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A Method for Gene Disruption That Allows Repeated Use of *URA3* Selection in the Construction of Multiply Disrupted Yeast Strains

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Manuscript received December 23, 1986
Revised copy accepted April 27, 1987

ABSTRACT

In this paper, we describe a 3.8-kb molecular construct that we have used to disrupt yeast genes. The construct consists of a functional yeast *URA3* gene flanked by 1.1-kb direct repeats of a bacterial sequence. It is straightforward to insert the 3.8-kb segment into a cloned target gene of interest and then introduce the resulting disruption into the yeast genome by integrative transformation. An appropriate DNA fragment containing the disruption plus flanking homology can be obtained by restriction enzyme digestion. After introducing such fragments into yeast by transformation, stable integrants can be isolated by selection for Ura$^+$. The important feature of this construct that makes it especially useful is that recombination between the flanking direct repeats occurs at a high frequency (10^{-4}) in vegetatively grown cultures. After excision, only one copy of the repeat sequence remains behind. Thus in the resulting strain, the Ura$^+$ selection can be used again, either to disrupt a second gene in similar fashion or for another purpose.

IN yeast molecular biology, gene replacement is a common procedure in which a cloned DNA segment introduced by transformation is substituted by homologous recombination for a related region in the recipient genome (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981). Often, the replacement event is monitored by including an appropriate selectable marker on the incoming molecule (SCHERER and DAVIS 1979). In many cases it is not easy to remove the marker and thus the same selection cannot be used for subsequent gene replacement events. In order to make repeated use of a convenient selectable marker, we have developed a molecular construct in which the *URA3* gene of *Saccharomyces cerevisiae* is flanked by direct repeats of a bacterial sequence. We anticipated that if such a construct were introduced into a cloned gene and integrated into the yeast genome, it would undergo frequent mitotic recombination between the direct repeats to eliminate the *URA3* gene and leave behind a single copy of the repeat sequence at the site of the original integration (Figure 1). Mitotic recombination between nontandem direct repeats is known to occur at high frequency and this property has been used extensively to move genetic markers back and forth between yeast sequences cloned into plasmid molecules and the yeast genome (SCHERER and DAVIS 1979; WINSTON, CHUMLEY and FINK 1983). Because these excision events would lead to a Ura$^-$ phenotype, derivatives that have undergone such events could be directly selected with 5-fluoro-orotic acid (5-FOA) (BOEKE, LACROUTE and FINK 1984).

We have constructed a 3.8-kb molecular construct that consists of the *URA3* gene flanked by direct repeats of Salmonella *hisG* DNA. We show here that when this construct is inserted into the *TRP1* locus of yeast, recombination between the direct repeats occurs at a 10^{-4} frequency in vegetatively grown cultures. The resulting *trp1* mutation bearing a single copy of the direct repeat sequence does not revert to Trp$^+$. Similar results have been obtained with insertions of this construct in other yeast genes. We therefore believe that this construct can be used easily and systematically to disrupt any cloned gene of interest. Furthermore, because it is easy to eliminate the Ura$^+$ marker, the Ura$^+$ selection can be used repeatedly, and the same 3.8-kb construct can be used to disrupt several different genes within a single strain. We show that a gene disruption carrying the 3.8-kb construct integrates efficiently into its normal chromosomal locus even when introduced into a strain that already carries copies of the bacterial repeat sequence at one or two other loci, and that an SK-1 diploid strain carrying four copies of the repeat sequence sporulates efficiently and gives the normal high frequency of asci containing four viable spores.

MATERIALS AND METHODS

Strains: *Escherichia coli* K12 strain MM294 (F^-, *endA, hsdR, supE44, thiA*) was used for all plasmid manipulations (GUARENTE *et al.* 1980). Yeast strain NKY 274 (*MATα, ho::LYS2, ura3, lys2*) was derived from the homothallic prototrophic strain SK-1 (KANE and ROTH 1974; WILLIAMSON *et al.* 1983; W. RAYMOND, L. CAO and N. KLECKNER, unpublished data).

