Yeast Functional Analysis Reports

Additional Modules for Versatile and Economical PCR-based Gene Deletion and Modification in *Saccharomyces cerevisiae*

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An important recent advance in the functional analysis of *Saccharomyces cerevisiae* genes is the development of the one-step PCR-mediated technique for deletion and modification of chromosomal genes. This method allows very rapid gene manipulations without requiring plasmid clones of the gene of interest. We describe here a new set of plasmids that serve as templates for the PCR synthesis of fragments that allow a variety of gene modifications. Using as selectable marker the *S. cerevisiae TRP1* gene or modules containing the heterologous *Schizosaccharomyces pombe his5*⁺ or *Escherichia coli kan*ʳ gene, these plasmids allow gene deletion, gene overexpression (using the regulatable *GAL1* promoter), C- or N-terminal protein tagging [with GFP(S65T), GST, or the 3HA or 13Myc epitope], and partial N- or C-terminal deletions (with or without concomitant protein tagging). Because of the modular nature of the plasmids, they allow efficient and economical use of a small number of PCR primers for a wide variety of gene manipulations. Thus, these plasmids should further facilitate the rapid analysis of gene function in *S. cerevisiae*. © 1998 John Wiley & Sons, Ltd.

KEY WORDS — epitope tagging; green fluorescent protein; functional analysis; overexpression studies; gene deletion; gene truncation; polymerase chain reaction

INTRODUCTION

In *Saccharomyces cerevisiae*, gene deletion and the modification of chromosomal genes by homologous recombination are now standard techniques. Among the useful gene modifications are placing a gene under control of a regulatable promoter for overexpression and/or protein-depletion studies and adding sequences encoding protein tags that allow facile protein detection and isolation. Recently, a PCR-mediated technique has been developed that allows single-step deletion and tagging of chromosomal genes (McElver and Weber, 1992; Baudin *et al.*, 1993; Lorenz *et al.*, 1995; Wach *et al.*, 1994, 1997, 1998). In this method, the PCR primers used have 5′-ends (~ 40 nucleotides) that correspond to the desired target gene sequences and 3′-ends (~ 20 nucleotides) that anneal to and allow amplification of the selectable marker gene (and, if included in the template, of sequences encoding a tag). The amplified DNA is

transformed directly into yeast, and homologous recombinants that carry the deleted or tagged target gene are identified. The method works best when the selectable marker itself cannot undergo homologous recombination with the host genome, and to this end heterologous marker modules based on the *Escherichia coli kan*^{r} gene (which confers resistance to G418/geneticin: Jiminez and Davies, 1980; Hadfield *et al.*, 1990) or the *Schizosaccharomyces pombe his5*$^+$ gene (which complements *S. cerevisiae his3* mutations) have been developed (Wach *et al.*, 1994, 1997).

Tagging of a protein has many uses. The *Aequorea victoria* green fluorescent protein (GFP) is proving to be invaluable for localizing proteins in both living and fixed cells (Prasher, 1995; Heim and Tsien, 1996; Niedenthal *et al.*, 1996), and both the influenza virus hemagglutinin (HA) epitope (Field *et al.*, 1988; Tyers *et al.*, 1993) and the c-*myc*-encoded Myc epitope (Evan *et al.*, 1985; Munro and Pelham, 1987) have been widely used both for protein localization by immuno-fluorescence and for the biochemical detection and isolation of proteins. The *Schistosoma japonicum* glutathione *S*-transferase (GST) (Smith *et al.*, 1986) is another useful protein tag that allows both the localization of proteins by immunofluorescence (Bi and Pringle, 1996; Longtine *et al.*, 1998) and the rapid, one-step biochemical purification of both the tagged protein and associated proteins using glutathione-conjugated agarose beads (Smith and Johnson, 1988; Ausubel *et al.*, 1995).

In this paper, we describe a set of plasmids that extend the range of the PCR-mediated gene-modification method in several ways. These plasmids allow a small number of PCR primers to be used for a wide variety of gene manipulations.

