	1	274
3	10	522 (0.93)	25	10	6	0	563
4	10	215 (0.88)	21	6	3	0	245
Totals		1287 (0.91)	86	28	16	1	1418
Control (no DNA)		1	0	0	0	0	1

Transformations were done using a lithium acetate procedure (SCHIESTL and GIETZ 1989). Each tube contained 200 μl cells (10^9 cells/ml), 0.5 μg YCp631 and 100 μg denatured salmon sperm DNA. After completion of the transformation procedure, washed cells were resuspended in 200 μl 10 mM Tris-HCl/1 mM EDTA and 0.1 volume of a 10× DNase I buffer (0.5 mM Tris-HCl, pH 8.0; 0.1 M $MgCl_2$) was added. Cells were divided evenly into two tubes and the amount of DNase indicated in the table was added to one of the tubes. Cells were incubated at room temperature for 15 min and then the entire contents of each tube were plated onto a single SC-Lys plate. The density of cells plated was comparable to that used in the reversion experiments. The numbers of colonies appearing per day are given; the numbers in parentheses following the day 3 data are the fraction of the total colonies. In the absence of DNase, 81% of the Lys$^+$ colonies were evident on day 3 (data not shown). In a separate experiment in which DNase was not used, cotransformation was assessed using 0.5 μg of YCp631 and an equivalent amount of a *URA3*-containing plasmid (YCp50). In this experiment, 74% of the total number of Lys$^+$ transformants were evident by day 3 and less than 1% of these were also Ura$^+$. The general observation that DNase reduces the phenotypic lag suggests that DNA can be taken up by cells after selective plating.

(SCHIESTL and GIETZ 1989) was used to introduce a *CEN* plasmid encoding the *LYS2* gene into strain SJR159. In this type of transformation, cells are not outgrown prior to selective plating, so the time of appearance of Lys$^+$ prototrophs should reflect the growth lag on selective medium of cells newly acquiring a functional *LYS2* gene. As can be seen in Table 2, 90% of the Lys$^+$ transformants appeared by day 3. Based on the transformation results, we conclude that there is not a significant phenotypic lag associated with the acquisition of a wild-type *LYS2* gene. It could be argued, however, that since the majority of revertants are not truly wild-type (*i.e.*, they are second-site intragenic revertants), some might exhibit a substantial phenotypic lag. This possibility is addressed below.

Statistical analysis indicates that reversion events occur after plating: As alluded to above, there are likely to be a variety of second-site nucleotide changes in the *lys2ΔBgl* allele that can result in lysine prototrophy. Since the resultant gene products are not truly wild-type and may not have full enzymatic activity, one can imagine that different classes of revertants might vary in the time required to produce sufficient gene product for growth to initiate on SC-Lys medium. Members of one class, for example, might uniformly begin growing 2 days after plating while members of another class might not initiate growth until 2 days later. This scenario predicts that the overall distribution of Lys$^+$ revertants should approximate a LEA and COULSON distribution, which it clearly does not (see Figure 1). While Poisson-like deviations from a theoretical LEA and COULSON distribution would be expected if reversion continues after selective plating, other explanations are possible. The observed narrowing of the distribution with time could be explained, for example, if some of the cells which initiate growth very slowly (corresponding to the late appearing revertants) were to die before growth could begin. Alternatively, one could hypothesize that some classes of revertants (especially the late appearing revertants) grow more slowly than others during the nonselective growth prior to plating. Given some sort of distribution-narrowing effect, it is possible to envision that the distribution of the total numbers of revertants from each culture might approximate the Poisson distribution, while the means of some of the within-culture distributions might differ statistically.

A specific prediction of the adaptive mutation model is that the independent cultures should be essentially the same with respect to the numbers of late appearing revertants. The numbers of colonies on plates derived from the same culture should be no more similar to each other than to the numbers of colonies on plates from different cultures. In contrast, independent cultures should be very different with respect to the numbers of early appearing Lys$^+$ revertants, since these represent mutations that occurred prior to selective plating. One would thus expect the numbers of early appearing revertants on plates from an individual culture to be more similar to each other than to the numbers of early appearing revertants on plates derived from a different culture. Stated another way, the late appearing revertants from independent cultures should belong to a single population if analyzed statistically while the early appearing revertants should not. A very powerful statistical test that we have used to address this issue is ANOVA. In ANOVA, one calculates an F value by comparing the mean variance within independent cultures to the variance among cultures, with the null hypothesis being that all of the cultures are part of a single population. A significant F value indicates that the means of some of the cultures are statistically different from each other.