Media: *E. coli* cells were grown in LB broth or LB agar (MILLER 1972). Ampicillin was supplemented at 100 μg/ml. Yeast were grown in either YPD or minimal medium (SHER-

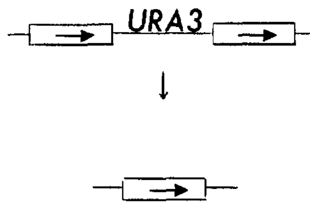

FIGURE 1.—Reduction of *URA3* gene flanked by direct repeats.

MAN, FINK and HICKS 1983). Minimal medium contained 0.7% yeast nitrogen base (Difco), 2% agar and 2% glucose, and 0.004% leucine, tryptophan, adenine and uracil were added according to strain requirements (SHERMAN, FINK and HICKS 1983). 5-Fluoro-orotic acid was purchased from SCM Specialty Chemicals, Gainesville, Florida. 5-FOA plates were prepared as described previously (BOEKE, LACROUTE and FINK 1984).

Yeast transformation: Lithium acetate transformations were performed by standard methods (ITO *et al.* 1983).

Nucleic acid techniques: All restriction enzymes, T4 DNA ligase and T4 DNA polymerase were purchased from New England Biolabs and used according to manufacturer's specifications. Plasmid DNA was isolated by a cleared lysate protocol; DNA manipulations were described previously (MANIATIS, FRITSCH and SAMBROOK 1982). Yeast chromosomal DNA isolations and Southern blottings using SP6 polymerase transcribed RNA probes were carried out as described by HOLM *et al.* (1986), MANIATIS, FRITSCH and SAMBROOK (1982), SOUTHERN (1975), and MELTON *et al.* (1984).

Plasmid constructions: All constructions were built from pNK294 (*Salmonella hisOGD*) (FOSTER *et al.* 1981; N. KLECKNER unpublished data), YRP7 (*TRP1, ARS1*) (SHERMAN, FINK and HICKS 1983), and YEP24 (2μ, *URA3*), (ROSE, GRISAFI and BOTSTEIN 1984; BOTSTEIN *et al.* 1979; New England Biolabs Catalog 1986). pNK294 bears the *hisOGD* region of the Salmonella histidine operon (BARNES 1981) with a *Bam*HI linker inserted at the *Pvu*II site of the *hisD* gene.

***pNKY51* (*his*G direct repeats separated by *URA3*):** pNKY51 was built in four steps. (1) The backbone, pNKY3 was made by deleting the 2μ DNA from YEP24 with *Eco*RI and by inserting a *Bgl*II linker at the remaining *Eco*RI site (*Eco*RI site is regenerated). (2) A 1.1-kb *Bgl*II-*Bam*HI fragment of pNK294 bearing Salmonella *hisG* DNA was inserted into the *Bgl*II site of pNKY3 to form pNKY49. (3) The same 1.1-kb *hisG* fragment as in (2) was inserted at the *Bam*HI site of pNKY49 to form pNKY50. (4) The *Eco*RI site at the 5′ end of *URA3* in pNKY50 was destroyed by fill-in and ligation reactions. The resulting plasmid, pNKY51, contains the 3.8 kb *hisG-URA3-hisG* fragment that can be gel isolated by a *Bgl*II and *Bam*HI digest. The complete structure of pNKY51 is shown in Figure 2.

***pNKY1009* (*TRP1* insertion vector):** pNKY1009 was built in two steps. (1) The *Bam*HI site in YRP7 was destroyed by fill-in and ligation reactions and a *Bam*HI linker was inserted into the *Eco*RV site to form pNKY1006. (2) The 3.8-kb *Bam*HI-*Bgl*II fragment of pNKY51, which contains the *hisG-URA3-hisG* construct, was inserted into the *Bam*HI site within the *TRP1* gene of pNKY1006 to form pNKY1009 (Figure 2).

RESULTS AND DISCUSSION

Construction and testing of pNKY51: We have made a molecular construct appropriate for the strategy described in the introduction: an *E. coli* plasmid vector containing the yeast *URA3* gene flanked by direct repeats of a sequence from the Salmonella histidine operon (pNKY51; Figure 2). Digestion of this plasmid with *Bam*HI and *Bgl*II produces a 3.8-kb fragment that can be inserted into any cloned gene or construct of interest. This fragment contains very few sites for "six cutter" restriction enzymes. Thus, after insertion of the construct into the target gene, it is usually easy to excise a fragment containing the construct and flanking target gene homology that is suitable for introduction into the yeast genome.