MATERIALS AND METHODS

Strains, growth conditions, and DNA methods

All *S. cerevisiae* strains were derived from YEF473 (**a**/α *ura3–52/ura3–52 his3Δ-200/his3Δ-200 trp1Δ-63/trp1Δ-63 leu2Δ-1/leu2Δ-1 lys2–801/lys2–801*) (Bi and Pringle, 1996). Yeast were grown at 30°C on YP solid medium, synthetic complete (SC) liquid or solid medium, or YM-P rich liquid medium (Lillie and Pringle, 1980; Guthrie and Fink, 1991) containing 2% dextrose, 1% raffinose, or 1% raffinose plus various concentrations of galactose (as indicated). YPD-G418 plates contained YP medium with 2% dextrose and 200 μg/

ml G418 (Life Technologies, Gaithersburg, MD). *E. coli* strain DH12S (Life Technologies) and standard media and methods (Ausubel *et al.*, 1995) were used for plasmid manipulations. Yeast genomic DNA was isolated according to the method of Hoffman and Winston (1987). Plasmid DNA was isolated from *E. coli* and from agarose gels using Qiagen kits (Qiagen, Santa Clarita, CA). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Yeastmaker DNA (Clontech Laboratories, Palo Alto, CA) was used as carrier DNA in yeast transformations.

DNA for plasmid constructions and yeast transformation was generated by PCR using the Expand system (Boehringer Mannheim, Indianapolis, IN) and HotStart 100 tubes (Molecular *Bio*-Products, San Diego, CA). The lower mix (final volume, 25 μl) contained 2·5 μl of Expand buffer with 17·5 mM-$MgCl_2$, 0·8 mM of each dNTP, 10 μg of BSA, and 2 μM of each primer. The upper mix (final volume, 75 μl) contained 7·5 μl of Expand buffer with 17·5 mM-$MgCl_2$, 0·75 μl of the Expand enzyme mixture, and 0·1 μg of template plasmid DNA. Reactions were run for 20 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min/kb of the desired product at 68°C; the 20 cycles were followed by a 10-min extension at 68°C.

PCR screening of transformants for integration by homologous recombination was done in 50 μl reactions containing 2 μM of each primer, 2 mM $MgSO_4$, 5 μg of BSA, 0·2 mM of each dNTP, 0·5 μl (2·5 units) of *Taq* DNA polymerase (Promega, Madison, WI), and 1 μl (~0·5 μg) of yeast genomic DNA. PCR reactions were run for 36 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min/kb of the desired product at 72°C; the 36 cycles were followed by a 10-min extension at 72°C.

