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# Characterization of the *trp5-27* allele used to monitor drug-induced mitotic gene conversion in the *Saccharomyces cerevisiae* tester strain D7

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**Mitotic gene conversions, among other recombinagenic events, can play an important role in the multistep process of carcinogenesis. The ability of chemicals to induce such gene conversions can easily be monitored in the *Saccharomyces cerevisiae* tester strain YHE2, a derivative of strain D7. For the detection of drug-induced gene conversions, two mutations in the *TRP5* locus are used, *trp5-12* and *trp5-27*. Here we report on the characterization of the stable allele *trp5-27*. Our analysis revealed two relevant mutations in *trp5-27*: (a) a transition C to T at position 121 after ATG that results in an amber stop codon and abolishes gene expression and (b) a transversion A to T at position 1555 that creates an ochre stop codon. Simultaneous amber and ochre suppression with the suppressors *SUP3* and *SUP11*, respectively, was capable of relieving the tryptophan-requiring phenotype of strains carrying the *trp5-27* allele. These findings have implications on the length of gene conversion tracts in conversion events between *trp5-12* and *trp5-27*: conversion tracts can cover several kilobases, if the site of the mutation in *trp5-12* lies outside of the positions mutated in *trp5-27*. Conversely, the maximal length is limited to 1435 bp, if the mutation in *trp5-12* is located between the positions mutated in *trp5-27*.**

## Introduction

Mitotic gene conversion is a process of nonreciprocal transfer of genetic information between homologous chromatid regions of chromosomes. In cells that are heterozygous for two alleles of a marker gene on the two homologous chromosomes, gene conversion within the region of the marker gene can result in the loss of heterozygosity, and a clone can develop that is homozygous for one of the marker alleles (Würgler, 1992). If in proliferating tissue a cell turns homozygous for a pre-existing mutation in a tumor suppressor gene that leads to the loss of gene function, this loss of heterozygosity will enable the phenotypical manifestation of the mutation and may result in increased proliferation of the cell (Sengstag, 1994). Thereby, gene conversions, among other recombinagenic events, represent a possible step in the multistep process of carcinogenesis (Fearon and Vogelstein, 1990). Chemical compounds that raise gene conversion frequencies are, therefore, thought to contribute considerably to the development of neoplasms. In addition to their effect on cancer induction, promotion and progression, gene conversion events leading to loss of heterozygosity can also affect other biological functions that influence health (Würgler, 1992). During the last few years, the awareness of the importance of such processes has substantially increased, and various test systems have been designed to identify the recombinational properties of substances which might be indicative of their carcinogenic potential (Sengstag, 1994). One of these test systems, the *Saccharomyces cerevisiae* tester strain D7 (Zimmermann *et al.*, 1975), allows the detection of gene conversions simultaneously with reversions and mitotic recombinations. As a unicellular eukaryote, *S.cerevisiae* provides a readily manipulable, yet genetically complex model organism suitable for the study of recombinational events (Zimmermann, 1992), and since its introduction the tester strain D7 has been extensively used for genotoxicity testing (Zimmermann *et al.*, 1984). As the yeast cells lack certain enzymatic activities that can be crucial for the activation of procarcinogens, various human enzymes have been heterologously expressed in a yeast strain derived from D7 (Eugster *et al.*, 1990, 1991, 1992) and are capable of conferring such metabolic competence to yeast (Eugster *et al.*, 1993; Eugster and Sengstag, 1993; Sengstag *et al.*, 1994). This approach has proved promising in broadening the applicability of the test system.

The process of gene conversion can easily be followed in heteroallelic diploid yeast strains that carry two defective alleles of the same gene locus (Zimmermann, 1975). In strain D7 (Zimmermann *et al.*, 1975), two non-complementing alleles of the tryptophan synthase gene *TRP5* (Zalkin and Yanofsky, 1982) are combined. Taken on their own, the two alleles *trp5-12* and *trp5-27* are completely stable (*trp5-27*) or exert low reversion frequencies (*trp5-12*, reversion frequency $10^{-6}$) that do not interfere with gene conversion (Zimmermann and Schwaier, 1967). Conversely, the tryptophan-requiring heteroallelic diploid D7 generates tryptophan nonrequiring convertants at a frequency of approximately $10^{-5}$. The conversion frequency can be increased by treatment of the cells with DNA-damaging agents (Zimmermann *et al.*, 1975). For our genotoxicity testing, we are working with a *ura3* derivative of D7, YHE2 (Eugster *et al.*, 1990). We were interested to characterize the *trp5* mutations present in our tester strain in order to gain more insight into the gene conversion events taking place between the two *trp5* alleles. Here we report the characterization of one of the alleles, *trp5-27*.

