A Tester System for Detecting Each of the Six Base-Pair Substitutions in *Saccharomyces cerevisiae* by Selecting for an Essential Cysteine in Iso-1-Cytochrome c

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**ABSTRACT**

A collection of isogenic yeast strains that is specifically diagnostic for the six possible base-pair substitutions is described. Each strain contains a single, unique base-pair substitution at the Cys-22 codon of the *CYC1* gene, which codes for iso-1-cytochrome c. These mutations encode replacements of the functionally critical Cys-22 and render each strain unable to grow on media containing nonfermentable carbon sources (Cyc). Specific base-pair substitutions, which restore the Cys-22 codon, can be monitored simply by scoring for reversion to the Cyc+ phenotype. These strains revert spontaneously at very low frequencies and exhibit specific patterns of reversion in response to different mutagens. Only true (Cyc+) revertants were recovered after 7 days on selection medium. The following mutagen specificities were observed: ethyl methanesulfonate and N-methyl-N'-nitro-N-nitrosoguanidine, G-C → A-T; 4-nitroquinoline-1-oxide, G-C → T-A and G-C → A-T; diepoxybutane, A-T → T-A, A-T → G-C and G-C → T-A; 5-azacytidine, G-C → C-G. Methyl methanesulfonate induced all six mutations, albeit at relatively low frequencies, with preference for A-T → T-A and A-T → G-C. Ultraviolet light was the most inefficient mutagen in this study, consistent with its preference for transition mutations at dipyrimidine sequences reported in other systems. This tester system is valuable as a simple and reliable assay for specific mutations without DNA sequence analysis.

**ANALYSIS** of mutagen specificity is essential for understanding the molecular basis of mutagenesis. The most convenient tester systems for determining mutagenic potential and specificity are based on genetic reversion analyses. YANOFSKY, ITO and HORN (1966) utilized a set of *trpA* mutants to investigate mutagenic mechanisms in *Escherichia coli*. Mutants from the extensive collection of *Salmonella typhimurium* histidine auxotrophs were developed by AMES, LEE and DURSTON (1973) for detecting both frameshift and base substitution mutations. Their system was especially significant for having established a correlation between mutagens and carcinogens (McCANN *et al.* 1975). More recently, some of their strains have been used to detect specific base substitutions either by phenotypic screening of *hisG* revertants (LEVIN and AMES 1986) or by colony hybridization to sequence-specific probes (MILLER and BARNES 1986). In yeast, PRakash and SHERMAN (1973) devised a tester system to detect specific mutations based on reversion of defined *cyc1* mutants. A more comprehensive picture of mutagen specificity is generally obtained from tester systems that detect forward mutations. COULONDRE and MILLER (1977) utilized the *E. coli* lacI gene for this purpose. Their system is based on generation of nonsense codons at 78 different sites, which are subsequently identified by genetic mapping and suppression analysis. More recently, lacI has been exploited to determine mutational specificity by direct DNA sequencing, both in *E. coli* (FARABAUGH *et al.* 1978; CALOS and MILLER 1981; MILLER 1985; BURNS, ALLEN and GLICKMAN 1986; BURNS, GORDON and GLICKMAN 1987; SCHAAPER, DUNN and GLICKMAN 1987; SCHAAPER and DUNN 1987), and in human cells (HSIA *et al.* 1989). A number of other forward mutation systems, based on convenient methods for retrieval and sequencing of DNA, have been developed. These include the lambda *cl* gene (WOOD, SKOPEK and HUTCHINSON 1984); hybrid M13lac (LECLERC and ISTOCK 1982); the *E. coli* gpt and supF genes in mammalian systems (HAUSER *et al.* 1986; ASHMAN and DAVISON 1987; GREENSPAN, XU and DAVISON 1988); and the endogenous *aprt* gene of Chinese hamster ovary cells (GROSOVSKY *et al.* 1988). Two forward mutation systems, both designed with methods to readily retrieve altered DNA, have been developed in yeast. One is based on inactivation of the *SUP4-o* nonsense suppressor gene (PIERCE, GIRoux and KUNZ 1987), and the
other on inactivation of the \textit{URA3} gene (Lee et al. 1988).