Plasmid constructions

Plasmids pFA6a-kanMX6, pFA6a-His3MX6, pFA6a-GFP(S65T)-kanMX6, and pFA6a-GFP(S65T)-HIS3MX6 have been described by Wach *et al.* (1997); plasmid pGTEP (Tyers *et al.*, 1993; M. Tyers, G. Tokiwa, and B. Futcher, personal communication) contains sequences encoding three tandem repeats of the influenza virus hemagglutinin epitope (3HA); plasmid pCR2.1 15X Myc (containing sequences encoding 15 tandem repeats of the Myc epitope) was kindly provided by O. Mondesert and P. Russell; plasmid pGEX-2T (Smith and Johnson, 1988) contains the
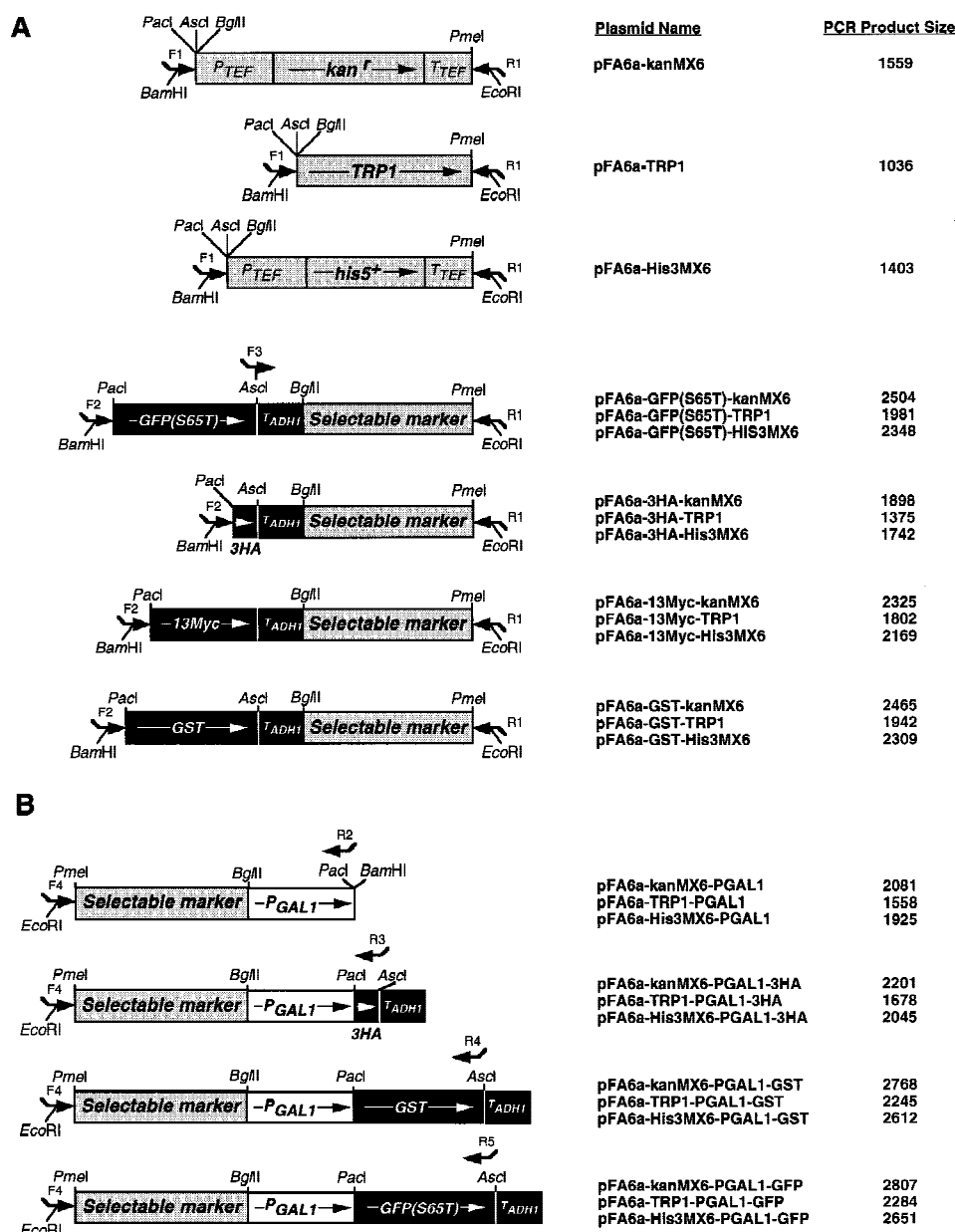
Figure 1. Modules for use as PCR templates to generate fragments for gene manipulation. Gray boxes: selectable markers including the *kanMX6* module (Wach *et al.*, 1994), the *S. cerevisiae TRP1* gene (see text), and the *His3MX6* module including the *S. pombe his5*+ gene (Wach *et al.*, 1997). Black boxes: protein-tagging modules consisting of the sequences encoding 3HA, 13Myc, GST, or GFP(S65T) together with the *S. cerevisiae ADH1* terminator. Arrows within the boxes indicate directions of transcription. Arrows outside the boxes indicate forward (F) and reverse (R) PCR primers (not to scale; see Table 1); the bent portions represent the regions of the primers homologous to the yeast target sequences. PCR product sizes are indicated assuming that each primer includes exactly 40 nucleotides of homology to the target sequence. Restriction sites used for cloning are indicated; note that the *AscI* site at the junction of 13Myc and T_{ADH1} sequences was lost during the construction of the pFA6a-13Myc plasmid series (see Materials and Methods). (A) Modules to be used for gene deletion, C-terminal protein tagging, or C-terminal protein truncation with or without protein tagging. (B) Modules to be used for placing a full-length or N-terminally truncated gene under control of the *GAL1* promoter (white boxes) with or without concomitant protein tagging.

Schistosoma japonicum gene encoding GST; and plasmid pBM272 (Johnston and Davis, 1984) contains the *S. cerevisiae GAL1/10* promoter region. Other plasmids (Figure 1) were constructed as follows; in these descriptions, italics in oligonucleotide primer sequences indicate a restriction enzyme site, underlining indicates the complement of a stop codon, and bold face indicates the complement of a start codon. Sequencing of all PCR products immediately after their initial cloning identified no mutations resulting from the PCR (except in the case of the 13Myc sequences; see below), and sequencing of insert junctions confirmed the preservation of the desired reading frames (where relevant).