## Materials and methods

*Yeast and bacterial strains, media and culture conditions*

The construction of the *S.cerevisiae* strains YHE2 (a/α, *ade2-40/ade2-119, ilv1-92/ilv1-92, trp5-27/trp5-12, ura3Δ5/ura3Δ5*), and YHE1 (α, *ade2-40, ilv1-92, trp5-27, ura3Δ5*), has previously been described (Eugster *et al.*, 1990). Strain YAE76 (a, *ade2-119, ilv1-92, trp5-111, ura3Δ5*) was constructed in this laboratory. Strain JRY1631 (α, *ade2-110, cyh2, his3Δ200, leu1, lys2-801, trp5, ura3-52*) was kindly provided by Dr J.Rine. Yeast transformations were performed as described by Klebe *et al.* (1983). The bacterial strain DH5αF′ was used for propagation of recombinant plasmids.

Yeast strains were grown in standard media. Minimal medium YM [0.67% yeast nitrogen base without amino acids (Difco), 2% glucose] was appropriately supplemented with amino acids as described by Sherman (1991). *Escherichia coli* was grown in LB medium which was supplemented with 150 μg/ml ampicillin where appropriate.

*Plasmid constructions*

Plasmid pAE158 is a derivative of YEp352 (Hill *et al.*, 1986) and contains in its *Bam*HI site the *TRP5* gene from pYAS1 (Dohmen *et al.*, 1989) as a 3.3 kb *Bam*HI fragment. In pAE158, *TRP5* has the same orientation as *lacZ*. Plasmid pAE268 is analogous to pAE158, but contains the *trp5-27* gene which was cloned

from YHE1 (see Results). Plasmids pAE470, pAE471, pAE475 and pAE491 are derived from pAE268 and have their *Bgl*II/*Sal*I, *Spe*I/*Sal*I, *Nco*I and *Bst*EII/*Sal*I *trp5-27* fragments, respectively, replaced by the corresponding *TRP5* sequence. Plasmids pRS313SUP3, pRS316SUP3 and pUN60SUP3 were constructed by introducing the 137 bp *SUP3 Bam*HI fragment of mWJ64 (Guo and Wu, 1982) into the *Bam*HI sites of pRS313, pRS316 (Sikorski and Hieter, 1989) and pUN60 (Elledge and Davis, 1988), respectively. To obtain pAE492, the blunt-ended *trp5-27 Bam*HI fragment of pAE268 was introduced into the *Xba*I/*Not*I cleaved, blunt-ended plasmid pRS316SUP3 to give pAE342. Subsequently, the *Pvu*II fragment of pAE342 containing *trp5-27* and *SUP3* was cloned in the *Pvu*II cut vector pRS313. Plasmid pAE496 was constructed by cloning the *Sac*I/*Sal*I *trp5-27* fragment of pAE268 in the *Sac*I/*Sal*I cleaved vector pRS313.

*Colony hybridization and detection*

Bacterial colonies representing the *Bam*HI/*Hin*dIII sublibrary of YHE1 in pRS316 was transferred to Pall Biodyne A transfer membranes, lysed and their DNA bound to the filters as described by Sambrook *et al.* (1989). For the detection of *TRP5*-containing colonies, the 3.3 kb *Bam*HI fragment of pAE158 was digoxygenin-labelled with the DIG DNA Labeling and Detection Kit from Boehringer. Labelling, hybridization and chemiluminescent detection were performed according to the supplier's protocols.

