A New Reversion Assay for Measuring all Possible Base Pair Substitutions in *Saccharomyces cerevisiae*

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ABSTRACT

A TRP5-based reversion system has been developed that allows the rates of all possible base pair substitutions to be measured when the TRP5 locus is in both orientations relative to a defined origin of replication. This system should be useful for a wide variety of mutation and repair studies in yeast.
Reversion assays that can detect specific base pair substitutions have proven extremely useful. One of the best known is the set of *E. coli* lacZ alleles that can individually detect all possible base pair substitutions (Cupples and Miller 1989). Hampsey developed a comparable tester system for yeast that can detect all possible base pair substitutions by reversion of various point mutations at the essential Cys-22 of the CYC1 gene (Hampsey 1991). Although the cyc1 reversion system has proven quite useful in a number of studies (Thomas *et al.* 1997; Earley and Crouse 1998; Bruner *et al.* 1998; Scott *et al.* 1999; Brachman and Kmiec 2003) there are several problems with this assay. As noted in the original paper, the reversion assay requires diploid strains (Hampsey 1991) for reasons that are not entirely clear. When genetic manipulation of the strain background is desired, the requirement for diploidy substantially increases the difficulty of strain construction. In addition to a hemizygous cyc1 mutant gene, it is necessary to perform the reversion analysis in a cyc7 deletion background in order to avoid gene conversion of the CYC1 gene from the similar CYC7 gene (Hampsey 1991). In work subsequent to our published work with this system, we observed strain isolates that would not revert under any circumstances (results not shown). The reason for this problem remains unknown, but the appearance of such strains greatly complicated the use of this system for genetic analysis. We report here the development of a series of strains that together can detect any possible base pair substitution in the Glu-50 residue of TRP5. Each strain reverts only via a true reversion event and revertants are easily assayed by plating on media lacking tryptophan. The only genetic requirement for the assay is that all genes of the Trp pathway other than TRP5 be functional, and either haploid or diploid strains can be used. Moreover, we have placed the mutant trp5 gene in both orientations close to a dependable origin of replication, so that the effect of replication orientation on
mutagenesis can be studied. We show here that these strains can be used to determine very easily the mutagenic specificity of various mutagens.

The difficulty in developing a specific reversion assay is finding an absolutely essential residue in a gene whose reversion can be easily scored. In addition, in order to be able to study all possible base pair reversion events in a similar sequence context, the residue needs to have a codon with unique first and second nucleotides. The *S. cerevisiae* TRP5 gene is homologous to the *E. coli* tryptophan synthase α and β chains, encoded by *trpA* and *trpB* respectively (Zalkin and Yanofsky 1982). It had been shown that the Glu-49 residue of *E. coli* trpA was essential for function and could not be substituted by any other amino acid (Yutani *et al.* 1987). An alignment of a variety of fungal and bacterial tryptophan synthases indicated that the residue corresponding to *E. coli* Glu-49 was conserved in all species and therefore was likely essential in the other enzymes (Fig. 1A). The *S. cerevisiae* TRP5 residue homologous to *E. coli* trpA Glu-49 is Glu-50. A convenient feature of the *S. cerevisiae* sequence is that this codon is part of a *TaqI* restriction site, which is destroyed by any change in positions 1 or 2 of the codon. Thus any mutation in nucleotide sequence would presumably give a strain that would be Trp− and that would lack the *TaqI* site (Fig. 1B). Correspondingly, any true revertant should restore the *TaqI* site. In order to place the mutant gene in an region near a reliable origin of replication, the TRP5 gene was first deleted from the genome and then inserted in both orientations near the *ARS306* origin which has been well characterized (Deshpande and Newlon 1992; Theis *et al.* 1999; Poloumienko *et al.* 2001). All strains with the introduced *trp5* mutations were phenotypically Trp−, indicating the essential nature of the Glu-50 residue.

Various *trp5* strains were grown and plated on Trp− plates, either with or without prior mutagen treatment. Revertants were chosen at random from a variety of strains and mutagen
treatments and the region surrounding the introduced mutation was amplified by PCR and digested with TaqI in order to determine if the revertant were a true revertant, or whether reversion could be obtained by second site mutations. Out of the 119 revertants tested, there were none that did not gain the TaqI site, indicating that reversion required the restoration of the Glu-50 residue. However, 12% of the tested revertants gave a PCR product that did not cut completely, even after repurification of the revertant colony. The partial digestion was not due to insufficient restriction enzyme or impurities in the DNA; in addition, there was usually approximately equal amount of digested and undigested product (results not shown). Sequence of two of these revertants showed approximately equal amounts of the original mutant sequence and the reverted wildtype sequence. Such revertants were seen rarely upon MMS treatment, more frequently with UV treatment, and none were observed under other conditions. Therefore it may be that replication blocking lesions can result in gene duplication events prior to reversion, but more work will have to be done to clarify the cause and mechanism of these reversion events.