To construct plasmid pFA6a-TRP1, the ~920-bp PCR product obtained using pRS304 (Sikorski and Hieter, 1989) as template, forward primer 5′-AAA*AGATCT*GTACAATCTTGATC CGGAGC-3′, and reverse primer 5′-AAA*GTTT AAAC*CTCCTTACGCATCTGTGCGG-3′ was digested with *Bgl*II and *Pme*I and ligated into *Bgl*II/*Pme*I-digested pFA6a-kanMX6, thus replacing the *kanMX6* module with the *S. cerevisiae TRP1* gene. To construct plasmid pFA6a-GFP(S65T)-TRP1, the *Bgl*II-*Pme*I fragment containing *TRP1* from pFA6a-TRP1 was ligated into *Bgl*II/*Pme*I-digested pFA6a-GFP(S65T)-kanMX6, thus replacing the *kanMX6* module with *TRP1*. To construct plasmid pFA6a-3HA-kanMX6, the PCR product obtained using pGTEP (see above) as template, forward primer 5′-AAAA*TTAATTAA* CATCTTTTACCCATACGATGTTCCT-3′, and reverse primer 5′-AAAA*GGCGCGCC*<u>TCA</u>G CACTGAGCAGCGTAATCTGGAAC-3′ was digested with *Pac*I and *Asc*I and ligated into P*ac*I/*Asc*I-digested pFA6a-GFP(S65T)-kanMX6, thus replacing *GFP(S65T)* with a 125-bp fragment encoding a 3HA epitope followed by a stop codon introduced by the primer. Plasmids pFA6a-3HA-TRP1 and pFA6a-3HA-His3MX6 were then created by digesting pFA6a-3HA-kanMX6 with *Bgl*II and *Pme*I and ligating in the corresponding fragments from pFA6a-TRP1 and pFA6a-His3MX6, respectively.

To construct plasmid pFA6a-13Myc-kanMX6, the PCR product obtained using pCR2.1 15X Myc (see above) as template, forward primer 5′-AAAA AA*TTAATTAA*CGGTGAACAAAAGCTAATC TCC-3′, and reverse primer 5′-AAAAAA*GGCG CGCC*<u>TCA</u>ATTCAAGTCTTCTTCTGAGAT - 3′ was cloned into pGEM-TA (Promega), yielding plasmid pGEM-TA/Myc. Sequence analysis of the

insert in pGEM-TA/Myc revealed that the *Pac*I site at one end was intact and that the insert encoded 13 Myc epitopes (the DNA encoding two Myc epitopes had apparently been lost during the PCR reaction) followed by the stop codon introduced by the primer. However, the *Asc*I site had been altered during the PCR reaction. Thus, pFA6a-13Myc-kanMX6 was constructed by digestion of pGEM-TA/Myc with *Eco*RI (at a site present in the pGEM-TA sequences), followed by blunting of the ends using T4 DNA polymerase and then digestion with *Pac*I. The resulting 594-bp fragment was purified and ligated to pFA6a-GFP(S65T)-kanMX6 that had been digested with *Asc*I, made blunt ended as above, and then digested with *Pac*I. Plasmids pFA6a-13Myc-TRP1 and pFA6a-13Myc-His3MX6 were constructed by replacing the *Bgl*II-*Pme*I fragment containing the *kanMX6* module of pFA6a-13Myc-kanMX6 with the corresponding fragments from pFA6a-TRP1 and pFA6a-His3MX6, respectively.

To construct plasmid pFA6a-GST-kanMX6, the ~700-bp PCR product obtained using pGEX-2T (see above) as template, forward primer 5′-AAAA*TTAATTAA*TATGTCCCCTATACTA GGTTATTG-3′, and reverse primer 5′-AAAA*GG CGCGCC*<u>TCA</u>ACGCGGAACCAGATCCGATT TTGG-3′ was digested with *Pac*I and *Asc*I and ligated into *Pac*I/*Asc*I-digested pFA6a-GFP(S65T)-kanMX6, thus replacing *GFP(S65T)* with sequences encoding GST followed by a stop codon introduced by the primer. To construct plasmids pFA6a-GST-TRP1 and pFA6a-GST-His3MX6, *Bgl*II-*Pme*I fragments from pFA6a-TRP1 and pFA6a-His3MX6, respectively, were ligated into *Bgl*II/*Pme*I-digested pFA6a-GST-kanMX6.

To construct plasmid pFA6a-kanMX6-PGAL1, the PCR product (containing the *GAL1* promoter) obtained using pBM272 (see above) as template, forward primer 5′-AAAA*AGATCT*GTAAAG AGCCCCATTATCTTA-3′, and reverse primer 5′-AAAA*TTAATTAA*AGA**CAT**TTTGAGATCC GGGTTTTTTCTCCTT-3′ was digested with *Bgl*II and *Pac*I, and the resulting 569-bp fragment was ligated into *Bgl*II/*Pac*I-digested pFA6a-kanMX6. To construct plasmids pFA6a-TRP1-PGAL1 and pFA6a-His3MX6-PGAL1, the *Bgl*II-*Pac*I fragment containing P_{GAL1} from pFA6a-kanMX6-PGAL1 was ligated into *Bgl*II/ *Pac*I-digested pFA6a-TRP1 and pFA6a-His3MX6, respectively. To construct plasmids containing sequences encoding 3HA, GST, and

Table 1. PCR primers used to amplify the transformation modules.