*DNA sequence analysis*

Fragments to be sequenced were subcloned in pBLKS and pBLSK (Stratagene). DNA sequence analysis was performed on single-stranded DNA by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase Version 2.0 DNA Sequencing Kit (US Biochemical Corporation) according to the manufacturer's specification. [$\alpha$-$^{35}$S]dATP was purchased from DuPont.

## Results

### *Cloning of the trp5-27 gene*

In order to clone the *trp5-27* gene, a genomic sublibrary of the *S.cerevisiae trp5-27* strain YHE1 was constructed in the *E.coli*/*S.cerevisiae* shuttle vector pRS316. To this end, total DNA isolated from YHE1 was double-digested with *Bam*HI and *Hin*dIII, which divides the *trp5-27* gene in two fragments of 1815 and 1516 bp length. DNA fragments in a size range of 1.4−1.9 kb were purified from an agarose gel and ligated to the *Bam*HI and *Hin*dIII cleaved vector pRS316. Four hundred and fifty individual ampicillin resistant *E.coli* transformants were used for a colony hybridization experiment (see Materials and methods). As a probe, the 3.3 kb digoxigenin-labelled *TRP5 Bam*HI fragment of pAE158 was taken. To confirm the identity of the 11 clones that gave rise to positive signals, their plasmid DNA was subjected to restriction analysis. By this procedure, two clones representing the 1.8 and 1.5 kb fragments of the *trp5-27* were chosen for further characterization. Subsequently, the complete *trp5-27* gene was reconstituted in the *E.coli*/*S.cerevisiae* shuttle vector YEp352 by triple-ligating the two individual *trp5-27 Bam*HI/*Hin*dIII fragments to the *Bam*HI cleaved YEp352 to give pAE268.

The phenotype conferred by the *trp5-27* plasmid pAE268 was determined by transforming strain YHE1 to uracil prototrophy with pAE268 and, as a control, with pAE158, which is a YEp352 derivative and carries the wild-type *TRP5* gene. YHE1 pAE268 and YHE1 pAE158 were streaked on supplemented minimal medium lacking uracil or uracil and tryptophan, and the plates were incubated for 3 days at 30°C (Figure 1A). In contrast to YHE1 transformed with the *TRP5*-carrying plasmid, YHE1 containing the *trp5-27* plasmid was unable to grow on medium lacking tryptophan. Thus, plasmid pAE268 does not confer tryptophan prototrophy to YHE1 and, therefore, presumably contains a mutation in its *trp5-27* allele that distinguishes it from pAE158.

### *Mapping and sequencing of an amber mutation in trp5-27*

Since knowledge of the underlying mutation(s) in the *trp5-27* and *trp5-12* alleles present in the *S.cerevisiae* strains D7 and YHE2 would give us a more detailed insight into the gene conversion

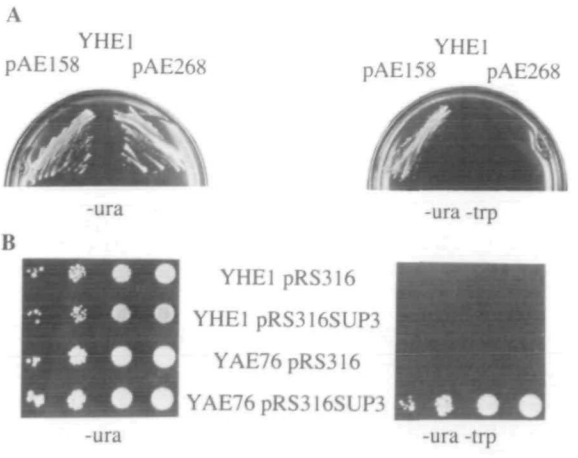


**Fig. 1.** (A) Comparison of the tryptophan phenotypes conferred to YHE1 by plasmids carrying *TRP5* (pAE158) and *trp5-27* (pAE268). YHE1 pAE158 and YHE1 pAE268 were streaked on supplemented minimal medium lacking uracil (−ura) or uracil and tryptophan (−ura −trp) and incubated for 3 days at 30°C. (B) Amber suppression in YHE1 (*trp5-27*) and YAE76 (*trp5-111*). The strains transformed with a vector control (pRS316) or an amber suppressor containing plasmid (pRS316SUP3) were pregrown in supplemented minimal medium to stationary phase and diluted to an absorbance at 600 nm of 0.3. 5 µl of these cell suspensions and of 1:10, 1:100 and 1:1000 dilutions were placed on supplemented minimal medium lacking uracil (−ura) or uracil and tryptophan (−ura −trp). Plates were incubated for 3 days at 30°C.