Results of the treatment of the trp5 strains with various mutagens are displayed in Fig. 2. Spontaneous reversion of the mutant strains is very low; in some cases no revertants were seen in multiple cultures. UV damage caused primarily GC→AT transitions, though there were increases in all types of mutations. UV targets primarily dipyrimidines (Friedberg et al. 1995). Thus, UV was a poor mutagen for reverting the cyc1 mutations in the Hampsey strains, because the proper sequence context was missing (Hampsey 1991). Here, however, the A149G strain provides a sequence context that matches a prime target for UV mutagenesis, and thus gives a high frequency of reversion. 5AZ was the most specific mutagen, giving almost exclusively CG→GC transversions, as also seen in the cyc1 gene (Hampsey 1991). EMS is known to be
highly specific in its mutagenicity, and this was seen in these experiments, as the only significant reversion frequencies were GC→AT transitions, as also observed in cyc1 reversion (Hampsey 1991). MMS gave significant numbers of several different types of revertants, with the greatest number being AT→GC transitions. Although similar in many aspects of the reversion spectrum, the relative sensitivities of cyc1 and trp5 to MMS-induced reversion by GC→AT transitions and GC→TA transversions differed. Many of the mutations caused by MMS are likely due to error prone synthesis across abasic sites (Glaab et al. 1999; Xiao et al. 2001); the most likely explanation for the difference in MMS mutation specificity between the two assays is sequence context effects. The base analogue HAP gave both AT→GC and GC→AT transitions, as has been found previously (Shcherbakova and Pavlov 1993; Pavlov et al. 1996; Kulikov et al. 2001), although in a different ratio. In E. coli, it was observed that HAP induced equal numbers of AT→GC and GC→AT transitions in the lacI gene (Pavlov et al. 1996). In yeast, in the URA3 gene, 21 out of 29 sequenced HAP mutations were GC→AT transitions and 5 out of 29 were AT→GC transitions (Shcherbakova and Pavlov 1993); in the LYS2 gene all transitions were GC→AT (Kulikov et al. 2001). In the present reversion assay, there were substantially more AT→GC transitions than GC→AT transitions. A strand and sequence context bias has been previously noted for HAP mutagenesis (Kulikov et al. 2001) and the differences in spectrum we see here are yet another indication of this bias.

Mutagens were tested on the trp5 mutants in both orientations relative to the nearby ARS306 origin of replication; in only two cases were the differences as much as two fold between the two orientations: in strain A149G with UV damage and strain G148A with HAP. In both cases, although the standard deviations do not overlap, the difference is less than two fold and it would be necessary to perform additional experiments to be sure that the observed
differences were a function of orientation of replication. For most of the mutagens, there have been no previous experiments in yeast that have shown any difference in mutation due to replication direction. However, it was observed that reversion of a ura3-29 allele by HAP showed a marked strand bias of 7 fold when the gene was in a similar location to our TRP5 gene (Pavlov et al. 2002). As mentioned above, HAP is known to display marked sequence context bias (Kulikov et al. 2001) and it may be that mutagenesis is affected by the replication strand only in certain contexts. The activity of the ARS306 origin is not known to be affected by strain background (Zhu et al. 1992; Deshpande and Newlon 1992) and the ARS306 origin in our strains has been found to fire efficiently (Ann Dershowitz and Carol Newlon, personal communication). Therefore we believe the difference we observe with previous data (Pavlov et al. 2002) is not due to the lack of directional replication through the TRP5 gene.

The set of mutations at the Glu-50 codon of TRP5 can be added to the small list of strains that can show all possible base pair reverions individually. The ease of revertant selection, lack of second site revertants, and simple genetic requirements should make this assay useful in a number of circumstances. Because a given strain will reveal only one type of reversion event and because the background of spontaneous reversion is so low, it is possible to observe mutational events that occur at such low frequencies they would rarely be revealed by sequencing of random events. This property makes it possible to study certain rare mutational events, even in the background of other types of events that are much more frequent. For example, given their frequency with UV damage, the mutational events occurring in the A149T strain would be expected to comprise less than 5% of total UV mutational events, yet these events can be studied in isolation in the A149T strain. These events are interesting, for the sequence at the mutant base does not contain the usual dipyrimidine target of UV (Friedberg et
One possible explanation for these reversion events is that they are due to error prone replication by a translesion polymerase engaged to bypass a thymine photoproduct produced at two adjacent thymidine bases just 3' of this target. Whether or not this explanation is correct, it is suggestive of the type of events that can be studied in such a reversion system that would be difficult to study in other contexts.