Primer	Purpose	Primer sequence[a]
F1	Deletion	5′-(gene-specific sequence) C*GGATCC*CCGGG*TTAATTAA*-3′[b]
R1	Deletion/C-terminal tagging	5′-(gene-specific sequence) *GAATTC*GAGCTC*GTTTAAAC*-3′ [b,c]
F2	C-terminal tagging	5′-(gene-specific sequence) C*GG ATC C*CC GGG *TTA ATT AA*-3′[c]
F3	C-terminal truncation (no tag)	5′-(gene-specific sequence) TGA *GGCGCGCC*ACTTCTAAA-3′[c]
F4	P$_{GAL1}$ introduction	5′-(gene-specific sequence) *GAATTC*GAGCTC*GTTTAAAC*-3′[d]
R2	P$_{GAL1}$ (no tag)	5′-(gene-specific sequence) **CAT** TTTGAGATCCGGGTTTT-3′[e]
R3	P$_{GAL1}$ with 3HA tagging	5′-(gene-specific sequence) GCA CTG AGC AGC GTA ATC TG-3′[f]
R4	P$_{GAL1}$ with GST tagging	5′-(gene-specific sequence) ACG CGG AAC CAG ATC CGA TT-3′[f]
R5	P$_{GAL1}$ with GFP(S65T) tagging	5′-(gene-specific sequence) TTT GTA TAG TTC ATC CAT GC-3′[f]

The primer combinations used for various manipulations and the orientations and locations of the forward (F) and reverse (R) primers relative to the plasmid templates are indicated in Figure 1. The reading frames for primers used to introduce protein tags are indicated by spacing in the sequences, and restriction enzyme sites included in the primers (see Figure 1) are indicated by italics: GGATCC, *Bam*HI; GAATTC, *Eco*RI; GTTTAAAC, *Pme*I; TTAATTAA, *Pac*I; GGCGCGCC, *Asc*I.

[a]The gene-specific sequences included in the primers used in this study were exactly 40 nucleotides in length. It is possible that using longer gene-specific sequences may improve the efficiency of homologous integration in some cases; note that this would slightly increase the sizes of the expected PCR products (see Figure 1).

[b]For deletions, the gene-specific sequences of the forward primer are typically chosen to end just upstream of the start codon, whereas those of the reverse primer are chosen to end just downstream of the stop codon.

[c]For tagging of full-length proteins, the gene-specific sequences of the forward primer are chosen to end just upstream of the stop codon, preserving the reading frame of the tag, whereas those of the reverse primer are chosen to end just downstream of the stop codon. For C-terminal truncations, the gene-specific sequences of the forward primer are chosen depending on the desired location of the truncation. If the protein is to be truncated without tagging, primer F3 (which includes a stop codon; underlining) is used with one of the plasmids of Figure 1A that contains the *Asc*I site at the junction of the tag and T$_{ADHI}$ sequences.

[d]The gene-specific sequences are typically chosen so as to delete ∼50 nucleotides upstream of the target gene start codon, but this can be varied depending on the desired extent of deletion of target gene promoter sequences and the perceived risk of affecting the expression of adjacent genes.

[e]The start codon (provided by its complement in the primer; bold face) lies 69 nucleotides downstream of the *GAL1* promoter transcriptional start. For P$_{GAL1}$-controlled expression of full-length genes, the gene-specific sequences are chosen to correspond to the complement of the N-terminal codons of the target gene, ending just downstream of the start codon; for N-terminal truncations, the gene-specific sequences are chosen from the region where the truncation is desired. In each case, the primer must maintain the reading frame of the start codon provided in the primer.

[f]For N-terminal tagging of full-length proteins, the gene-specific sequences are chosen to correspond to the N-terminal codons of the target gene; they may or may not include its start codon but must maintain the indicated reading frame. For N-terminal truncation with protein tagging, the gene-specific sequences are chosen from the region where the truncation is desired, maintaining the indicated reading frame. Each tag requires a unique primer that corresponds to the region just upstream of the stop codon present in the DNA encoding the protein tags.

GFP(S65T) under control of the *GAL1* promoter, pFA6a-kanMX6-PGAL1, pFA6a-TRP1-PGAL1, and pFA6a-His3MX6-PGAL1 were digested with *Pac*I and *Bam*HI and ligated to *Pac*I-*Bgl*II fragments carrying sequences encoding the tags from pFA6a-3HA-kanMX6, pFA6a-GST-kanMX6, and pFA6a-GFP(S65T)-kanMX6.