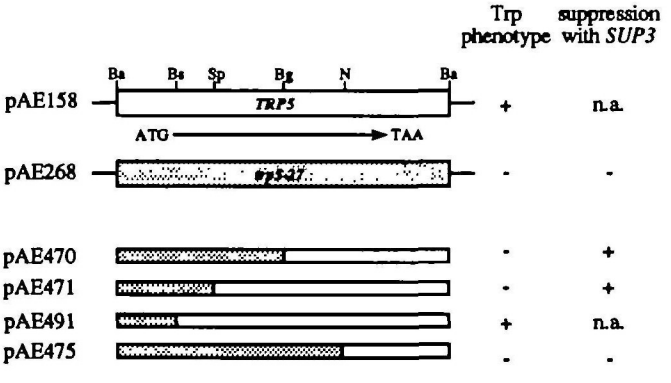


**Fig. 2.** Replacement of plasmid-borne *trp5-27* segments by wild-type *TRP5* and determination of the phenotype conferred by these plasmids. The white and grey boxes represent the *TRP5* and *trp5-27* sequences, respectively. The phenotypes conferred by the plasmids were determined in strain YHE1 and are indicated as (+) for tryptophan prototrophy and (−) for tryptophan auxotrophy. By testing the tryptophan phenotype of JRY1631 cotransformed with respective *trp5* plasmids and pRS313SUP3, novel *trp5* alleles derived from *trp5-27* were identified whose tryptophan conferring phenotype was (+) or was not (−) amber suppressible (n.a., not applicable). The direction and approximate length of the *TRP5* open reading frame are indicated by the arrow. Selected restriction sites are indicated (Ba = *Bam*HI, Bg = *Bgl*II, Bs = *Bst*EII, N = *Nco*I, Sp = *Spe*I).

events, we were interested in localizing the site of the mutation(s) in the *trp5-27* allele. Therefore, we replaced fragments of increasing length of the *trp5-27* plasmid pAE268 by the corresponding *TRP5* fragment and tested the tryptophan phenotype conferred by the new constructs (Figure 2). For this purpose, YHE1 was transformed with each novel construct and Ura$^+$ transformants were streaked on supplemented minimal medium lacking uracil or tryptophan and uracil. A tryptophan non-requiring phenotype was only observed when *trp5-27* sequences downstream of the *Bst*EII site were replaced by the corresponding wild-type sequences, while replacement of the sequences downstream of the *Spe*I site was not sufficient to confer tryptophan

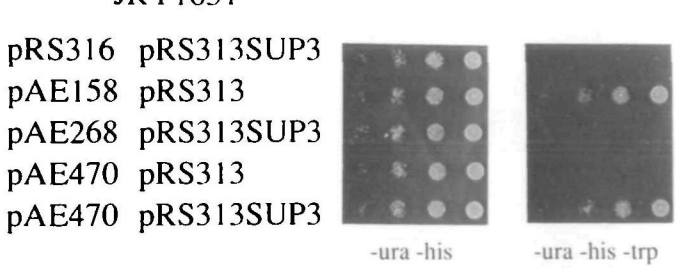


**Fig. 3.** Amber suppression of various *trp5* alleles in JRY1631. JRY1631 cotransformed with the indicated plasmids was grown to stationary phase in liquid medium and diluted to $3 \times 10^6$ cells/ml; 5 µl of these and of 1:10, 1:100 and 1:1000 dilutions were spotted on supplemented minimal medium lacking uracil and histidine (−ura −his) or uracil, histidine and tryptophan (−ura −his −trp). The plates were incubated for 2 days at 30°C.

prototrophy. This allowed us to map the site(s) of the mutation(s) in *trp5-27* to a 380 bp *Bst*EII/*Spe*I fragment. To elucidate the exact nature of the mutation(s) assumed to lie in this DNA segment, the DNA sequence of the corresponding *trp5-27* fragment was determined. The fragment was cloned into pBLKS and pBLSK, single-stranded DNA was prepared and sequenced according to the dideoxy method. Comparison of the *trp5-27* sequence with the wild-type *TRP5* sequence revealed a transition C to T at nucleotide position 121 after ATG. This mutation results in an amber stop codon TAG in *trp5-27* and represents a potential cause for the $Trp^-$ phenotype observed for the *trp5-27* allele.