A drawback to the tester systems described above is that in no case can mutational alterations be ascertained without additional genetic or biochemical manipulations. A method to unambiguously define mutations based on an easily scorable mutant phenotype would be extremely valuable. Recently, two such systems were described in \textit{E. coli}. Foster et al. (1987) established an assay for G-C \rightarrow T-A transversions based on selection for an essential serine in plasmid-encoded \( \beta \)-lactamase. Miller and coworkers described a set of \textit{lez}2 mutants to detect all six base substitutions (Cupple and Miller 1989), as well as specific frameshift mutations (Cupple et al. 1990), based on selection for essential active site residues in \( \beta \)-galactosidase.

In this paper I describe a convenient system to detect each of the six base substitutions in yeast. The system is based on the critical requirement for cysteine at position 22 of iso-1-cytochrome \( c \), encoded by the \textit{CYCI} gene. Six isogenic strains, each containing a unique, single base substitution in codon-22, were constructed. These strains do not grow on media containing glycerol as the sole carbon source (\textit{Cyc}~) and revert to \textit{Cyc}+ only by defined substitutions that restore the \textit{Cys}-22 codon. Therefore, reversion to the \textit{Cyc}+ phenotype is diagnostic for specific base substitutions.

\section*{MATERIALS AND METHODS}

\textbf{Yeast strains, media and chemicals:} Strains YM1-1YM7 are isogenic derivatives of strain S260-11B (\textit{MATa cy1-706:CYH2 cy6-67 ura3-52 leu2-3, 112 cyh2}) and differ from each other only by single base substitutions within codon-22 of the \textit{cy1} gene. Strain S260-11B and the \textit{cy1}-706:CYH2 allele were described previously by Baim and Sherman (1988). Diploid strains YM151-1YM57 were constructed by crossing strains YM1-1YM7, respectively, with strain B-7462 (\textit{MATa cy1-1 cy6-77 ura3-52 his1-1 can1-100}) and selecting for diploids on minimal medium supplemented with uracil. The \textit{cy1-1} (Singh and Sherman 1978; Stiles et al. 1981) and \textit{cy6-67} (T. Cardillo and F. Sherman, unpublished data) alleles are complete deletions of their respective structural genes. The symbol Cyc is used to denote phenotypes, corresponding to either the ability (\textit{Cyc}+) or inability (\textit{Cyc}−) to grow on media containing glycerol as the sole carbon source.

Revertants of strains YM152-1YM57 were selected on YPGD medium, consisting of 1% yeast extract, 2% peptone, 3% glycerol, 0.1% glucose and 2% agar. Glucose is important in this medium to obtain efficient and reproducible reversion frequencies. Initial growth under nonselective conditions apparently stimulates reversion frequencies, an observation reported initially by Fink and Lowenstein (1969); this effect presumably reflects a requirement for DNA replication in order to convert mutagen-induced lesions into mutations. Although Sherman et al. (1974) reported that initial growth on nonselective medium did not increase reversion frequencies of \textit{cy1} mutants, this is probably a consequence of the residual growth afforded by \textit{CYC7}-encoded iso-2-cytochrome \( c \) in their strains. Glucose at 0.1% seemed to be optimal in that higher concentrations did not further stimulate reversion frequencies, but resulted in higher background growth of the mutants.YPD, YPG and minimal media containing either 2% glucose or 3% glycerol are standard yeast media (Sherman, Fink and Hicks 1983). Minimal medium was supplemented with uracil (20 \( \mu \)g/ml) and leucine (30 \( \mu \)g/ml) as required. Medium containing \( 5' \)-fluoroorotic acid was described by Boeke et al. (1987).

\textbf{Chemical mutagens} were purchased from Sigma Chemical Co. and were used without additional purification.