Transformation of yeast and screening for homologous integration at the target gene

PCR was performed using one of the plasmids shown in Figure 1 as template and appropriate target-gene-specific primer pairs designed as indicated in Figure 1 and Table 1. The products from six to eight PCR reactions were pooled, extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated, and resuspended in 10 μl of water. This concentrated DNA was transformed into *S. cerevisiae* cells using a lithium acetate procedure (Gietz *et al.*, 1992). G418-resistant transformants (containing the *kanMX6* module) were selected essentially as described previously (Wach *et al.*, 1994; Wach, 1996). Briefly, the transformed cells were washed once with 1 ml of water, resuspended in 200 μl of water, and spread on two YPD plates (100 μl per plate). These plates were incubated at 30°C for 2–3 days and replica-plated to YPD-G418 plates. To identify stable transformants, the YPD-G418 plates

were incubated at 30°C for 2–3 days and then replica-plated to fresh YPD-G418 plates, and G418-resistant colonies were picked and streaked on YPD-G418 plates. Trp$^+$ and His$^+$ transformants were selected by standard procedures (Guthrie and Fink, 1991). To identify transformants in which the module had indeed integrated by homologous recombination with the target gene sequences, genomic DNA was prepared and used as the template in PCR reactions (see above) using one primer that annealed within the transformation module and a second primer that annealed to the target gene locus outside the region altered. A PCR product of the expected size confirmed homologous integration; all transformants segregated 2:2 for the selectable marker.

Morphological observations

Differential interference contrast and epifluorescence microscopy were performed using a Nikon Microphot SA microscope. Cells were prepared for immunofluorescence as described by Pringle *et al.* (1991). Monoclonal anti-HA epitope (HA.11) and monoclonal anti-c-Myc epitope (9E10) antibodies were purchased from Berkeley Antibody Company (Richmond, CA). FITC and rhodamine-labeled secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Plasmid requests

Send plasmid requests to Mark Longtine (fax: (+1) 919/962 0320; e-mail: mlunc@isis.unc.edu). Investigators planning to use one or more of the plasmids for commercial purposes should state this in their requests. To receive a DNA Strider file of the plasmid sequences, send a Macintosh-formatted disk. For plasmids containing the *GFP(S65T)* allele, a Howard Hughes Medical Institute material transfer agreement must be signed. To obtain this document, contact Roger Y. Tsien, Howard Hughes Medical Institute, Cellular and Molecular Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093–0647 (fax: (+1) 619/534 5270) and state that you want to obtain the pFA-series plasmids with *GFP(S65T)* registered to A. Wach and P. Philippsen. A copy of the material transfer agreement must be received before plasmids containing *GFP(S65T)* can be shipped.

RESULTS AND DISCUSSION

PCR template plasmids for gene deletion and gene tagging

Wach *et al.* (1994, 1997) have described a set of plasmids that can be used as templates in PCR reactions to generate DNA fragments that can be used for targeted modification of chromosomal genes. These plasmids use either the *kanMX6* module (which confers resistance to G418) or the *His3MX6* module (containing the *S. pombe his5$^+$* gene, which complements *S. cerevisiae his3* mutations) as selectable marker and allow either gene deletion or generation of fusion genes encoding proteins with GFP (wild-type or the S65T mutant version) fused to their C-termini. In the work reported here, we have expanded this collection of plasmids in three ways.

First, we have created plasmids that allow use of the *S. cerevisiae TRP1* gene as the selectable marker for gene deletion and the generation of C-terminal GFP(S65T) fusions (Figure 1A). A disadvantage of using the *TRP1* marker in this method is that it can recombine efficiently with the endogenous *trp1* locus; thus it is only practical for use in strains that contain *TRP1* deletions such as *trp1*Δ-63 (Sikorski and Hieter, 1989). However, because the PCR products obtained using the *kanMX6* and *His3MX6* modules are homologous for long regions at their ends due to the shared *A. gossypii TEF* promoter and terminator sequences (Figure 1A), transformation of a strain containing one of these modules with a PCR product containing the other (to modify a second gene) may result in recombination with the previously integrated selectable marker rather than with sequences at the new target gene. Because the *TRP1*-containing PCR products have only ∼20 bp of homology with either the *kanMX6* or the *His3MX6* module, this problem is avoided.

