Yeast vectors for integration at the *HO* locus

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ABSTRACT

We have constructed new yeast vectors for targeted integration of desired sequences at the *Saccharomyces cerevisiae HO* locus. Insertion at *HO* has been shown to have no effect on yeast growth, and thus these integrations should be neutral. One vector contains the KanMX selectable marker, and integrants can be selected by resistance to G418. The other vector contains the *hisG-URA3-hisG* cassette, and integrants can be selected by uracil prototrophy. Subsequent growth on 5-FOA permits identification of colonies where recombination between the hisG tandem repeats has led to loss of the *URA3* marker and return to uracil auxotrophy. We also describe several new bacterial polylinker vectors derived from pUC21 (ampicillin resistance) and pUK21 (kanamycin resistance).

INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* is widely used as an experimental organism. Many genetic screens are facilitated by the stable integration of sequences into the yeast genome. For instance, one may want to insert a reporter gene needed for the screen so that it is stably maintained in the absence of growth on selective media and with no variation in copy number. Integration of an additional copy of a wild-type gene before conducting a mutant screen is another use of chromosomal integration. For example, we conducted a pilot screen for mutations that affect repression by LexA-Sin3 and identified numerous mutations in the *RPD3* gene (1). We then integrated a second copy of *RPD3* at the *URA3* locus and thus were able to eliminate this common mutation in a subsequent screen. One might want to integrate a cassette that conditionally expresses a gene, either the wild-type or a dominant negative version, in order to screen for suppressors (2–5).

Integration of a sequence into the yeast genome is often done by cloning a DNA fragment into a Yeast Integrating (YIp) plasmid, such as YIp5 (which has a *URA3* marker). Cleavage of the plasmid within the *URA3* sequence and transformation into a *ura3* strain usually leads to integration of the plasmid at the *URA3* locus (6). This integration frequently results in the plasmid sequences being present between two copies of *URA3* sequence, one being wild-type *URA3* and the other the original mutant *ura3* allele.

There are two difficulties with this common integration strategy. The first problem is that it uses up one of the markers, such as *ura3*, which may be needed for plasmids, libraries or disrupting specific genes. A second problem is that recombination can occur between the tandemly repeated sequences (i.e. *URA3* and *ura3* flanking the plasmid sequences), leading to loss of the integrated DNA fragment (6). Depending on exactly where recombination occurs, the strain that has excised the YIp plasmid may be *URA3* or *ura3*, and thus continuous selection for uracil prototrophy does not guarantee maintenance of the integrated plasmid.

Loss of the integrated DNA sequences can cause problems for some genetic screens involving selections. For example, suppose one has integrated a *YFG1-URA3* reporter gene for a screen in which the promoter of the *YFG1* (Your Favorite Gene) gene is driving expression of the *URA3* gene. One would mutagenize this strain and select for growth on 5-FOA (a uracil analog that selectively kills cells expressing the Ura3 enzyme; 7) to identify mutations that reduce expression of the *YFG1* gene. However, the recombination between the repeats flanking the reporter gene will result in excision of the reporter gene and an undesired 5-FOA-resistant colony. In a second example we have integrated a DNA fragment with a *GAL-YFG1** cassette. This fragment expresses the dominant negative *YFG1** allele from the inducible *GAL1* promoter, and thus the strain is unable to grow on galactose medium. A suppressor screen would be to look for either second site mutations or multicopy plasmids that allow this strain to grow on galactose. However, loss of the *GAL-YFG1** cassette through recombination between the flanking repeats would also result in growth on galactose, if YIp-type integration constructs are used.