\textbf{In vitro mutagenesis:} Oligonucleotide-directed \textit{in vitro} mutagenesis was performed according to the technique of Kunkel, Roberts and Zakour (1987) using bacteriophage M13-CYC1-B/H. M13-CYC1-B/H is M13mp19 containing the 2.5-kb \textit{BamHII}-HindIII \textit{CYCI}+ DNA fragment. Single-stranded, uracil-containing DNA was obtained from bacteriophage M13-CYC1-B/H propagated in \textit{E. coli} strain CJ236 (\textit{dut1 ung1 thi1 relA1 p}r)C105 (Cmr). Sequence changes in the \textit{Cys}-22 codon were directed with the following four oligonucleotides:

\begin{align*}
\text{ORR-6} & : TCTCAATCTCCACACCCG; \\
\text{ORR-7} & : TCTCAATCGCCACACCGT; \\
\text{ORR-8} & : CTGAAAGCCACACCGT; \\
\text{ORR-9} & : CTGAAAAGCCACACCGT.
\end{align*}

The underlined bases indicate the directed mutations. Oligonucleotides were synthesized using the Biosearch model 8650 DNA Synthesizer and were purified by polyacrylamide gel electrophoresis as described by Lo et al. (1984). After completion of the mutagenesis procedure, mutations were confirmed by DNA sequencing.

\textbf{Gene transplacements:} The \textit{CYC1} gene and the four site-directed mutations in M13-CYC1-B/H were subcloned from RF DNA as 2.5-kb \textit{BamHII}-HindIII fragments into the yeast integrating vector YIp5 (Struhl et al. 1979). The two \textit{Cys}-22 alterations, previously isolated as 246-bp EcoRI-KpnI fragments in M13mp19 (Hampsey, Das and Sherman 1986), were first transferred as EcoRI-KpnI fragments to pM19 and then subcloned as 2.5-kb \textit{BamHII}-HindIII DNA fragments into YIp5. pM19 contains the \textit{cy1} \textit{BamHII}-HindIII fragment with a unique \textit{SacI} site between the EcoRI and KpnI sites (M. Hampsey, unpublished) that was used as a marker to confirm EcoRI-KpnI fragment replacements.

Transplacement of the \textit{cy1} alleles into the normal chromosomal locus was performed by integrative transformation and subsequent excision as described by Baim and Sherman (1988), except that eviction of the resident \textit{CYC1-706:CYH2} allele and flanking plasmid DNA was obtained by first selecting for 5'-fluoroorotic acid resistance (Boeke et al. 1987) and subsequently screening for cycloheximide resistance. Single copy transplacement at the \textit{cy1} locus was determined for each strain by Southern blot analysis and the codon-22 mutations were confirmed by reisolating and sequencing \textit{cy1} DNA fragments (data not shown).

\textbf{Retrieval of DNA fragments encompassing \textit{cy1} codon-22:} The 246-bp EcoRI-KpnI fragments, which include the coding information for amino acids 2-83, were cloned directly from total yeast DNA into M13mp19 as described previously (Hampsey, Das and Sherman 1986). Alternatively, DNA was amplified from total yeast DNA by the polymerase chain reaction, using the GeneAmp kit and DNA Thermal Cycler manufactured by Perkin Elmer Cetus and synthetic oligonucleotides oJN-24 and oRB-27. (oJN-24 is the \textit{CYC1} noncoding strand sequence from \textit{-74} to \textit{-57} and oRB-27 is the coding strand sequence from \textit{391} to \textit{372}; +1 is the A of ATG initiation codon.) Amplified DNA was
then subcloned as 246-bp EcoRI-KpnI fragments into M13mp19.

DNA sequence analysis: Single-stranded DNA was isolated from M13mp19 derivatives containing either 2.5-kb BamHI-HindIII or 246-bp EcoRI-KpnI cyclin DNA fragments. Sequencing was performed according to the dideoxynucleotides method, following the protocol supplied with the United States Biochemical sequencing kit. At least two independent clones from PCR-amplified DNA were sequenced to avoid potential PCR artifacts. Codon-22 sequences were determined from BamHI-HindIII clones using the primer oJN-24 (described above); EcoRI-KpnI fragments were sequenced using the M13 universal primer.