*Attempted suppression of the trp5-27 amber mutation*

To determine whether the amber mutation found on *trp5-27* plasmid pAE268 was the only mutation responsible for the tryptophan auxotrophic phenotype conferred by the plasmid, we attempted to suppress this $Trp^-$ phenotype with the amber suppressor *SUP3*. In order to achieve this objective, *S.cerevisiae* strain JRY1631 with the relevant genotype *trp5*, *his3Δ200*, *ura3-52* was cotransformed with either YEp352 or pAE268 as *URA3* plasmids and either pRS313 or pRS313SUP3 as *HIS3* plasmids. $Ura^+/His^+$ transformants were grown in liquid culture to stationary phase. Aliquots of serial dilutions were spotted on supplemented minimal medium lacking uracil and histidine or tryptophan, uracil and histidine, and the plates were incubated at 30°C for 2 days (Figure 3). The *trp5* mutation of JRY1631 was not suppressible with *SUP3* as shown by the inability of JRY1631 pRS316 pRS313SUP3 to grow without tryptophan. Surprisingly, JRY1631 carrying pAE268 and the *SUP3*-containing plasmid was also unable to grow without tryptophan, indicating that the $Trp^-$ phenotype conferred by pAE68 was not amber-suppressible.

To make sure that the inability to amber-suppress JRY1631 pAE268 was not due to a cloning artefact on pAE268, but that the cloned *trp5-27* allele was identical with the allele present in YHE1, we attempted to suppress the tryptophan auxotrophic phenotype of YHE1 with the amber suppressor *SUP3*. YHE1 was transformed with the control vector pRS316 and with pRS316SUP3. In parallel, strain YAE76, which has an amber-suppressible tryptophan-requiring phenotype (A.Ehrenhofer-Murray and C.Sengstag, unpublished), was transformed with the same plasmids in order to confirm functional expression of *SUP3* in pRS316SUP3. $Ura^+$ transformants were grown in liquid culture to stationary phase and diluted to $3 \times 10^6$ cells/ml. Aliquots of serial dilutions were spotted on supplemented minimal medium lacking uracil or tryptophan and uracil, and the plates were incubated at 30°C for 3 days (Figure 1B). Only the amber suppression control strain YAE76 containing the *SUP3* plasmid could grow without tryptophan, while *trp5-27* strain YHE1 containing *SUP3* was unable to grow. Therefore, as found for the *trp5-27* allele on pAE268, the *trp5-27* mutation of YHE1 is not suppressible with the amber suppressor *SUP3*. These results suggested either that *SUP3* was not the appropriate amber suppressor for *trp5-27*, or that there was a second mutation present in *trp5-27* that restricted its amber suppressibility.

*Amber suppression of novel trp5 alleles and identification of a second mutation in trp5-27*

To rule out the possibility that the inability of *SUP3* to suppress the $Trp^-$ phenotype of *trp5-27* was due to the nature of this suppressor, we attempted to amber-suppress novel *trp5* alleles derived from *trp5-27*. This was achieved by exchanging fragments of pAE268 for the corresponding wild-type *TRP5* fragment, cotransforming JRY1631 with the new constructs and either pRS313 or pRS313SUP3 to uracil and histidine prototrophy and determining the tryptophan phenotype of the transformants by streaking them on medium lacking uracil and histidine or tryptophan, uracil and histidine (Figure 2; c.f. also Figure 3). A *trp5* allele present on pAE470 with the sequence downstream of the *Bgl*II site replaced by wild-type sequences was suppressible with *SUP3*, while substitution of the *trp5-27* sequences downstream of the *Nco*I site conferred no suppressible phenotype. These results demonstrated that the suppressor *SUP3* was in fact capable of suppressing certain *trp5* alleles derived from *trp5-27*. In addition, these results strongly suggested the presence of a further mutation within a 606 bp *Bgl*II/*Nco*I fragment of *trp5-27* that interferes with amber suppression. In order to identify this potential second mutation, the *trp5-27 Bgl*II/*Nco*I fragment was subjected to DNA sequence analysis. This revealed a transversion A to T at nucleotide position 1555 after ATG. This mutation creates an ochre stop codon TAA in *trp5-27* that presumably was responsible for the inability to amber-suppress *trp5-27*.