For ANOVA, 22 independent overnight cultures containing 10^9 cells each were washed, concentrated and divided into 10 aliquots which were plated individually onto SC-Lys medium. Newly arising Lys$^+$ colonies were tabulated daily for each of the 10 plates from each of the 22 independent cultures (Table 1 gives the daily sum for each culture). ANOVA for day 3 data gave a highly significant F value of 28.9 (P

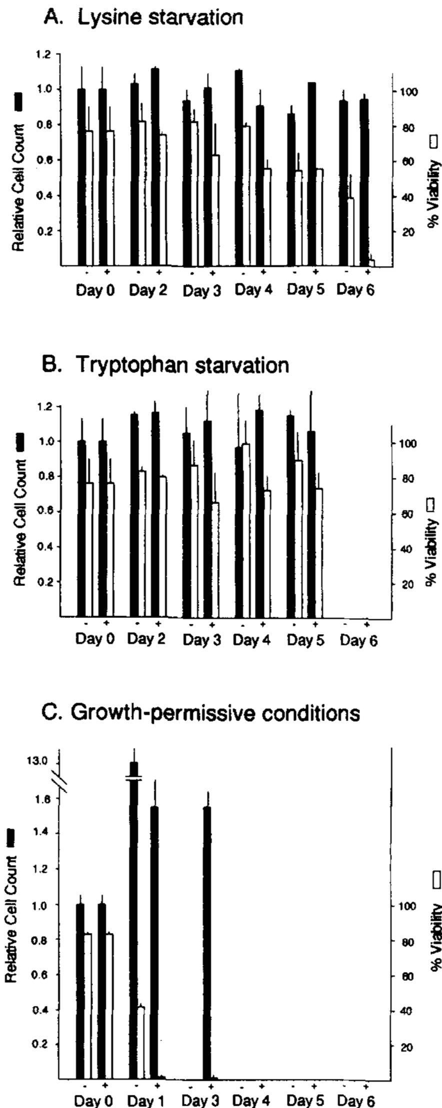


FIGURE 2.—Assessment of cell growth on different types of media. Data were obtained by plating cells on the following media: (A) SC-Lys-Arg or SC-Lys-Arg+Can for lysine starvation, (B) SC-Trp-Arg or SC-Trp-Arg+Can for tryptophan starvation, and (C) SC-His-Arg or SC-His-Arg+Can for growth-permissible conditions. Cells were washed off the plates with sterile H_2O at the indicated times after plating; prototrophic colonies were excised from the plates before washing. Day 0 corresponds to 0.5–1 h after plating; the same plates served as the day 0 control for both the "+" and "−" canavanine experiments. An appropriate dilution was counted in a hemocytometer to estimate the total number of cells per plate; all cell counts for a given type of medium have been normalized to those obtained on day 0. Day 0 absolute cell counts per plate were 1.36×10^8 for the lysine and tryptophan starvation experiments and 1.62×10^8 for the growth-permissible experiment. The numbers of viable cells were determined by plating appropriate dilutions on YPD medium and the viability was calculated as the ratio of viable cells to the hemocytometer cell counts. It should be noted that cells plated in the absence of canavanine formed colonies within 2 days when replated on rich medium, while cells washed from the canavanine plates were slow to form colonies and were counted after 6 days. Each bar represents an average obtained by washing cells from two to five plates (data from only one plate was available for day 5 in the lysine starvation experiments); the line above each bar corresponds to the standard deviation. Solid and open bars correspond to the relative cell counts and viabilities, respectively. "+" indicates canavanine present; "−" indicates no canavanine present. Less than 1% of the cells exposed to canavanine were viable in experiment (C), thus accounting for the apparent absence of the open bar for these days.

< 0.001), confirming that the cultures are not part of a single population with respect to the early appearing revertants. That is, the mean number of revertants varied greatly from culture to culture, as predicted if mutations occurred at variable times during nonselective growth. ANOVA using the day 4 data yielded a barely significant F value of 2.08. ANOVA of the days 5, 6, 7 and 8 data gave F values of 1.42, 0.94, 0.76 and 1.34, respectively; these F values are not significant. The variance among the independent cultures with respect to the late appearing revertants is not different from the mean variance within the independent cultures, indicating that all revertants are members of a single population. This result strongly supports the conclusion that some of the reversion events leading to the Lys$^+$ phenotype occurred after selective plating.

Lys$^-$ cells do not show detectable growth after selective plating: The occurrence of late appearing Lys$^+$ revertants documented above could be explained if the auxotrophic cells continued to divide after selective plating. Growth on the SC-Lys medium was assessed by washing cells off the plates immediately after plating (day 0) and at various days post-plating. Cells were counted in a hemocytometer to determine the total number of cells and appropriate dilutions were plated on YPD plates to determine the total number of viable cells. While no growth was detected on the SC-Lys medium in these experiments (see Figure 2), it still could be argued that cell division is occurring. One can imagine that cells die under the starvation conditions used and that each cell that dies is replaced by one new cell. This scenario assumes cannibalism of dead cells by cryptically growing cells, a possibility we feel is unlikely because cells which fail to form colonies on rich medium are nevertheless visible in the microscope.