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FIGURE LEGENDS

**Figure 1.** A. Alignment of the most conserved region of the *S. cerevisiae* TRP5 protein with various fungal and bacterial homologues. Alignment was done using CLUSTAL W (1.74) multiple sequence alignment (Thompson *et al.* 1994). The residues shared in gray are similar, and those highlighted in yellow are identical in all species compared. The arrow marks the Glu-50 residue chosen for mutagenesis in the present study. B. Sequence surrounding the Glu-50 codon (GAA) of Trp5. The *TRP5* wild type sequence has a *Taq*I site overlapping the Glu-50 codon, but replacement of the base at nucleotide position 148 or 149 by any base other than wildtype (N) destroys the restriction site, as well as renders the strain Trp<sup>−</sup>.

**Figure 2.** Reversion frequencies of the various *trp5* mutant strains when treated with different mutagens. All strains were derived from the S288C derivative SJR828a (from Sue Jinks-Robertson), MATα *his3Δ200 ura3-52 leu2Δ1*. Gene deletion and mutagenesis was done using the pCORE plasmid and *delitto perfetto* strategy (Storici *et al.* 2001). The *TRP5* gene was first deleted from its normal location in the genome; a PCR product of the *TRP5* gene was then used to replace *RNQ1*, located near *ARS306*, in either orientation, after which the desired *trp5* mutations were introduced. For measuring revertants, after mutagenesis strains were plated on SD media lacking tryptophan (SD-trp). Usually fewer than 1-2 X 10<sup>8</sup> cells per 100 mm diameter plate were plated, but plating at twice that density did not seem to interfere with the number of revertant colonies obtained. For measurement of UV mutagenesis, strains were plated on SD-trp plates at a density of about 5 X 10<sup>7</sup> cells per 100 mm diameter plate and then exposed to 20 J/m<sup>2</sup> UV light in an Ultra-Lum ultraviolet crosslinker (for an average survival of 74% ± 9%). For 5–azacytidine (5AZ) mutagenesis, cultures were grown overnight in YPD medium and approximately 2 X 10<sup>7</sup> cells were transferred to SD-complete medium containing 5 mg/ml 5AZ, grown overnight and then plated on SD-trp plates. For
the methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) mutagenesis assays, cells were washed and resuspended in three times the original culture volume of 50 mM potassium phosphate buffer, pH 7. At that point, EMS was added to a final concentration of 2.0% (for an average survival of 79% ± 11%) or MMS to a final concentration of 0.5% (for an average survival of 11% ± 4%) and cells were incubated at room temperature for one hour before being plated on SD-trp plates, after inactivation of the mutagen by the addition of an equal volume of sterile 10% sodium thiosulfate. Mutagenesis with 6-N-hydroxylaminopurine (HAP) was as described (Shcherbakova et al. 1996), using HAP at a concentration of 100 µg/ml. In all cases, YPD plates for determination of viable cells were counted after 2 days of incubation, and SD-trp plates after 5 days. YPD plates for growth of trp5 strains were routinely supplemented with 40 mg/l tryptophan. Reversion frequencies were determined from at least 3 cultures per strain and were averaged. If there were more than 10 revertants per culture, the standard deviation was calculated and is indicated with error bars on the graph. Forward orientation of the TRP5 gene is indicated by $\text{\textregistered}$ and reverse orientation by $\text{\textregistered}$. In the forward orientation, TRP5 is oriented left to right on Chromosome III and is to the left of ARS306.
Figure 1

A

- S. cerevisiae TRP5
- A. nidulans
- S. pombe
- N. crassa
- Coprinus cinereus
- Hellobacillus mobilis
- P. aeruginosa
- P. putida
- Caulobacter crescentus
- Rhodobacter sphaeroides
- P. syringae
- E. coli trpA

ATC GAA TTG
TAG CTT AAC

Trp⁺, TaqI⁺

B

- ATC NAA TTG
- TAG NTT AAC
- ATC GNA TTG
- TAG CNT AAC

Wild type

Trp⁻, TaqI⁻