To overcome these problems we have constructed plasmids that target integration of desired sequences at the *HO* locus without generation of any tandem duplication, using either the KanMX or *hisG-URA3-hisG* selectable markers. The *hisG-URA3-hisG* marker confers uracil prototrophy, but recombination between the repeated hisG sequences results in loss only of the *URA3* marker (8). Growth on 5-FOA can be used to select for strains that have returned to uracil auxotrophy. We have selected the *HO* locus as the target for integration for several reasons. *HO* encodes an endonuclease that initiates interconversion of the mating-type locus and promotes diploidization of haploid strains (9). The *HO* locus is not required for growth, and nearly all laboratory strains have a mutation at the *HO* locus. In fact, Baganz *et al.* (10) have quantitatively demonstrated that disruption of the *HO* gene has no effect on growth rate.

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MATERIALS AND METHODS

Plasmid construction

Plasmid pUC21ΔBB was constructed by cleaving pUC21 (11) with *Bam*HI and *Bgl*II followed by ligation to delete the fragment between the two sites; the resulting plasmid has neither a *Bam*HI nor a *Bgl*II restriction site. Plasmid pUC21-NotI was constructed by inserting the 123 bp *Not*I polylinker fragment from plasmid pFA6b (12) into pUC21ΔBB that had been cleaved with *Not*I. A similar strategy was used to construct equivalent vectors with a Kanamycin resistance marker, pUK21ΔBB and pUK21-NotI, starting with plasmid pUK21 (11).

Plasmid *HO*-poly-*HO* was constructed in several steps. A 912 bp fragment (*HO*-L) with sequences from the *HO* promoter (–2720 to –1814 upstream from the ATG) was made by PCR with primers designed to generate a fragment with *Hin*DIII and *Bsi*WI overhang sequences (see below). This fragment was inserted into *Hin*DIII–*Bsi*WI digested pUC21-NotI. A 507 bp fragment (*HO*-R) with sequences from the 3′ end of the *HO* gene (+1199 to +1699 downstream of the ATG) was made by PCR with primers designed to generate a fragment with *Eco*RI and *Sfi*I overhang sequences (see below). This fragment was inserted into *Eco*RI–*Sfi*I digested pUC21-NotI. Plasmid *HO*-poly-KanMX4-*HO* was constructed by inserting the 1494 bp *Bsi*WI–*Eco*RI fragment with KanMX4 from plasmid pFA6-KanMX4 (12) into *Bsi*WI–*Eco*RI digested *HO*-poly-*HO*. Plasmid *HO-hisG-URA3-hisG*-poly-*HO* was constructed by inserting the 3853 bp *Bam*HI–*Bgl*II fragment with the *hisG-URA3-hisG* cassette (8) into *Bam*HI digested *HO*-poly-*HO*.

The PCR amplification of the *HO*-L was performed using the 'sticky end PCR cloning' method (13) using four primers, F852–F855 (Table 1). Briefly, this method involves performing two PCR reactions followed by DNA melting and annealing. PCR with primers F852 and F854 results in a blunt-ended fragment where each end contains 5 bp of either the *Hin*DIII or *Bsi*WI recognition sites. In a separate reaction, PCR with primers F853 and F855 results in a blunt-ended fragment where each end contains 1 bp needed for the *Hin*DIII or *Bsi*WI recognition sites. The two PCR reactions are mixed, melted at 100°C and then slow cooled to allow DNA annealing. This DNA annealing results in four types of DNA products, with different ends. One-quarter of the DNA products have overhangs for cloning into the vector digested with *Hin*DIII and *Bsi*WI. This method eliminates the sometimes problematic step of restriction digestion of DNA products after PCR amplification. The same method was used to prepare the *HO*-R fragment using primers F856–F859.

