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**Mis-targeting of multiple gene disruption constructs containing hisG**

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**Abstract** Gene targeting by homologous recombination occurs in *Saccharomyces cerevisiae* efficiently when there are as few as 30 base pairs of sequence homology at both ends of the targeting construct. Multiple gene disruptions within a single cell are possible using the hisG cassette, which allows recovery of the marker but leaves a single hisG sequence imbedded in the disrupted gene(s). We use an integration hisG construct, which has limited homology to the target at one end, to show that a single genomic copy of hisG decreases the percentage of integration at the target locus from 44% to 4.5% and two genomic hisG copies decrease it to less than 1%. Enlarging the homology at the disruption construct abolishes this effect. Thus competition between endogenous hisG sequences and successive hisG cassette transformations occurs if there is limited homology at one end of the targeting construct. Therefore, methods using limited homology, such as PCR-mediated gene targeting, are inefficient when significant internal homology exists.

**Key words** Gene targeting · *Saccharomyces* · hisG · Homology

**Introduction**

In *Saccharomyces cerevisiae*, targeted gene disruption and deletions have been obtained with PCR-based strategies using as few as 30 base pairs of homology to the target gene at both ends of the integrating DNA molecule (Gietz et al. 1992). In order to achieve efficient targeting, it is important that the transforming PCR fragment contains homology at the ends. Where the genome contains homologous marker sequences, gene conversion with the incoming marker DNA occurs in competition with the regions of homology at the fragment ends. Complete deletion of the marker gene in the host strain is one way around this problem (Replogle et al. 1999), unless multiple gene disruptions are required.

Multiple gene disruptions can be performed using constructs that enable the selectable marker to be removed following integration into the genome, thus conserving the available selectable markers. A common example is the use of hisG repeats flanking *URA3* (Alani et al. 1987). Another such system utilizes the 2-μm site-specific recombination system to delete the marker sequence (Storici et al. 1999). Typically the hisG construct is imbedded within flanking DNA sequences containing homology to the target gene within the genome. Following targeted integration into the genome, recombination events between the repeated hisG elements and excision events of the *URA3* gene can be selected for by growth on medium containing 5-fluoroorotic acid (Boeke et al. 1984). The resulting disruption contains a single remaining hisG element at the site of integration and allows for another locus to be disrupted using the hisG cassette. An integration attempt at a second locus using a construct containing the hisG cassette contains internal sequence homology to the hisG element residing at the first disrupted locus.

Here we describe the effect of different sizes of homology on specific targeting at one locus using the hisG-*URA3*-hisG cassette in strains already containing one or more hisG DNA sequence elements at other loci.

**Materials and methods**

**Yeast strains**

Y433: MATα ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ98

JDY20: Isogenic to Y433 except for apn1::hisG

JDY27: Isogenic to Y433 except for apn1::hisG and ntl2::hisG
Plasmid construction

pJD47 was created by PCR amplification of the OGG1 ORF using primers containing BamHI and EcoRI tails (OGG1up 5'-GGGGATCCATCGTCTTTTGAATTCGCCAA-3' and OGG1low 5'-GGGGATCCTAAAAATTCTGAGC-3'). Restriction enzyme digestion with EcoRI and BamHI allowed cloning into the multi-cloning site of pUC19, to create pJD4. The blunt-end MscI restriction site, which is located 71 base pairs into the OGG1 ORF, was cut and a BglII linker was inserted, creating pJD5. The 3.85-kb hisG-URA3-hisG cassette was released from pNKY51 by digestion with BamHI and BglII (Aliani et al. 1987), followed by ligation into pJD5 BglII site to create pJD47. Digestion with BamHI/EcoRI released the 4.9-kb transforming fragment (Fig. 1).

pJD52 was created from pJD47 by insertion of a XbaI/EcoRI PCR fragment containing 993 base pairs adjacent to the 71 base pairs of homology on the 3' side of the pJD47 construct (Fig. 1).