*Simultaneous amber and ochre suppression of trp5-27*

To confirm that the amber and ochre mutations of *trp5-27* at positions 121 and 1555 after ATG were the only mutations responsible for its tryptophan-requiring phenotype, we undertook experiments to suppress both mutations simultaneously. To this end, the *HIS3* plasmids pAE496 and pAE492 were constructed that contain the *trp5-27* gene from YHE1 alone or with the amber suppressor *SUP3*, respectively. JRY1631 was cotransformed with pAE496 or pAE492 as *HIS3* plasmids and pRS316 or pUN60 as *URA3* plasmids. Plasmid pUN60 carries the ochre suppressor *SUP11* (Elledge and Davis, 1988). Histidine and uracil prototrophic transformants were subsequently streaked on supplemented minimal medium lacking histidine and uracil or tryptophan, histidine and uracil, and the plates were incubated for 3 days at 30°C (Figure 4). The $Trp^-$ phenotype of JRY1631 was not suppressed with *SUP3* or *SUP11* alone or in combination (data not shown). Similarly, a JRY1631 transformant containing the *trp5-27* plasmid and either *SUP3* or *SUP11* was unable to grow without tryptophan, whereas a transformant expressing *trp5-27* and both *SUP3* and *SUP11* showed a tryptophan prototrophic phenotype. This result clearly demonstrated that simultaneous amber and ochre suppression was necessary and sufficient to cure the $Trp^-$ phenotype of *trp5-27*. Similarly, the $Trp^-$ phenotype of YHE1 was suppressed by a plasmid containing both suppressors *SUP3* and *SUP11* (data not shown).

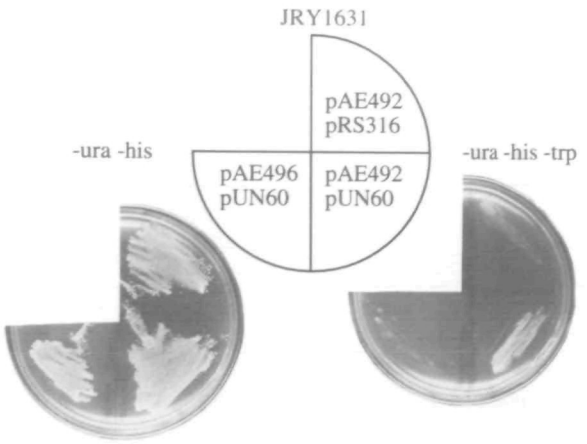


**Fig. 4.** Simultaneous amber and ochre suppression of *trp5-27*. JRY1631 transformants were streaked on supplemented minimal medium lacking uracil and histidine (−ura −his) or uracil, histidine and tryptophan (−ura −his −trp). The plates were incubated at 30°C for 3 days.

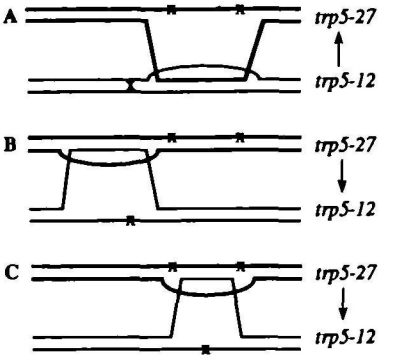


**Fig. 5.** Schematic representation of possible gene conversion events between *trp5-27* and *trp5-12* that result in $Trp^+$ convertants in D7. The approximate site of the relevant mutations in *trp5-27* and one possible location of a mutation in *trp5-12* are designated by crosses (X). The directions of information transfer during gene conversion are indicated by arrows. Conversion tracts can cover several kilobases, if the site of the mutation in *trp5-12* lies outside of the positions mutated in *trp5-27* (A and B). Conversely, the maximal tract length is limited to 1435 bp, if the mutation in *trp5-12* is located between the positions mutated in *trp5-27* (C).