Estimation of iso-1-cytochrome c levels: Iso-1-cytochrome c amounts were determined by low temperature (−196°C) spectroscopic examination of intact yeast cells as described by SHERMAN and SLONIMSKI (1964). The spectroscope was obtained from Central Scientific Co. (Franklin Park, IL) and was mounted on a standard monocular laboratory microscope. A 1000 W projector lamp was used as the light source.

Reversion analysis: Strains YMH52–YMH57 were grown for 2 days to approximately 7 x 10^7 cells/ml in YPD medium at 30°C, harvested, and resuspended at 1 x 10^8 cells/ml in 50 mM potassium phosphate buffer, pH 7.0 (KP buffer). Chemical mutagenesis (except 5-AZ) was performed at concentrations of 1 x 10^8 cells/ml in KP buffer at 23°C as described below for each mutagen. Cell survival following each mutagenic treatment was monitored by plating appropriately diluted cells on YPD plates and scoring colonies after 2–3 days at 30°C. Spontaneous and induced revertants were selected on YPGD medium at 2–5 x 10^7 cells per plate and scored after 7 days at 30°C.

EMS, MMS and DEB were added to cell suspensions at final concentrations of 2, 0.5 and 0.2%, respectively. After incubation for the indicated times, samples were withdrawn and added to an equal volume of sterile 10% sodium thiosulfate to inactivate the mutagens. Cells were collected by centrifugation, washed twice, and spread on YPGD plates. MNNG and NQO were dissolved in acetone at 10 mg/ml immediately before use, diluted in KP buffer to 4 mg/ml and 1 mg/ml, respectively, and added to cell suspensions at final concentrations of 40 µg/ml and 10 µg/ml. Following incubation for the indicated times, samples were withdrawn, treated, washed as described above for the other mutagens, and spread on YPGD plates.

UV mutagenesis was done by irradiating cells spread directly on YPGD medium. Irradiation was performed in the dark using a 254-nm lamp (UVP, Inc., San Gabriel, California) mounted 15 cm above the surface. Plates were incubated in the dark to avoid photoreactivation.

5-AZ was prepared immediately before use at 10 mg/ml in minimal medium (2% glucose) supplemented with uracil, and filter sterilized. The indicated concentrations of 5-AZ were prepared by dilution using the same medium. Cultures were inoculated with approximately 10^6 cells and incubated with vigorous aeration for 24 hr to densities of 2–4 x 10^7 cells/ml. Cultures were collected by centrifugation, washed twice, and spread on YPGD plates.

RESULTS

Cys-22 mutants: Seven isogenic yeast strains, differing only by single point mutations in the Cys-22 codon of CYCl, were constructed by molecular genetic techniques, as described in the MATERIALS AND METHODS. These strains are presented in Table 1. Strain YMH1 contains the normal amount of iso-1-cytochrome c observed in a CYCl strain, as determined by low temperature (−196°C) spectroscopic analysis, and exhibits a Cyc+ phenotype. Strains YMH2–YMH7 are completely deficient in iso-1-cytochrome c and are phenotypically Cyc−. Hemizygous (cycl/cycl-1) diploid derivatives, designated YMH51–YMH57, were obtained by crossing each haploid strain with strain B-7462 (Table 1). Consistent with the haploid phenotypes, strain YMH51 is Cyc− and strains YMH52–YMH57 are Cyc−.

Generation of revertants: Strains YMH52–YMH57 were tested by monitoring reversion to Cyc+ in response to seven different mutagens. Spontaneous reversion was measured as a control in each mutagenesis experiment. In total, approximately 1.2 x 10^9 untreated cells for each strain were plated on selective medium during the course of these experiments from which spontaneous reversion frequencies were determined. All six strains revert spontaneously only at very low frequencies (Table 2). The G-C → A-T transition and G-C → T-A transversion exhibited the highest spontaneous reversion rates, occurring at frequencies of 1.3 x 10^-8 and 1.8 x 10^-8. The A-T → G-C transition and G-C → C-G transversion arose at barely detectable frequencies, occurring at about 2 x 10^-9 and 1 x 10^-9, respectively. No A-T → T-A or A-T → C-G transversions were detected during the course of these experiments, corresponding to spontaneous reversion frequencies of <1 x 10^-9. GIROUX et al. (1988) reported the same pattern of spontaneous base substitutions at the SUP4-o locus, suggesting that this particular region of cycl does not undergo aberrant spontaneous mutation.