Table 1. Primers

F852	AGC TTA ATT ATC CTG GGC ACG AGT
F853	TAA TTA TCC TGG GCA CGA GT
F854	GAC GCC ATT TTA AGT CCA AAG
F855	GTA CGA CGC CAT TTT AAG TCC AAA G
F856	AAT TCC TGG GGG AAC AAC TTC AC
F857	CCT GGG GGA ACA ACT TCA C
F858	CAT AGG CCA CTG TAA GAT TCC GCC ACA T
F859	AGG CCA CTG TAA GAT TCC GCC ACA T

RESULTS AND DISCUSSION

Construction of pUC plasmids with different polylinkers

Beginning with plasmid pUC21 (11), we constructed two new plasmids, pUC21ΔBB and pUC21-NotI, with different polylinkers (Fig. 1 and Table 2). The *Not*I restriction endonuclease has an 8 bp recognition sequence, and thus cuts rarely in genomic DNA. pUC21-NotI has two *Not*I sites with a large polylinker in between. Thus, it is likely that any fragment inserted in this region can be excised with *Not*I. We also made two related vectors, pUK21ΔBB and pUK21-NotI, which have Kanamycin resistance markers (Table 2). All of these plasmids maintain a continuous open reading frame through the polylinker and the lacZ' coding region, and can thus be used for blue/white screening for inserts during cloning. However, all of these plasmids turn blue more slowly than traditional pUC vectors due to a change in the spacing between the –10 and –35 regions of the bacterial promoter driving lacZ' expression (11).

Construction of plasmids for integration at HO

Plasmid *HO*-poly-*HO* (Fig. 1 and Table 2) was constructed by inserting two fragments from the *HO* gene into pUC21-NotI. These two fragments are 912 and 507 bp, respectively, and fragments of this size are very effective at directing homology-mediated recombination (14). These fragments were chosen for the lack of restriction sites present in the polylinker. The integration cassette can be excised by *Not*I cleavage. If the inserted fragment contains a *Not*I site there are several other sites in the polylinker that could be used.

Although we have not tested this, one could use this plasmid lacking a selectable marker for targeted integration at *HO*. One first inserts the *URA3* gene into the *HO* gene, and then transforms this strain with the *HO*-poly-*HO* plasmid with an insert. Selection on 5-FOA should identify transformants where ho::*URA3* has been replaced by the integrating vector, allowing retention of more available markers.

Plasmids *HO*-poly-KanMX4-*HO* and *HO-hisG-URA3-hisG*-poly-*HO* (Fig. 1 and Table 2) contain the KanMX4 and *URA3* selectable markers, respectively. KanMX, which confers resistance to G418, is rarely used for maintaining YCp or YEp plasmids, and use of this marker here allows the use of other markers (i.e. *URA3*, *HIS3*, etc.) for other genetic manipulations. Selection for uracil prototrophy can be used to identify integration of the *HO-hisG-URA3-hisG*-poly-*HO* plasmid at the *HO* locus. Moreover, one can select for cells that have returned to the ura3 state by selection on 5-FOA, identifying yeast in which recombination has occurred between the hisG repeats flanking the *URA3* gene (8). Thus, these cells which have the integrated sequences that are stably maintained in the absence of selection can be transformed with a *URA3* plasmid.

There are a number of other markers available as cassettes that could be that inserted into the *HO*-poly-*HO* plasmid, including nutritional markers such as *HIS3*, *LEU2* or *TRP1* (15), as well as dominant drug resistance markers (16).