To provide more rigorous evidence that growth/cannibalism is not occurring under the selective conditions used, we plated cells under conditions where cells dividing in the absence of lysine should be selectively killed. For these experiments the arginine analogue canavanine was added to the standard SC-Lys medium and arginine was omitted (SC-Lys-Arg+Can medium). In the absence of arginine, canavanine is efficiently taken up by cells and incorporated into proteins, eventually causing cell death (SIKORSKI and BOEKE 1991). To detect cell death due to growth, the numbers (hemocytometer counts) and viabilities (colonies formed on rich medium) of cells plated selectively in the presence of canavanine (SC-Lys-Arg+

Can) were compared with those of cells plated selectively in the absence of canavanine (SC-Lys-Arg). As a control to confirm the efficiency of canavanine killing, cells were plated under conditions where they should grow (histidine-deficient medium; SJR159 is His$^+$) in both the presence (SC-His-Arg+Can) and absence of canavanine (SC-His-Arg). Growth was also examined in the absence of tryptophan (SC-Trp-Arg and SC-Trp-Arg+Can media).

The data obtained in these experiments are presented in Figure 2, where the hemocytometer counts and viability measurements for day 0 and days 2–6 post-plating are illustrated. It is clear from the lysine (and tryptophan) starvation data that, although the presumptive nongrowing cells eventually succumbed to the effects of canavanine, there was very little loss in viability before day 6. Given that starving cells very probably metabolize protein, it is not surprising that canavanine eventually killed these cells. If, as the previously described data indicate, it takes 3 days for colony formation after a reversion event occurs, then the day 8 colony counts (see Table 1) correspond to events occurring on day 5 when there was very little killing by canavanine and, we would argue, very little, if any cell division. In the control experiments, less than one cell division (a 1.5-fold increase in cell number) was sufficient to kill 99% of the cells (Figure 2C).

The mutation rate during nonselective growth prior to selective plating was estimated by empirical curve-fitting (see MATERIALS AND METHODS) using the data in Table 1 to be approximately 4.9×10^{-9} per cell division. Similarly, the mutation rate per cell during the 8-day time period after selective plating (*i.e.*, the Poisson component of the total mutant distribution) was estimated to be 3.0×10^{-8}. Multiplying the average of 8.8×10^{8} cells per culture by the estimated post-plating mutation rate per cell, the mean number of late appearing Lys$^+$ revertants per culture that appear to have arisen after selective plating is approximately 26. If the assumption is made that the late appearing revertants actually resulted from cell division, then the mutation rate per division (4.9×10^{-9}) can be used to estimate that 2.8 doublings (a sevenfold increase in cell number) would be required to produce the 26 post-plating revertants per culture. While we do not believe that it is possible at this point to completely exclude any cell growth on lysine-deficient medium, the experiments presented above indicate that if any growth is occurring, it is certainly much less than one doubling. Therefore, cryptic growth cannot account for the numbers of late appearing Lys$^+$ colonies.

The time-dependent appearance of Lys$^+$ revertants is adaptive: To investigate whether the time-dependent increase in Lys$^+$ revertants was due to a nonspecific effect of amino acid deprivation on mutation rates, YPD-grown cultures were held for 3 days under tryptophan or leucine starvation conditions prior to plating for Lys$^+$ revertants. In the tryptophan starvation experiments, aliquots of nonselectively grown cultures of SJR159 were either plated selectively on SC-Lys to examine reversion (control plates) or were plated on SC-Trp to starve for tryptophan. The SC-Trp plates were incubated at 30° for 3 days and Trp$^+$ colonies were excised from the plates. The remaining cells were washed off the plates with H_2O and then replated on SC-Lys medium. If Lys$^+$ revertants accumulated nonspecifically during the 3-day amino acid starvation, then the number of revertants from the tryptophan-deprived plates 3 days after plating on SC-Lys should be similar to the number of revertants from the control culture after 6 days on SC-Lys. If, on the other hand, the Lys$^+$ revertants accumulate only under selective conditions, then the time-dependent appearance of revertants after plating on SC-Lys medium should be similar in the tryptophan-deprived and control cultures. As shown in Table 3A, the numbers of revertants appearing on days 3 through 7 after selective plating on SC-Lys were similar when the control and tryptophan-starved cells were compared, indicating that Lys$^+$ revertants failed to accumulate during tryptophan starvation. In other experiments, minimal liquid medium lacking leucine (liquid medium was used because viability was very low after starvation for leucine on plates) was used as the starvation medium. As in the tryptophan starvation experiments, the time-dependent appearance of the Lys$^+$ revertants from the leucine-starved cells was similar to that from the unstarved, control cells (Table 3B).

The directed mutation hypothesis suggests that cells "select" and fix advantageous mutations, with the criterion for selection being that a mutation permits cellular growth. Thus one would predict that Lys$^+$ revertants would arise under conditions of lysine starvation but not under conditions of starvation for an amino acid in addition to lysine, since reversion to Lys$^+$ would not permit growth in the latter case. We thus compared the effects of simultaneous starvation for tryptophan and lysine with the effects of starving for lysine alone on reversion of *lys2ΔBgl*. Aliquots of overnight cultures were plated either on SC-Lys-Trp for starvation or directly onto SC-Lys. After 3 days of starvation on the SC-Lys-Trp medium, cells were washed off the plates and transferred to SC-Lys medium to monitor the appearance of Lys$^+$ revertants. As is evident in Table 3C, holding cells on medium lacking both tryptophan and lysine for an extended period had no effect on the time of appearance of revertants once cells were replated for the detection of reversion events. This is consistent with the notion that the so-called adaptive reversion events do not