The properties of this construct in yeast have been examined. The 3.8-kb *Bam*HI-*Bgl*II fragment of pNKY51 was inserted into the yeast *TRP1* gene at the *Eco*RV restriction site to yield pNKY1009 (Figure 2). A 4.7-kb fragment of pNKY1009 containing the construct plus flanking *TRP1* homology was generated by digestion with enzymes *Eco*RI and *Bgl*II and then

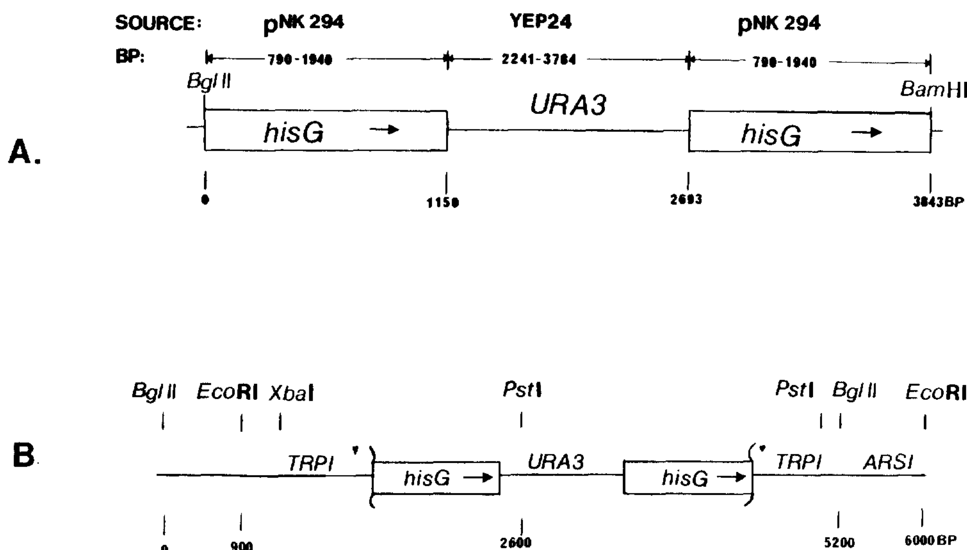

FIGURE 2. A, Restriction map of 3.8-kb construct that consists of the *URA3* gene flanked by direct repeats of *Salmonella hisG* DNA. Construction is described in the Materials and Methods. The following "six cutter" enzymes do not cut within the 3.8-kb *Bgl*II-*Bam*HI fragment: *Eco*RI, *Bgl*II, *Bam*HI, *Hpa*I, *Pvu*II, *Sal*I, *Sph*I, *Xba*I, *Xho*I, *Sac*I, *Sac*II. B, Restriction map of the chromosomal *TRP1* locus bearing a *hisG-URA3-hisG* insertion. In order to obtain this integrant, pNKY1009 was digested with *Eco*RI and *Bgl*II and a 4.6 kb fragment bearing the *TRP1* disruption was introduced into NKY274 by lithium acetate transformation (ITO *et al.* 1983). Trp$^-$, Ura$^+$ colonies were identified and characterized by Southern blotting.

TABLE 1

Frequency of *URA3* gene loss within a transformant colony

Strain	Relevant genotype	No. of colonies: Growth on minimal medium plus uracil	No. of colonies: Growth on 5-FOA plates	Frequency of Ura^-
NKY290	*ura3, trp1::hisG*	6×10^6	6×10^6	1
NKY537	*ura3, trp1::hisG-URA3-hisG*	A_0 4×10^6	68	2×10^{-5}
		A_0 7×10^5	62	9×10^{-5}
		B_0 2×10^5	94	5×10^{-4}
		B_0 2×10^6	22	1×10^{-5}

Colonies from two *trp1::hisG-URA3-hisG* transformants of NKY274 were plated onto minimal glucose plates plus uracil and minimal glucose plates plus uracil and 5-FOA (BOEKE, LACROUTE and FINK 1984).