EMS and MNNG are alkylating agents whose primary mutagenic lesions are believed to be O6-ethylguanine and O6-methylguanine, respectively. These mutagens induce primarily G-C → A-T transitions in several different organisms (PRakash and SHERMAN 1973; COULONDRE and MILLER 1977; BURNS, ALlen and GLICKMAN 1986; KOHALMI and KUNZ 1988;

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### Table 1

<table>
<thead>
<tr>
<th>Haploid strain</th>
<th>Diploid strain</th>
<th>Codons 21–23</th>
<th>Amino acid 22</th>
<th>Mutational specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>YMH1</td>
<td>YMH51</td>
<td>CAA TGC CAC</td>
<td>Cys</td>
<td>G-C → A-T</td>
</tr>
<tr>
<td>YMH2</td>
<td>YMH52</td>
<td>CAA GGC CAC</td>
<td>Arg</td>
<td></td>
</tr>
<tr>
<td>YMH3</td>
<td>YMH53</td>
<td>CAA AGC CAC</td>
<td>Ser</td>
<td>A-T → T-A</td>
</tr>
<tr>
<td>YMH4</td>
<td>YMH54</td>
<td>CAA GGC CAC</td>
<td>Gly</td>
<td>G-C → T-A</td>
</tr>
<tr>
<td>YMH5</td>
<td>YMH55</td>
<td>CAA TCC CAC</td>
<td>Ser</td>
<td>G-C → C-G</td>
</tr>
<tr>
<td>YMH6</td>
<td>YMH56</td>
<td>CAA TTC CAC</td>
<td>Phe</td>
<td>A-T → C-G</td>
</tr>
<tr>
<td>YMH7</td>
<td>YMH57</td>
<td>CAA TAC CAC</td>
<td>Tyr</td>
<td>A-T → G-C</td>
</tr>
</tbody>
</table>

* DNA sequence changes in codon 22 are underlined. Codons 21 and 23 are included to depict the immediate sequence context flanking the mutational targets. For the complete sequence of the CYCl structural gene see HAMPSEY, DAS and SHERMAN (1986).
be rather nonspecific with respect to induction of base induced both mutations at >1000-fold above back-response to EMS and MNNG. Indeed, a 2-hr exposure predicts that YMH52 should exhibit the strongest response to MNNG, resulting in stronger reversion to reversion of YMH52 at 9.4 T-A transversion and A-G C tran-

ground. These data suggest that the primary mutagenic lesion induced by MMS occurs at A-T base-pairs.

In the most comprehensive study of UV specificity in yeast to date, Kunz et al. (1987) found that 93% of UV-induced mutations at the SUP4-o locus are single base substitutions and that 85% of these are transitions with a clear preference for G-C → A-T over A-T → G-C. Furthermore, UV-induced mutations are sequence context dependent. Ninety-nine percent of all substitutions occurred at sites of adjacent purines with the mutation arising primarily at the 3' position of the dipyrimidine (Kunz et al. 1987). Similar specificity for UV has been found at other yeast loci (Ivanov et al. 1986; Lee et al. 1988) and in other organisms (Wood, Skopek and Hutchinson 1984; Miller 1985; Schaefer, Dunn and Glickman 1987; Hsia et al. 1989). Inspection of the sequence context flanking the target base-pairs within codon-22 (Table
1) reveals that neither of the transition-specific sites is part of a dipyrimidine sequence. Each of the four transversion-specific sites is part of a dipyrimidine sequence, although only the sites specific for G→C and A→T occupy the 3' position. These are the two most infrequently detected base substitutions identified by Kunz et al. (1987), representing only 3.2% of all UV-induced base substitutions at SUP4+o. This information suggests that the cycl tester strains should exhibit only a weak response to UV. Indeed, none of the six strains was efficiently reverted, even at a UV dose that resulted in only 35% cell survival (Table 2).