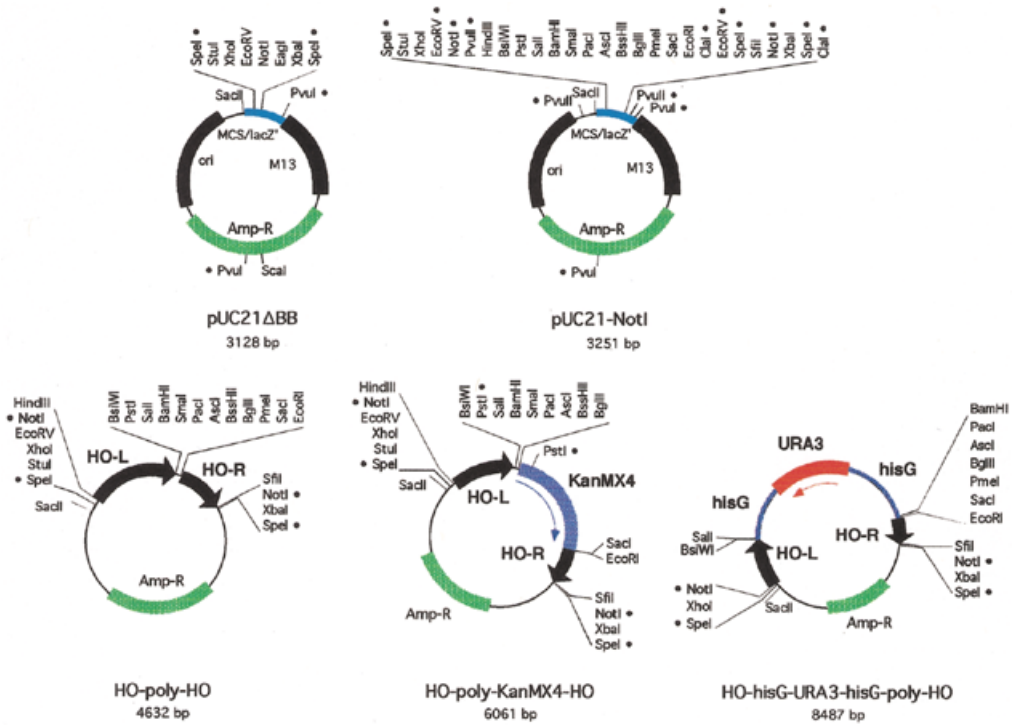

Figure 1. Plasmid maps. Each plasmid map is drawn to scale, with restriction sites indicated. A bullet (•) next to a restriction site indicates that it is not unique.

Efficiency of integration at HO

Several experiments were performed to determine the efficiency of integration at *HO*. Strain DY131, which has a *HO-lacZ* reporter integrated at the *HO* locus, was transformed with *Not*I cleaved *HO*-poly-KanMX4-*HO* and G418 resistant colonies were identified. The parent strain expresses lacZ from the *HO* promoter and this strain is blue on the chromogenic substrate X-gal. In contrast, integration of *HO*-poly-KanMX4-*HO* at the *HO* locus will eliminate the *lacZ* gene, and these colonies should be white on X-gal. We tested 14 transformants and found that all were white, showing that integration was very efficient. A similar experiment was performed with the *HO-hisG-URA3-hisG*-poly-*HO* plasmid, and we found that seven of eight Ura+ transformants were white.

In another test of the *HO*-poly-KanMX4-*HO* integrating vector, three separate genes were cloned into the polylinker. The plasmid was digested with *Not*I and the 10.6 kb *Not*I fragment was transformed into yeast. Ten independent G418-resistant colonies were examined, and for all 10 isolates PCR analysis demonstrated that the upstream and downstream endpoints of the integration were as predicted. For four of these 10 isolates, further PCR analysis was performed to characterize the internal sequences of the integrating fragment. The results showed that correct integration had occurred for all four. We conclude that integration is an efficient process.

Finally, we wish to draw attention to the 'sticky end PCR cloning' method (13) that we used in preparing these vectors (see Materials and Methods). One might have a fragment that one wishes to insert into these vectors, but there is a problem if the insert contains restriction sites for all of the sites in the polylinker. The sticky end PCR cloning method eliminates this problem, as there is no need to cleave the ends of the DNA after PCR amplification.