Transformation with pJD52 or pJD47

Strains Y433, JDY20 and JDY27 were transformed by the LiAC method (Gietz et al. 1992), with 1 μg of the 5.9-kb transforming BamHI/XbaI fragment released from pJD52 (Fig. 1) or the 4.9-kb BamHI/EcoRI fragment released from pJD47. The transformation efficiency was calculated as number of Urata transformants/μg DNA. The same number of cells was transformed in each case. Urata transformants were picked at random and single colony PCR amplification was used to check for correct integration at the OGG1 ORF using primers to OGG1 (5'ATGGTCTATAAAATTGCGC-3') and URA3 (5'-TATCAGTTTACCCGGGAA-3').

Southern blot of mis-targeted Urata colonies in JDY20

Southern blot was performed using standard protocols, and visualized using a DIG kit (Boehringer Mannheim). DNA was isolated using a genomic DNA isolation kit (Promega). It was then cut with PvuII restriction enzyme and probed with the APN1 ORF, which had been PCR-amplified using primers APN13' 5'-TATCTATGAAAGTGGCAACAGC-3' and APN15' 5'-TGACAAAATCTCCGAATAAGAACA-3', before being labeled with DIG (Boehringer Mannheim).

PCR integration of LYS2

PCR integration of LYS2 were performed using the primers for LYS-G and DNA substrate previously reported (Manivasakam et al. 1995).

**Fig. 1** Targeting constructs. Two constructs targeting the OGG1 locus were made each with identical 1,064 base pairs (bp) of homology to the OGG1 locus on one side of the hisG-URA3-hisG cassette. The short construct obtained by digestion of pJD47 with MscII and EcoRI contains 71 bp of homology to the OGG1 locus on the other side of the hisG-URA3-hisG cassette. The long construct obtained by digestion of pJD52 with BamHI and XbaI has 1,034 bp on the other side of the hisG-URA3-hisG cassette.

Results and discussion

In attempts to sequentially disrupt different genes involved in cellular responses to oxidative stress using the hisG system, we encountered a sharp reduction in the targeting efficiency, which became increasingly more severe with the increasing number of hisG sequences in the genome. To further characterize this competition, we determined the exact frequencies of integration using constructs that differed only in the length of homology at one end, in cells containing 0, 1 and 2 genomic hisG copies.

Table 1 shows that a transforming hisG-URA3-hisG vector containing homologies of 71 base pairs and 1,064 base pairs to OGG1 (pJD47, Fig. 1) yields successful integration events into the OGG1 locus in 44% (4/9) of the transformants, as determined by PCR.

Generally, one can expect 80–90% correct homologous recombination frequencies when using constructs with >1-kb flanking homologies. The lower frequency of homologous recombination at the OGG1 locus observed when the pJD47 short construct was integrated into the Y433 background may be due to competition between integration at OGG1 and the ura3–52 locus. As the transformation frequency of the short construct was approximately 19-fold lower than that obtained with the long construct (Table 1), the higher mis-targeting frequency may reflect an inability to integrate at OGG1 rather than an increase in targeting elsewhere.

With one copy of hisG at another locus in the genome, the number of successful integrations into the OGG1 locus decreased to approximately 5% (1/22). With two genomic hisG copies at other different locations, this number decreased further to less than 1% (<1/100). When the homology of the transforming construct was increased on the side containing limited homology from 71 base pairs to 1,034 base pairs (pJD52, Fig. 1), the transformation frequency increased from 60–90%. Thus, the majority of the Urata transformants obtained when using the longer construct (pJD52) were correctly targeted to the OGG1 locus, regardless of the number of genomic hisG copies (Table 1).

**Table 1** Frequency of integration at targeted OGG1 loci. Strains Y433, JDY20 and JDY27 were transformed with short or long constructs (Fig. 1). Urata colonies were selected and re-streaked on SC-Ura minimal media. Single-colony PCR was performed to confirm integration at the OGG1 locus. Values in parentheses indicate overall transformation efficiency, expressed as number of transformants/μg DNA.

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<th>Number of hisG copies in genome</th>
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<tr>
<td></td>
<td>0</td>
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<tr>
<td>Long homology</td>
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<tr>
<td>pJD52</td>
<td>18/22</td>
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<td>(2.6 x 10^3)</td>
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<tr>
<td>Short homology</td>
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<tr>
<td>pJD47</td>
<td>4/9</td>
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<td>(1.4 x 10^3)</td>
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