TABLE 3

Effect of tryptophan, leucine or lysine plus tryptophan starvation on the frequency of SJR159 Lys⁺ revertants

	Cumulative number of Lys⁺ revertants by day after transfer to SC-lys				
	3	4	5	6	7
A. Tryptophan starvation (3 days on plates)[a]					
Unstarved control	92	135	161	178	189
Tryptophan-starved	60	119	132	134	147
Starved/unstarved ratio	0.65	0.88	0.82	0.75	0.78
B. Leucine starvation (3 days in liquid)[b]					
Unstarved control	55	94	102	112	118
Leucine-starved	30	78	101	106	112
Starved/unstarved ratio	0.55	0.83	0.99	0.95	0.95
C. Lysine/tryptophan starvation (3 days on plates)[c]					
Unstarved control	39	55	72	80	
Lys/Trp-starved	34	46	56	67	
Starved/unstarved ratio	0.87	0.84	0.77	0.84	

There consistently seems to be some loss of Lys⁺ revertants during nonspecific starvation, although the differences between the day 3 starved and day 3 unstarved samples are not statistically significant. This could be due either to a lag in growth of Lys⁺ cells after transfer from the starvation conditions or it could reflect a very early adaptive component that is lost when the cells are nonspecifically starved.

[a] Numbers given are the sums from ten independent cultures. 18.6×10^8 (two aliquots from each of the 10 cultures) and 8.72×10^8 (one aliquot from each of the 10 cultures) viable cells were plated from the unstarved and starved cultures, respectively. The numbers of prototrophic colonies are per 18.6×10^8 cells plated. Two-way ANOVA using paired starved-unstarved numbers from each culture gave an insignificant F value of 2.2 when day 3 comparisons were done. Comparing the day 6 control to the day 3 tryptophan-starved cultures yielded a highly significant F value of 60.3 ($P < 0.01$).

[b] Numbers given are the sums from plating a single aliquot from each of nine cultures; one culture had a jackpot of revertants during nonselective growth in YPD and so was omitted from the analysis. 14.0×10^8 and 13.3×10^8 viable cells were plated from the unstarved and starved cultures, respectively. The numbers of prototrophic colonies are per 14.0×10^8 cells plated. Two-way ANOVA using paired starved-unstarved numbers from each culture gave an insignificant F value of 1.92 when day 3 comparisons were done. Comparing the day 3 starved to the day 6 unstarved yielded a highly significant F value of 17.2 ($P < 0.01$).

[c] Numbers given are the sums from plating a single aliquot from each of 12 cultures. 9.31×10^8 and 8.02×10^8 viable cells were plated from the unstarved and starved cultures, respectively. The numbers of prototrophic colonies are per 9.31×10^8 cells plated. Two-way ANOVA using paired starved-unstarved numbers from each culture gave an insignificant F value of 1.25 when day 3 comparisons were done. Comparing the day 3 starved to the day 6 unstarved yielded a highly significant F value of 35.2 ($P < 0.01$).

TABLE 4

Effect of UV irradiation on the time-dependent appearance of SJR159 Lys⁺ revertants

A. Induction of revertants by UV irradiation[a]

Treatment before plating	Newly arising colonies on day:				
	3	4	5	6	7
No UV	51	41	24	14	17
UV	283	381	248	139	100
-Fold induction	5.5	9.3	10.3	9.9	5.9
No UV, 8 hr growth	42	40	31	24	22
UV, 8 hr growth	148	60	63	46	24
-Fold induction	3.5	1.5	2.0	1.9	1.1

B. Effect of nonspecific starvation on the accumulation of UV-induced revertants[b]

Plating conditions	Newly arising colonies at day after UV irradiation:					
	3	4	5	6	7	8
SC-Lys	267	738	451 (1456)	266	126	50
2 days on SC-Lys-Trp prior to plating on SC-Lys			888	324	139	48

[a] In the first data set, aliquots of cells from overnight cultures were irradiated with UV light and immediately plated on selective medium. Control aliquots from each culture were plated without prior exposure to UV light. Numbers given represent the sum of revertants derived from 10 independent cultures. A total of 2.2×10^9 viable "no UV" control cells (three aliquots per culture) and 7.6×10^8 viable "UV" experimental cells (one aliquot per culture) were plated; numbers given are per 2.2×10^9 viable cells. The second data set was generated by diluting a portion of the control or UV-irradiated cells 10-fold into YPD and allowing cells to grow for 8 hr before selective plating. Numbers represent the sum of revertants in 11 cultures. Numbers of revertants are per 1.7×10^9 viable cells plated. 1.7×10^9 control and 1.0×10^9 experimental cells were plated.