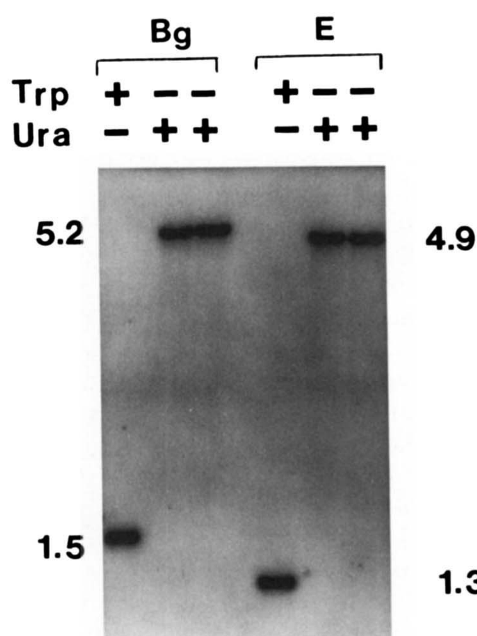

FIGURE 3.—Southern blot of DNA extracted from NKY274 (*MATα, ura3, lys2, ho::LYS2*) and two *trp1::hisG-URA3-hisG* transformants thereof. Restriction enzyme digested chromosomal DNA was probed with *TRP1* sequences. Lambda DNA digested with *Hind*III was used as a size marker (band sizes are in kb). E = *Eco*RI, Bg = *Bgl*II.

transformed into the strain NKY274 by selection for growth in the absence of uracil. Four out of four Ura^+ transformants were tryptophan auxotrophs.

Ura^- derivatives of two Ura^+, Trp^- transformants were initially obtained by patching onto 5-FOA plates, which are selective for *ura3* strains (uracil plus 5-FOA) (BOEKE, LACROUTE and FINK 1984). Quantitative plating experiments subsequently showed that the frequency of Ura^- derivatives in isolated single colonies of two Ura^+ transformants was about 0.01% (Table 1). None of the Trp^-, Ura^- derivatives gave rise to Trp^+ revertants at a detectable frequency ($<10^{-9}$ in cultures grown from single colonies).

Integration of the construct into the *TRP1* locus was confirmed by complementation analysis of one Ura^- derivative, NKY290. We transformed NKY290 (*MATα, trp1::hisG, ura3, lys2, ho::LYS2*) with a 2μ vector bearing both the *URA3* and *TRP1* genes or with a 2μ vector bearing only the *URA3* gene and selected for growth in the absence of uracil. Only transformants containing the *TRP1* plasmid could grow on medium lacking tryptophan.

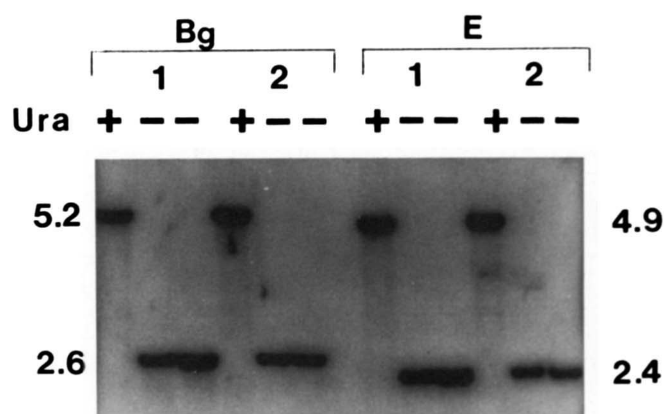

FIGURE 4.—Southern blot of DNA extracted from *trp1::hisG-URA3-hisG* transformants and 5-FOA^r derivatives. 1 and 2 represent two independent Trp^-, Ura^+ transformants and their 5-FOA^r derivatives. Restriction enzyme digested chromosomal DNA was probed with *hisG* sequences. Lambda DNA digested with *Hind*III was used as a size marker (band sizes are in kb). E = *Eco*RI, Bg = *Bgl*II.