Second, we have created plasmids that allow use of any of the three selectable markers to generate fusion genes encoding proteins fused at their C-termini to a triple HA epitope, a 13Myc epitope, or GST (Figure 1A). These protein tags have all been widely used; commercially available monoclonal antibodies directed against the HA or Myc epitope (see Materials and Methods) work well for immunofluorescence, Western-blot analysis, and immunoprecipitation, and reagents for isolating GST-fusion proteins are also commercially available. In addition, we have had success in localizing GST-fusion proteins in yeast by
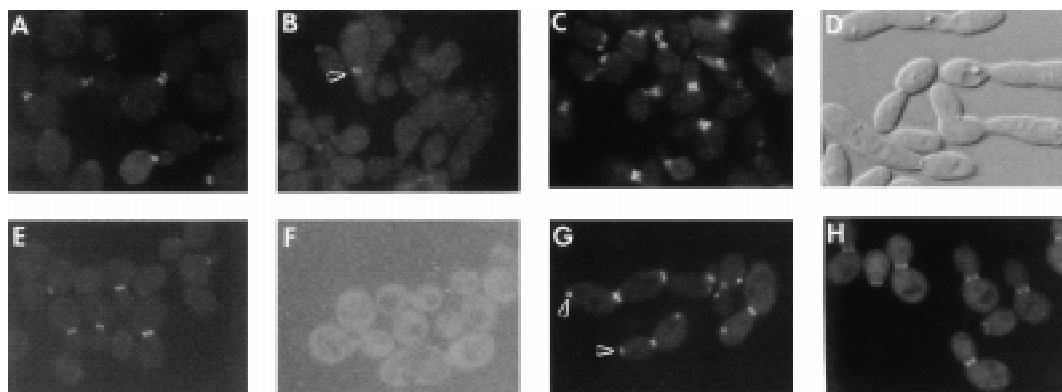
Figure 2. Tests of the PCR-template modules. (A–C) Immunofluorescence staining with anti-HA antibodies of strains M-651 (**a**/α *GIN4–3HA:kanMX6/GIN4–3HA:kanMX6*) (A), M-687 (**a** *TRP1:P_{GAL1}-GIN4–3HA:kanMX6*) (B), and M-711 (**a**/α *TRP1:P_{GAL1}-GIN4–3HA:kanMX6/GIN4*) (C) that had been grown on glucose (A, B) or on 1% raffinose with 2% galactose added 4 h before fixation (C). (D) Strain M-711 was grown for 18 h on 1% raffinose plus 2% galactose and visualized by differential interference contrast microscopy. (E–G) Fluorescence visualization of a Myo1p-GFP fusion protein in strain M-780 (**a** *MYO1-GFP(S65T):kanMX6*) grown on glucose (E) and in strain M-900 (**a** *His3MX6:P_{GAL1}-MYO1-GFP(S65T):kanMX6*) grown on glucose (F) or on 1% raffinose with 2% galactose added 4 h before viewing (G). (H) Fluorescence visualization of GFP-Cdc3p in strain M-899 (**a** *His3MX6:P_{GAL1}-GFP-CDC3*) grown to exponential phase on 1% raffinose plus 0·5% galactose. Arrowheads in (B) and (G) indicate features discussed in the text.

immunofluorescence using anti-GST antibodies (Bi and Pringle, 1996; Longtine *et al.*, 1998). Appropriate design of the forward primer allows the same plasmids to be used for C-terminal protein truncation with or without the inclusion of a protein tag (see primers F2 and F3 in Figure 1A and Table 1).

Third, to allow the regulated expression and/or overexpression of full-length or N-terminally truncated proteins with or without N-terminal 3HA, GST, or GFP(S65T) tags, as well as of tagged or untagged full-length or C-terminally truncated proteins, we constructed a set of plasmids in which each of the selectable markers is cloned upsteam of the *GAL1* promoter (P_{GAL1}) with or without associated tag sequences (Figure 1B). Appropriate design of the reverse primer (see Table 1) allows the generation of the N-terminal truncations.

Validation of the system

The system described here has been tested by manipulating several genes using the plasmids shown in Figure 1 as templates, primers designed as described in Table 1 (see also Figure 1), and the procedures described in Materials and Methods. For example, we tagged the 3′ end of *GIN4* (Longtine *et al.*, 1998) with 3HA sequences using the module from plasmid pFA6a-3HA-kanMX6. The normal cell morphology of the resulting

strains indicated that Gin4p-3HA was functional, and it localized normally to the mother-bud neck (Longtine *et al.*, 1998), as shown by immunofluorescence using anti-HA antibodies (Figure 2A).