[b] The accumulation of Lys⁺ revertants under the two different plating conditions was assessed by examining the numbers of colonies apparent on the indicated days after UV irradiation. Aliquots of cells from overnight cultures were plated on SC-Lys or SC-Lys-Trp medium and irradiated with UV light in a Stratalinker 2400 set at 5 mJ. Cells plated on SC-Lys-Trp were transferred to SC-Lys medium after two days of the nonspecific starvation. 9.3×10^8 cells (two aliquots plated from each of six independent cultures) were plated directly on SC-Lys medium; 8.3×10^8 cells (as above, but one plated aliquot was lost due to contamination) were plated on SC-Lys after 2 days of starvation on SC-Lys-Trp. Numbers of revertants are per 9.3×10^8 cells plated. The number in parentheses under the SC-Lys data corresponds to the cumulative number of Lys⁺ revertants present on day 5.

accumulate unless they afford an immediate selective advantage.

Effect of UV light irradiation on the time-dependent appearance of Lys⁺ revertants: UV irradiation experiments were done in order to examine the time-dependent appearance of prototrophic revertants when the potential for reversion was increased at the time of plating. In these experiments, aliquots of overnight cultures of strain SJR159 were exposed to UV light immediately prior to plating on selective SC-lys medium. Untreated aliquots of the same overnight cultures were also plated selectively for comparison purposes. As is evident in Table 4A, UV treatment substantially increased the number of Lys⁺ colonies appearing each day throughout the period of the reversion assay. The growth rates of UV-induced revertants making their appearance at different times were comparable in reconstruction experiments (data not shown). If the UV treatment were simply increasing the number of Lys⁺ revertants present at the time of plating, one would expect to see an increase only in the numbers of early appearing revertants instead

of the observed increase in late appearing revertants as well. This observation can be interpreted in one of two ways. First, one could argue that all of the prototrophic revertants were indeed induced by UV light at the time of plating, but that the time of colony appearance simply reflected a time-dependent phenotypic lag. This interpretation, however, is not consistent with our various reconstruction and transformation experiments. An alternative interpretation is that UV irradiation created lesions in DNA which were repaired in a time-dependent fashion under starvation conditions. Below we address whether the UV-induced, time-dependent revertants are adaptive in nature.

As a control for the above UV experiments, irradiated cells were diluted into rich medium and allowed to grow for 8 hr before selective plating. We predicted that we would see an initial increase in the number of prototrophic revertants, but that the numbers of revertants appearing at late times should be equal to those appearing at late times in the unirradiated cultures. This prediction was realized, indicating that one can increase the initial numbers of revertants without affecting the appearance of late revertants. Somewhat surprisingly, the absolute numbers of UV-induced revertants were less when growth was allowed before selective plating. This may reflect a difference in DNA repair mechanisms under different environmental conditions or may reflect a slower growth rate of those cells sustaining the DNA damage that led to prototroph formation.

In order to gauge whether there may be an adaptive component to the UV-induced, time-dependent Lys$^+$ revertants, we compared the accumulation of revertants under different starvation conditions. Aliquots from nonselectively grown, independent cultures were plated both on SC-Lys medium and on SC-Lys-Trp medium, and then were irradiated with UV. On the SC-Lys medium, Lys$^+$ revertants can immediately form colonies, whereas on the SC-Lys-Trp medium, Lys$^+$ revertants cannot grow because of the tryptophan auxotrophy in strain SJR159. Two days after the UV irradiation, cells were washed off the SC-Lys-Trp medium and replated on SC-Lys. Lys$^+$ colonies were counted daily as they appeared. As is evident in Table 4B, UV-induced Lys$^+$ revertants did accumulate during the nonspecific starvation conditions (compare the 267 colonies present on day 3 when cells were directly plated on SC-Lys *vs.* 888 colonies when cells were nonspecifically starved before giving them 3 days to form colonies on SC-Lys). The relevant point from this experiment, however, is that significantly *more* Lys$^+$ revertants had accumulated by the 5th day after UV irradiation when cells were plated directly on SC-Lys than when they were held for 2 days on SC-Trp-Lys prior to plating on SC-Lys (1456 and 888 Lys$^+$ colonies, respectively, evident by day 5 after UV treatment). Analysis of the day 5 data using two-way ANOVA for the paired case gave a highly significant F value of 67.6 ($P < 0.001$). It should be noted that after day 5 post-irradiation, the numbers of revertants arising daily were statistically the same for the two plating regimes (plating directly on SC-Lys *vs.* holding for 2 days prior to selective plating). Thus it appears that during the holding period on SC-Lys-Trp, a fraction of the Lys$^+$ revertants was specifically lost rather than simply being delayed with respect to time of appearance. While other explanations may be possible, we interpret these data as evidence that repair of UV-induced DNA damage, and presumably other DNA lesions as well, can be adaptive.