NQO-induced mutagenesis has been studied mainly in the lacI (Coulondre and Miller 1977) and cycl (Prakash and Sherman 1974; Prakash, Stewart and Sherman 1974) systems. In both studies NQO was reported to induce primarily G→A transversions and G→C→T→A transversions. Similar specificity is reported here. Two hours of exposure to 10 μg/ml NQO resulted in a 500-fold induction of G→C→T→A and a 210-fold induction of G→C→A→T. Little or no reversion of the other strains was observed. Interestingly, the relative G→C→T→A and G→C→A→T response is opposite to that reported in lacI and at the other sites at cycl, suggesting that sequence context can affect the relative efficiency of NQO-induced mutations.

DEB is a bifunctional alkylating agent (reviewed by Ehrenberg and Hassain 1981) and an effective mutagen in yeast (Olszewski and Kilrey 1975). Although its mutagenic potential has been investigated in yeast rad mutants (Zuk et al. 1980; Zaborowska, Zuk and Swietlinska 1982; Lecka-Czernik et al. 1984), the specificity of DEB-induced mutations has not been reported. The bifunctional nature of DEB suggests that it may induce a broader spectrum of mutations than the monofunctional alkylating agents. Following a 2-hr exposure to 0.2% DEB, three of the tester strains were efficiently reverted. The A→T→G→C transition was induced at a frequency of 10−5 and the A→T→T→A and G→C→T→A transversions at about 5×10−6 (Table 2). These frequencies represent >5000-fold induction of A→T→G→C and A→T→T→A and about 250-fold induction of G→C→T→A. Essentially no stimulation of the other three substitutions occurred.

The base analog 5-AZ was reported by Zimmermann and Schell (1984) to induce both mitotic recombination and point mutation in yeast. In the S. typhimurium tester systems, 5-AZ induced exclusively G→C→G transversions (Levin and Ames 1986; Miller and Barnes 1986). The same specificity, along with much weaker induction of G→C→T→A transversion, was found in the lacZ tester system (Cuples and Miller 1989). These results suggest that strain YM55 should respond to 5-AZ, which was indeed observed (Table 2). Very weak stimulation of G→C→T→A also occurred, at a rate barely above background.

Analysis of revertants: If reversion analysis of the strains described here is to be diagnostic for specific mutations it is essential either that all revertants are true (CYCI+) revertants or that true revertants can readily be distinguished from pseudorevertants. This consideration was addressed by examining iso-1-cytochrome c levels for many of the revertants isolated in this study. The rationale here is that true revertants will display the normal amount of iso-1-cytochrome c, whereas suppression of Cys-22 defects by second-site mutations is likely to be partial and would yield pseudorevertants that contain diminished amounts of iso-1-cytochrome c. Low temperature (~196°) spectroscopic examination of over 400 revertants that arose after 7 days of incubation on glycerol medium, including representative revertants of each strain induced by each of the mutagens in this study, revealed only strains containing the normal amount of iso-1-cytochrome c with normal spectral properties (data not shown). Furthermore, sequence analysis of CYCI DNA isolated from 21 independent revertants, including revertants induced by each of the mutagens used in this study, revealed only the CYCI+ sequence (data not shown).