## Discussion

The *trp5-27* allele, one of the two defective alleles of the *TRP5* locus (Zalkin and Yanofsky, 1982) in the diploid *S.cerevisiae* genotoxicity strain D7 (Zimmermann *et al.*, 1975), has found extensive use in the detection and quantification of drug-induced gene conversion events. Despite its broad application, the mutation in *trp5-27* so far has not been characterized and, thus, little is known about the nature of gene conversion events in D7 that produce tryptophan prototrophic convertants.

In this study, we have identified two relevant mutations in *trp5-27* that are responsible for its stable tryptophan auxotrophic phenotype. At nucleotide position 121 after ATG, *trp5-27* contained a transition C to T that results in an amber stop codon. This stop codon presumably terminates translation, giving rise to a truncated and probably abortive Trp5 gene product. The fact that suppression of this mutation with the amber suppressor *SUP3* (Guo and Wu, 1982) did not suffice to cure the $Trp^-$ phenotype was not due to inherent characteristics of *SUP3*, since other, novel *trp5* alleles derived from *trp5-27* were in fact suppressible with *SUP3*. It rather indicated the presence of a second mutation in the *trp5-27* gene. It was subsequently detected as a transversion A to T at nucleotide position 1555 after ATG and creates an ochre stop codon. Ochre suppression alone with *SUP11* (Elledge and Davis, 1988) in *trp5-27* did not result in tryptophan prototrophy. Conversely, simultaneous amber and ochre suppression was capable of relieving the tryptophan requirement of strains carrying *trp5-27*. Therefore, the two stop codons were the mutations relevant for the $Trp^-$ phenotype of *trp5-27*.

This finding has implications concerning the length of gene conversion tracts in conversion events between *trp5-27* and *trp5-12* that are successful in forming $Trp^+$ convertants in D7. In *S.cerevisiae*, gene conversion tracts are usually continuous, information being transferred as a single block from one chromosome to another (Borts and Haber, 1989). If transfer is to be directed from *trp5-12* to *trp5-27* (Figure 5A), a contiguous DNA stretch has to cover both mutations of *trp5-27* and thus requires a minimal length of 1435 bp. Consequently, the assumed mutation in *trp5-12* would lie outside of the positions mutated in *trp5-27*, and therefore would be located either upstream of position 121 or downstream of position 1555, presumably within the *TRP5* open reading frame that ends at position 2619. In such a case, the conversion tract could expand over several kilobases downstream or upstream of the *trp5-12* mutation, respectively. Likewise, if information transfer proceeds from *trp5-27* to a *trp5-12* allele whose mutant site lies outside of the positions mutated in *trp5-27*, the conversion tract could also span several kilobases (Figure 5B). Conversely, its size would be limited to a maximum of 1435 bp, if the mutation in *trp5-12* was located between the positions mutated in *trp5-27* (Figure 5C). Judd and Petes (1988) measured the tract length of mitotic conversions of the *ura3-3* allele at the *URA3* locus and reported a minimum average conversion tract length of 0.9 ± 0.8 kb. In 40% of the cases, though, the average tract length was between 4 and 10 kb. We therefore favour the model of the mutated site in *trp5-12* lying upstream of position 121 or downstream of position 1555 after ATG (Figure 5A and B), also since the conversion frequency of D7 ($10^{-5}$; Zimmermann *et al.*, 1975) compares well to the frequency in the *ura3-3/URA3* strain ($4 \times 10^{-6}$; Judd and Petes, 1988).

In light of these results, the characterization of the *trp5-12* allele of D7 represents an important task and will give more precise information on the direction and extent of information transfer during gene conversion between the two alleles.

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