Table 2. Plasmids

Plasmid	Features	Marker in *Escherichia coli*	GenBank accession no.	ATCC number
pUK21ΔBB	Bacterial vector	Kan	AF324725	87799
pUK21-NotI	Bacterial vector	Kan	AF324726	87800
pUC21ΔBB	Bacterial vector	Amp	AF324723	87801
pUC21-NotI	Bacterial vector	Amp	AF324724	87802
HO-poly-*HO*	Integrating vector	Amp	AF324727	87803
HO-poly-KanMX4-*HO*	Integrating vector, KanMX	Amp	AF324728	87804
HO-hisG-URA3-hisG-poly-*HO*	Integrating vector, *URA3*	Amp	AF324729	87805

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REFERENCES

1. Dorland,S., Deegenaars,M.L. and Stillman,D.J. (2000) Roles for the *Saccharomyces cerevisiae SDS3*, *CBK1* and *HYM1* genes in transcriptional repression by *SIN3*. *Genetics*, **154**, 573–586.
2. Burke,D., Gasdaska,P. and Hartwell,L. (1989) Dominant effects of tubulin overexpression in *Saccharomyces cerevisiae*. *Mol. Cell Biol.*, **9**, 1049–1059.
3. Akada,R., Yamamoto,J. and Yamashita,I. (1997) Screening and identification of yeast sequences that cause growth inhibition when overexpressed. *Mol. Gen. Genet.*, **254**, 267–274.
4. Liu,H., Krizek,J. and Bretscher,A. (1992) Construction of a *GAL1*-regulated yeast cDNA expression library and its application to the identification of genes whose overexpression causes lethality in yeast. *Genetics*, **132**, 665–673.
5. Sandrock,T.M., Brower,S.M., Toenjes,K.A. and Adams,A.E. (1999) Suppressor analysis of fimbrin (Sac6p) overexpression in yeast. *Genetics*, **151**, 1287–1297.
6. Rothstein,R. (1991) Targeting, disruption, replacement and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.*, **194**, 281–302.
7. Boeke,J.D., LaCroute,F. and Fink,G.R. (1984) A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.*, **197**, 345–346.
8. Alani,E., Cao,L. and Kleckner,N. (1987) A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. *Genetics*, **116**, 541–545.
9. Herskowitz,I., Rine,J. and Strathern,J. (1992) Mating-type determination and mating-type interconversion in *Saccharomyces cerevisiae*. In Jones,E.W., Pringle,J.R. and Broach,J.R. (eds), *The Molecular and Cellular Biology of the Yeast Saccharomyces*. Cold Spring Harbor Press, Plainview, NY, pp. 583–656.
10. Baganz,F., Hayes,A., Marren,D., Gardner,D.C. and Oliver,S.G. (1997) Suitability of replacement markers for functional analysis studies in *Saccharomyces cerevisiae*. *Yeast*, **13**, 1563–1573.
11. Vieira,J. and Messing,J. (1991) New pUC-derived cloning vectors with different selectable markers and DNA replication origins. *Gene*, **100**, 189–194.
12. Wach,A., Brachat,A., Pohlmann,R. and Philippsen,P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*, **10**, 1793–1808.
13. Zeng,G. (1998) Sticky-end PCR: new method for subcloning. *Biotechniques*, **25**, 206–208.
14. Ma,H., Kunes,S., Schatz,P.J. and Botstein,D. (1987) Plasmid construction by homologous recombination in yeast. *Gene*, **58**, 201–216.
15. Jones,J.S. and Prakash,L. (1990) Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. *Yeast*, **6**, 363–366.
16. Goldstein,A.L. and McCusker,J.H. (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast*, **15**, 1541–1553.

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F855	GTA CGA CGC CAT TTT AAG TCC AAA G
F856	AAT TCC TGG GGG AAC AAC TTC AC
F857	CCT GGG GGA ACA ACT TCA C
F858	CAT AGG CCA CTG TAA GAT TCC GCC ACA T
F859	AGG CCA CTG TAA GAT TCC GCC ACA T

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HO-poly-KanMX4-*HO*	Integrating vector, KanMX	Amp	AF324728	87804
HO-hisG-URA3-hisG-poly-*HO*	Integrating vector, *URA3*	Amp	AF324729	87805