Trp$^+$ revertants also accumulate in a time-dependent manner: Since nonsense mutations frequently revert extragenically and had been used in earlier studies (Cairns, Overbaugh and Miller 1988), we also examined the reversion of an undefined *trp1* amber mutation present in strain SJR159. We note that Von Borstel (1978) has previously reported that *trp1-1$_{am}$* revertants accumulate over a prolonged time period, but attributed the accumulation to cell turnover. In our experiments, Trp$^+$ revertants made their initial appearance after 3 days on selective medium lacking tryptophan (SC-Trp) and were counted daily until day 8 post-plating. These data are presented in Table 5. The average increase in the number of revertants (ratio of total revertants to those present on day 3) was 28.8-fold. As with the Lys$^+$ revertants, the distribution of Trp$^+$ revertants was intermediate between the theoretical Lea and Coulson and Poisson distributions (Figure 3A), while the distribution of late appearing Trp$^+$ revertants more closely approximated a Poisson distribution than a Lea and Coulson distribution (Figure 3B). No net cellular growth was evident on the selective medium through day 5 post-plating (see Figure 2B). In contrast to the situation seen with the Lys$^+$ revertants, genetic analysis of the Trp$^+$ revertants indicated that at least one-third could be attributed to the acquisition of extragenic suppressor tRNAs (data not shown). In addition, reconstruction experiments with Trp$^+$ revertants revealed that, while the majority of the early appearing revertants were fast growing, approximately one-third of late appearing colonies failed to produce colonies in the 3-day window allowed. Thus, it is likely that a sizeable fraction of the late appearing Trp$^+$ revertants were late appearing because of a slow growth rate or an extended lag phase rather than because they arose post-plating in response to the selective pressure imposed.

Analysis of the Trp$^+$ revertants was further complicated by the results of starvation experiments designed to examine whether the reversion events oc-

TABLE 5

Time-dependent appearance of SJR159 Trp⁺ revertants

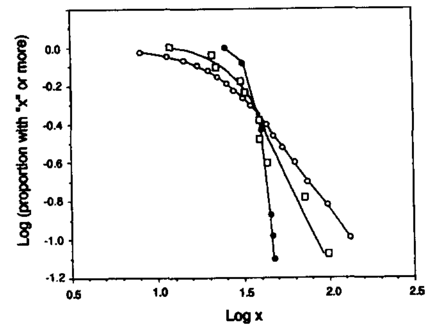
Cells plated ($\times 10^7$)	Newly arising colonies on day:						-Fold increase
	3	4	5	6	7	8	
92	22	21	61	136	335	334	41.3
92	98	35	66	116	236	617	11.9
76	30	39	44	112	217	344	26.2
84	71	55	67	117	276	187	10.9
90	22	42	69	200	350	277	43.6
91	35	32	30	100	179	238	17.5
84	39	25	50	124	172	291	17.9
76	12	28	53	191	258	451	82.8
81	43	53	96	302	265	367	26.2
86	32	28	46	88	213	271	21.2
112	21	16	38	87	195	335	33.0
79	38	28	46	67	161	173	13.5
Mean							28.8

Data obtained from 12 independent cultures are given. For each culture, a single colony was inoculated into 5 ml YPD and grown overnight at 30°. Each overnight culture was washed with H_2O and resuspended in a final volume of 1 ml H_2O. The entire culture was then plated in 0.1-ml aliquots on ten selective SC-Trp plates; there were thus approximately 10^8 cells per plate. Each number given represents the sum of the colonies on all ten plates for a given day post-plating. The -fold increase for each culture is the ratio of the cumulative number of colonies through day 8 to the number of colonies counted on day 3. Possible dependence of the number of late appearing revertants (days 4–8) on the number of early appearing revertants (day 3) in individual cultures was assessed by calculating the correlation coefficient (r) for these data. An insignificant value (−0.021) was obtained. Similarly, the numbers of revertants newly appearing on day 8 were not influenced by the numbers of previously appearing colonies ($r = 0.157$, $P > 0.05$).

curring on SC-Trp medium were, in fact, adaptive. In these experiments cells were held for 2–4 days on SC-Lys plates prior to plating on SC-Trp medium. The total numbers of Trp⁺ revertants present by day 7 post-plating were no different from the numbers present in control, unstarved cultures plated directly on SC-Trp (data not shown). The time-dependent distribution of colonies, however, in the lysine-starved cultures was quite different from that in the unstarved cultures. The numbers of colonies appearing at early times (3–5 days post-plating) were as much as twice the numbers appearing in the unstarved, control cultures. We interpret the increase in the numbers of early appearing Trp⁺ colonies in the lysine-starved cultures as being consistent with a lag in expression of the Trp⁺ phenotype in some of the revertants arising during nonselective growth in YPD. The phenotypic lag exhibited by some revertants when cells were directly plated on SC-Trp presumably would be overcome during lysine starvation prior to selective plating. For example, those Trp⁺ revertants attributable to acquisition of an extragenic suppressor likely would make less gene product than intragenic revertants and hence might take longer to accumulate enough gene product to initiate growth.