I wish to note that a second class of revertants of strain YM52 arose after prolonged incubation on selective medium, discernable as tiny colonies after 12-15 days. They did not arise spontaneously but were induced by each of the mutagens in this study except 5-AZ. The strongest response was elicited by 2-hr exposure to 2% EMS, resulting in revertants at a frequency of about 10−6. These revertants were phenotypically distinct from the other revertants and from the CYCI+ strain, YM51, in that they grew markedly slower on YPG medium and contained less than 5% of the normal amount of iso-1-cytochrome c. Their slow growth phenotype allowed them to be readily distinguished from true revertants. Therefore, they did not interfere with the specificity of strain YM52 for detecting the G→C→A→T transition and they are not represented in Table 2. Further characterization of one of these revertants revealed that it retains the sequence CCG at codon-22 with no other changes in the structural gene (R. Berroteran and M. Hampsey, unpublished data). No pseudorevertants of strains YM53-YM57 were found, even after prolonged incubation on selective medium.

Discussion

The critical requirement for cysteine at position 22 of iso-1-cytochrome c is a fundamental premise of this system. This requirement is supported by the follow-
ing evidence. First, Cys-22 is evolutionarily invariant throughout the phylogenetic series of nearly 100 eukaryotic cytochromes c (HAMPSEY, DAS and SHERMAN 1986, 1988). Second, the crystal structure of iso-1-cytochrome c (LOUIE, HUTCHEON and BRAYER 1988; LOUIE and BRAYER 1990) shows that Cys-22 forms a covalent thioether linkage to the heme prosthetic group, providing a structural basis for the Cys-22 requirement and suggesting that this requirement is critical. Third, all known replacements at position 22, which include serine, glycine, arginine, phenylalanine and tyrosine (HAMPSEY, DAS and SHERMAN 1986, 1988; Table 1), abolish iso-1-cytochrome c function. This information suggests that only cysteine at position 22 will allow efficient growth on media containing nonfermentable carbon sources. The observation that all revertants of strains YMH52–YMH57 that arise after 7 days on selective medium contain either the CYCI+ DNA sequence and/or contain iso-1-cytochrome c that is indistinguishable in amount and spectral characteristics from that of CYCI+ strains validates this premise.

The cycI tester strains were designed to minimize the possibility of obtaining Cyc+ revertants by mutational pathways other than the single base substitutions that restore the normal Cys-22 codon. Each of the base substitutions in codon-22 is a missense mutation (Table 1) and is therefore not suppressible by classical nonsense suppressors. These strains are homozygous for the cyc7–67 allele, which is a deletion of the entire structural gene encoding iso-2-cytochrome c (T. CARDILLO and F. SHERMAN, unpublished data); therefore, revertants can not arise either by mutations that result in overexpression of iso-2-cytochrome c or by recombination between the cycI and CYC7 genes, both of which have been described previously as classes of revertants of cycI mutants (MC-KNIGHT, CARDILLO and SHERMAN 1981; ERNST, STEWART and SHERMAN 1981). Furthermore, revertants also can not arise by reversion of the cycI–1 allele or by recombination between cycI and cycI–1, since cycI–1 is a deletion of the entire CYC1 region (SINGH and SHERMAN 1978; STILES et al. 1981).

This system is preferable to the one described by RAKASH and SHERMAN (1973) for detecting specific substitutions in yeast. Their system, also based on reversion of cycI mutants, was comprised of nine translation initiation codon mutants and two nonsense mutants. Although their system provided the first detailed characterization of mutational specificity in a eukaryotic organism, each of their strains could revert by more than one pathway. Consequently, reversion of specific mutants did not necessarily correlate with specific base substitutions. Rather, they had to rely on patterns of reversion and protein sequence analysis to elucidate mutational specificities. By contrast, the Cys–22 missense mutants described here revert by only a single pathway. Furthermore, these strains are isogenic, allowing for the relative rates of all six base substitutions to be compared in a single genetic background.

The response of these strains to several different mutagens was determined (Table 2). Predictable patterns of reversion were obtained with mutagens whose specificities have been described previously. Furthermore, the low spontaneous reversion frequencies for all six strains provided sufficient sensitivity to detect relatively weak stimulation of other base substitutions. For example, although EMS induced primarily the G·C → A·T transition, the other five base substitutions were also detected (Table 2). These strains should therefore be valuable for detecting the potential and specificity of relatively weak mutagens, although a more extensive analysis than is presented here would be required to accurately determine induction over background.