To test the *GAL1* promoter and *TRP1* selectable-marker constructs, we then replaced the *GIN4* promoter of one of the *GIN4–3HA:kanMX6* strains by the module from plasmid pFA6a-TRP1-PGAL1. In the resulting strains, the expression of Gin4p-3HA was regulated by the carbon source, as expected. During growth on glucose, very few cells had detectable Gin4p-3HA (Figure 2B); rarely, weak Gin4p-3HA staining was visible (Figure 2B, arrowhead). The addition of galactose to cultures growing on raffinose rapidly induced the expression of Gin4p-3HA (Figure 2C), and long-term overexpression by growth on galactose resulted in abnormal cell morphologies (Figure 2D) like those observed after overexpression of normal Gin4p (Longtine *et al.*, 1998). Similar results were obtained when the expression of a Myo1p-GFP fusion protein, which localizes to the mother-bud neck (Figure 2E; E. Bi and J. R. Pringle, unpublished results), was placed under control of the *GAL1* promoter using the module from plasmid pFA6a-His3MX6-PGAL1 (Figure 2F, G). Interestingly, additional patches of Myo1p-GFP signal, which were often at or near the bud tips, were observed in many of the galactose-induced cells

(Figure 2G, arrowheads). It is not known if these patches represent an aspect of normal Myo1p localization or are purely an artifact of overexpression of the Myo1p-GFP fusion protein.

To test the modules for introducing the *GAL1* promoter with simultaneous N-terminal tagging, we replaced the promoter of the essential gene *CDC3* with P_{GAL1}-GFP sequences using the module from plasmid pFA6a-His3MX6-PGAL1-GFP. As expected, GFP-Cdc3p was not detectable when cells of a heterozygous diploid (*His3MX6:P_{GAL1}-GFP-CDC3/CDC3*) were grown on glucose, but it was localized properly to the mother-bud neck (Kim *et al.*, 1991) when the cells were grown on galactose-containing medium (data not shown). Similarly, haploid *His3MX6:P_{GAL1}-GFP-CDC3* segregants were viable on galactose-containing medium and localized GFP-Cdc3p normally to the neck (Figure 2H), but they were inviable on glucose-containing medium.

Taken together, the results indicate that the various modules shown in Figure 1 indeed function as expected.

Conclusions

In this paper, we describe a set of plasmids useful as templates for PCR-mediated gene modifications in *S. cerevisiae*. Using any of three selectable markers, the plasmids allow gene deletion, gene overexpression (using the regulatable *GAL1* promoter), C- or N-terminal protein tagging [with GFP(S65T), GST, or the 3HA or 13Myc epitope], and partial N- or C-terminal deletions (with or without concomitant protein tagging). Because of the modular nature of the plasmids (Figure 1; Table 1), they allow a wide variety of gene manipulations using a small number of PCR primers. The efficiency of integration by homologous recombination at the desired target site is typically high; in our hands, it has usually been >75% (ranging from ~20% to >95%).

The tagging of proteins often provides a rapid and effective route to their localization and isolation. Most proteins appear to localize normally and retain function when tagged at their C-termini, and proteins that are not functional with one C-terminal tag are often functional with another or with an N-terminal tag. A particular attraction of protein tagging for localization studies is the ease of doing double staining. Because high-quality mouse monoclonal antibodies to the HA and Myc epitopes are available (see Materials and Methods), a protein tagged with one of these epitopes should generally be localizable in combination with a protein for which a non-mouse antibody is available. Moreover, the commercial availability of rabbit antibodies to both HA (Berkeley Antibody Company) and GST (Molecular Probes, Eugene OR; see also Bi and Pringle, 1996) allows double staining of two tagged proteins.

The versatility of this system for PCR-mediated gene modifications is far from exhausted. In particular, it could clearly be extended by the addition of other selectable markers (e.g., see Längle-Rouault and Jacobs, 1995; van den Berg and Steensma, 1997), additional promoters (e.g., see Niedenthal *et al.*, 1996), and/or other protein tags [including new GFP variants with altered emission spectra (Heim and Tsien, 1996) that could perhaps be used in combination with GFP(S65T)-tagged proteins to allow double labeling in living cells]. The approach can also be used to introduce a reporter gene for assays of promoter function (e.g., see Niedenthal *et al.*, 1996; Wach *et al.*, 1998). Another useful variation of this methodology allows the PCR-mediated introduction of site-directed mutations into chromosomal genes (Längle-Rouault and Jacobs, 1995). Finally, transformation of yeast with PCR-generated fragments can also be used to introduce sequences encoding a protein tag into a gene carried on a resident plasmid (A. McKenzie and J. R. Pringle, unpublished results); the plasmid carrying the tagged gene can be isolated by electroporation into *E. coli*, selecting for kanamycin resistance, and then retransformed into yeast.

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