As expected from the relatively broad distribution

A. Day 3



B. Days 4-8

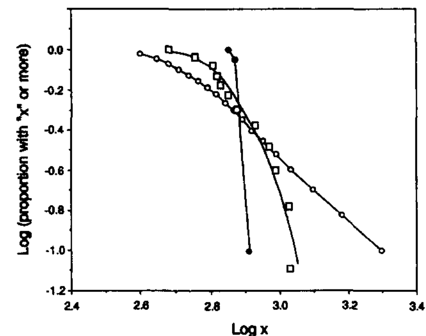


FIGURE 3.—Distributions of the numbers of Trp⁺ revertants. The theoretical and experimental distributions of the numbers of Trp⁺ revertants were obtained by plotting log (x) *vs.* log (proportion of cultures with x or more mutants), where "x" is the number of mutants per culture (see CAIRNS, OVERBAUGH and MILLER 1988). The squares represent experimental values calculated using the data in Table 5, the open circles are values for the LEA and COULSON distribution and the closed circles are values for the Poisson distribution.

of Trp⁺ revertant numbers, ANOVA using the Trp⁺ data in Table 5 supports the interpretation that many of the late appearing revertants were present at the time of plating. Whereas ANOVA results with the late appearing Lys⁺ revertants from independent cultures indicated that all were part of a single population, ANOVA using even the latest appearing Trp⁺ revertants gave highly significant F values (data not shown). While adaptive reversion at the *TRP1* locus may well be occurring in strain SJR159, the physiological differences between revertants make detection and study of the phenomenon nearly impossible.

DISCUSSION

The occurrence of what appear to be adaptive mutations has attracted considerable attention and the phenomenon now seems to be fairly well established in *E. coli* (CAIRNS, OVERBAUGH and MILLER 1988; HALL 1990, 1991; CAIRNS and FOSTER 1991). Our examination of the reversion of a *lys2* frameshift mutation in yeast provides evidence for adaptive mutation in a simple eukaryote as well. In a haploid yeast strain, Lys⁺ prototrophic revertants accumulate over a protracted period of time in the absence of detect-

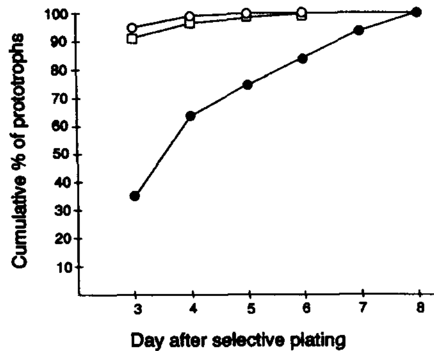


FIGURE 4.—Time-dependent appearance of Lys+ colonies. The cumulative percentage of prototrophs on days 3 through 8 post-plating for the reversion experiments (closed circles), reconstruction experiments (open circles) and transformation experiments (squares) are shown. To lessen the effects of jackpots on the early reversion data, the average cumulative percentages for each day were calculated using the data in Table 1.

able cell growth on selective media; they do not accumulate if direct selection for the revertant phenotype is not imposed. The growth rates of the Lys+ revertants in reconstruction assays are generally too high to be consistent with the presence of a population of slow growing, late appearing revertants at the time of initial plating. The results of transformation experiments with a *LYS2*-containing plasmid provide further support for the notion that late appearing revertants are the products of events occurring on the selective medium. Figure 4 summarizes the results of the Lys+ reversion analyses, the reconstruction experiments done using Lys+ revertants and transformation experiments done with a *LYS2* gene. This figure clearly illustrates that a much larger proportion of the Lys+ prototrophs appear late in the reversion assay than can be accounted for by physiologically determined delays in growth initiation alone.

In addition to the experimental results cited above, an examination of the distribution of the total numbers of Lys+ revertants demonstrated that it is much narrower than would be predicted assuming that reversion events occur only prior to selective plating (see Figure 1). The distribution falls between the theoretical LEA and COULSON and Poisson distributions, suggesting that the experimental data represent a composite of random and adaptive reversion events. While the deviation from the theoretical curves is consistent with the occurrence of adaptive mutations, we feel that little weight can be given to this particular observation on its own. There are a multitude of possibilities other than adaptive reversion that can be invoked to explain the observed deviations from theoretical curves (LENSKI, SLATKIN and AYALA 1991; CHARLESWORTH, CHARLESWORTH and BULL 1988; TESSMAN 1988). In the context of the data as a whole, however, and especially in light of ANOVA results indicating that the late appearing Lys+ revertants are members of a single population, we conclude that reversion to a Lys+ phenotype occurs in the absence of significant cell division when selectively advantageous.

In addition to examining the reversion of a *lys2* frameshift allele, we also examined the reversion of a *trp1* amber allele in the same strain. In our initial reversion experiments in which daily counts of Trp+ colonies were made, there were approximately 30-fold more late appearing revertants than early appearing revertants. As in the case of the *lys2* reversion experiments, the overall distribution of the numbers of Trp+ revertants was intermediate between the theoretical LEA and COULSON (random mutation) and Poisson (adaptive mutation) distributions. As suggested by reconstruction and starvation experiments and confirmed by ANOVA analysis, however, many of the late appearing Trp+ revertants were probably present at the time of selective plating and thus represent random rather than adaptive reversion events. Analysis of Trp+ revertants illustrates the extreme care that must be taken when examining possible adaptive mutation events and underscores the inherent danger in relying solely on the distributions of the mutants.