The physical structures of two Ura^+, Trp^- transformants and two 5-FOA resistant derivatives from each transformant were confirmed by Southern blot analysis. Chromosomal DNA derived from these transformants and the parental strain NKY274 was digested with *Eco*RI or *Bgl*II. Southern blotting of NKY274 chromosomal DNA with a *TRP1* probe revealed 1.3 and 1.5 kb bands for the *Eco*RI and *Bgl*II digests, respectively (Figure 3). Southern blots of Ura^+, Trp^- transformants probed with either *TRP1* or *hisG* sequences revealed the pattern of fragments expected for a simple gene replacement at *TRP1* with the *trp1::hisG-URA3-hisG* fragment: 5.2- and 4.9-kb bands were detected for the *Bgl*II and *Eco*RI digests, respectively (Figures 3 and 4). The 5-FOA^r, Trp^- transformants displayed the bands expected from a deletion of the *URA3* and one of the *hisG* sequences. *Bgl*II and *Eco*RI digests of chromosomal DNA ob-

TABLE 2

Integration of *leu2::hisG-URA3-hisG* fragment into strains containing 0, 1 and 2 copies of *his*G sequence

Strain	Relevant genotype	No. of copies of *hisG*	No. of Ura^+ transformants	No. of Ura^+, Leu^- transformants	Percent Ura^+, Leu^-
NKY274	*ura3, ho::LYS2, lys2*	0	92	66	72
NKY290	*ura3, ho::LYS2, lys2, trp1::hisG*	1	23	14	61
NKY289	*ura3, ho::hisG, lys2*	1	54	21	39
NKY536	*ura3, ho::hisG, lys2, trp1::hisG*	2	139	81	58

The above strains were transformed with a DNA fragment containing an insertion of the *hisG-URA3-hisG* sequences at the *Eco*RI site in *LEU2*.

tained from the 5-FOA^r derivatives yielded 2.6- and 2.4-kb bands, respectively, when probed with *hisG* sequences.

Construction of multiply marked strains using the 3.8-kb construct: We have thus far used the pNKY51 construct to make disruptions and/or deletions of the *TRP1, SPO13, HO, RAD50, URA3,* and *LEU2* yeast genes. Multiply marked strains can be made using these constructs in either of two ways: by transforming different construct disruptions into separate strains and generating the desired combinations by mating and sporulation, or by serial transformation of different construct disruptions into a single strain using repeating cycles of Ura^+ and Ura^- selections. We have used the first approach to construct haploid and diploid SK-1 derivatives containing as many as four copies of the *hisG* repeat sequence (not shown). The presence of these four inserts has no detectable effect on either vegetative growth or sporulation; the strains grow at normal rates and give the normal high proportion (>90%) of asci containing four viable spores. We do not know whether meiotic recombination between these ectopic repeat sequences (JINKS-ROBERTSON and PETES 1986; LICHTEN, BORTS and HABER 1987) occurs at some low level. We have also verified that it is feasible to transform strains bearing zero, one or two copies of the *hisG* sequence with a DNA fragment containing a *hisG-URA3-hisG* insert at the *LEU2* gene. The presence of *hisG* copies in the transformed strain does not interfere with integration of a construct disruption into its normal locus, as the proportion of Leu^- transformants is approximately 50% in all four strains (Table 2).

The *hisG-URA3-hisG* construct described here can easily be used to introduce gene disruption constructs into a wide variety of strain backgrounds. Selection for the desired integration event requires only the complementation of an appropriate Ura^- mutant by the *URA3* gene on the construct, and it is straightforward to select such Ura^- derivatives from any Ura^+ strain using 5-FOA.

Additional direct repeat constructs: We have constructed a vector analogous to pNKY51 in which the *URA3* gene is flanked by direct repeats of a different 1-kb sequence (derived from insertion sequence IS*10*) (HALLING *et al.* 1982). This second vector may be useful in combination with the first for construction of strains carrying large numbers of construct insertions or for special cases where having two copies of the *hisG* sequence is not desirable. It should also be straightforward to make new gene disruption constructs by substituting other selectable markers for the *URA3* gene in pNKY51. Most conveniently, the marker should be one for which both positive and negative genetic selections exist; the *LYS2* gene an obvious example (CHATTOO *et al.* 1979). If other types of markers are used, excision/reduction events must be obtained by genetic screening rather than by direct selection.

We gratefully acknowledge WENDY RAYMOND for critical comments on the manuscript and A. FERRI for help in manuscript preparation. E. A. was supported by a National Institutes of Health predoctoral genetics training grant to the Department of Biochemistry and Molecular Biology.

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Communicating editor: E. W. JONES