The specificities of 5-AZ and DEB in yeast had not been described previously, although recent studies using S. typhimurium (LEVIN and AMES 1986; MILLER and BARNES 1986) and E. coli (CUPPLES and MILLER 1989) demonstrated that 5-AZ induced either primarily or exclusively G·C → C·G transversions. 5-AZ displayed the same specificity in this tester system, inducing almost exclusively reversion of strain YMH55 (Table 2). Interestingly, DEB efficiently induced three different base substitutions, A·T → T·A, A·T → G·C and G·C → T·A; little or no stimulation of the other substitutions was detected (Table 2). These data suggest that DEB induces at least two different DNA lesions and that alkylation of A·T base-pairs may be the primary mutagenic lesion.

A limitation to any reversion-based tester system is that mutational alterations are detected in the context of a single DNA sequence. Sequence context is known to affect mutational spectra in response to certain mutagens, first recognized in studies of the bacteriophage T4 rII locus by BENZER (1961). A less than ideal context presumably accounts for the weak effect of UV in this study (see RESULTS). In general, a detailed analysis of mutagen specificity should rely on detection of mutations at multiple sites and is most reliably determined in forward mutation systems. Nonetheless, the potential problem associated with context effects can be diminished in reversion systems by scoring for mutations at multiple sites. The known structural requirements for functional iso-1-cytochrome c provides a basis for expanding the cycI tester system for this purpose. Both Cys-19 and Cys-22 covalently bind heme in thioether linkages, and His-23 and Met-85 function as axial ligands to the heme iron. The structural roles of these residues and their evolutionary conservation strongly suggest that all four
amino acids are critical for iso-1-cytochrome c function. [The only phylogenetic variance at these four positions is in the cytochromes c from Crithidia and Euglena (Hampsey, Das and Sherman 1986) where alanine occupies the equivalent of position 19. However, alanine does not functionally replace Cys-19 in iso-1-cytochrome c (M. Hampsey, unpublished data.)] Since histidine and cysteine are encoded by only two codons and methionine is encoded by a single codon, all of which require at the DNA level both A-T and G-C base pairs, additional mutants specific for detection of all six base substitutions can be constructed. Accordingly, simple reversion analysis would score for mutational specificity in four different sequence contexts. Construction and testing of these strains are in progress.

Hemizygous (cyc1/cyc1-1) diploids were used in this study in order to mask the effects of recessive mutations that could interfere with the reversion analyses. Diploid strains YMH2-YMH57 were found to be more resistant than haploid strains YMH2-YMH7 (Table 1) to the lethal effects of the various mutagens (haploid data not shown). These strains also yielded revertants of more uniform colony size. Moreover, reversion frequencies were more reproducible using the diploid strains (haploid reversion frequencies are not presented), an effect also reported by Prakash and Sherman (1973). Haploid strains, on the other hand, have the potential to be used to uncover recessive mutations identifying genetic loci that affect mutagenic potential and specificity. For example, haploid strains could be used to identify mutator genes that affect specific base substitutions. Miller and co-workers have exploited their lacZ tester system for this purpose. By screening for colonies with increased reversion rates to Lac⁺, they have recovered two new mutator loci in E. coli, mutM (Cabrera, Ngheim and Miller 1988) and mutY (Ngheim et al. 1988), that generate G-C → T-A transversions. Only recently has a mutator gene specific for a single base substitution been described in a eukaryotic system. The yeast rad18 mutator was shown by Kunz, Kang and Kohalmi (1991) to specifically increase G-C → T-A transversions.

In summary, the cyc1-based tester system described here offers a simple, inexpensive, and sensitive means to survey mutagen specificity with respect to single base substitutions in a eukaryotic organism. This system can be used to rapidly screen a large number of potential mutagens or to screen for mutagens that induce specific base substitutions. The effects of different physiological variables on mutagenic potential and specificity can readily be evaluated. This system may also prove to be valuable for isolating new mutator strains that could provide additional insight into the components affecting the fidelity of DNA replication and repair.

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LITERATURE CITED


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