That mutations can be induced under starvation conditions when cells are stressed is not particularly surprising. What is surprising, however, is that new mutations seem to arise only when they are directly advantageous to the cell. Although it was originally suggested that the selective pressure imposed somehow "induces" mutations and that the flow of information might proceed from phenotype to genotype (CAIRNS, OVERBAUGH and MILLER 1988), it seems more likely that the selective pressure simply "fixes" what might otherwise be transient mutations. At present there is little information concerning the origin of these mutations: whether they result from stress-specific changes in cellular metabolism or whether they reflect normal cellular processes. Several models have been advanced to explain the origin of adaptive mutations after a selective pressure has been imposed. HALL (1990) has suggested that a small subset of cells enter a hypermutable state under starvation conditions. Supportive evidence for this model was the occurrence of auxotrophies of no apparent utility in 1.8% (2/110) of his adaptive revertants; no increase in auxotroph frequency was observed among the starved cells as a whole. In our experiments, we failed to detect additional auxotrophies in over 500 Lys+ and 1000 Trp+ late appearing revertants analyzed (data not shown). Also, in more recent work, HALL (1991) has presented data which are not consistent with the hypermutation model.

An alternative explanation for adaptive reversion has been put forth by DAVIS (1989). He suggested that induction of transcription under starvation con-

ditions might be mutagenic and this could, therefore, explain why mutations appear to be adaptive. In general, amino acid starvation (as employed by HALL 1990, 1991) induces the transcription of genes whose products are involved in the biosynthesis of the relevant amino acid. Similarly, in the absence of glucose, addition of an alternative carbon source such as lactose (as in the CAIRNS, OVERBAUGH and MILLER 1988 experiments) results in high levels of transcription of those genes whose products are involved in the metabolism of the sugar. While transcription could be evoked to explain adaptive mutations in these experiments, it does not account fully for the more recent bacterial data of CAIRNS and FOSTER (1991) in which adaptive reversion was observed using a constitutive *lac2* allele. Similarly, in our yeast experiments, the *lys2* allele was under control of the *CYC1* promoter, a promoter that responds to carbon source (ZITOMER *et al.* 1979) and heme (GUARENTE and MASON 1983) but would not be expected to be affected by the various amino acid starvation conditions. It should be noted, however, that we have observed that a *ura3* frameshift mutation reverts more frequently when fused to the strong *GAL1–10* promoter than when under control of its own, relatively weak promoter (S. JINKS-ROBERTSON, unpublished observations). In addition, KOROGODIN *et al.* (1991) have reported that the frequency of reversion of *ade2* and *leu2* alleles in yeast is related to their levels of transcription during residual growth after the initiation of specific starvation for these compounds. Indeed, it is possible that the increase in the reversion frequency at the *LYS2* locus reported here might be the result of a very large increase in the reversion frequency during very limited growth after selective plating.

The model we most favor to explain the phenomenon of adaptive mutation is that proposed by STAHL (1988). He suggested that DNA metabolism continues and chromosomal nicks and gaps accumulate under starvation conditions. Repair synthesis of the nicks and gaps by DNA polymerase would be expected to introduce mismatches into the DNA which, when incorporated into the transcribed strand, would occasionally result in the production of a functional gene product and hence would allow cell growth to begin. If the mismatch repair systems function poorly (or, alternatively, if DNA polymerase is particularly error prone) under starvation conditions, then the synthesis of functional product and the resulting growth might out-pace mismatch repair. This, in turn, would result in the fixation of selectively advantageous mutations.

In yeast, we have observed that the introduction of DNA lesions by irradiation with UV light immediately prior to plating increases reversion frequencies at all subsequent time points assayed. Lesions in DNA would be expected to increase DNA metabolism in starving cells and hence the corresponding probability that a growth-conferring DNA replication/repair error will occur as the cells starve on selective medium. Furthermore, we have presented evidence that holding the UV-irradiated cells for 2 days on medium where Lys$^+$ revertants cannot initiate growth reduces the accumulation of revertants, suggesting that the repair of UV-induced damage can be adaptive. We view these result as being consistent with the model proposed by STAHL.

While it seems that adaptive mutations do indeed occur, the underlying mechanism(s) remains to be elucidated. CAIRNS and FOSTER (1991) have demonstrated that certain *recA* and *lexA* alleles can affect reversion frequencies on selective medium, but no specific roles for these genes in the process are immediately obvious. We are currently assaying mutations affecting mismatch repair (KRAMER *et al.* 1989) and error prone DNA repair (see FRIEDBERG 1988) for their effects on adaptive mutation in yeast.

We appreciate the interest, support and suggestions of our colleagues at Emory University throughout this project. We thank FRANK STEWART for the computer program used to generate theoretical curves for the distributions of mutants and BOB MALONE for suggesting the use of DNase in transformation experiments. A special thanks goes to FRANK STAHL, BRUCE LEVIN, JOHN CAIRNS, ED LOUIS and RHONA BORTS for helpful and insightful comments on the manuscript. This work was supported by National Institutes of Health grant GM38464 to S.J.-R.

Note added in proof: Adaptive reversion at a different locus in yeast was reported during review of this paper (HALL, B. G., 1992 Selection-induced mutations occur in yeast. Proc. Natl. Acad. Sci. USA **89:** 4300–4303).

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