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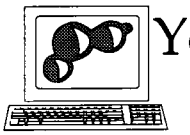

Yeast Functional Analysis Reports

Heterologous *HIS3* Marker and GFP Reporter Modules for PCR-Targeting in *Saccharomyces cerevisiae*

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We have fused the open reading frames of *his3*-complementing genes from *Saccharomyces kluyveri* and *Schizosaccharomyces pombe* to the strong *TEF* gene promotor of the filamentous fungus *Ashbya gossypii*. Both chimeric modules and the cognate *S. kluyveri HIS3* gene were tested in transformations of *his3 S. cerevisiae* strains using PCR fragments flanked by 40 bp target guide sequences. The 1·4 kb chimeric *Sz. pombe* module (*HIS3MX6*) performed best. With less than 5% incorrectly targeted transformants, it functions as reliably as the widely used geniticin resistance marker *kanMX*. The rare false-positive His^+ transformants seem to be due to non-homologous recombination rather than to gene conversion of the mutated endogenous *his3* allele. We also cloned the green fluorescent protein gene from *Aequorea victoria* into our pFA-plasmids with *HIS3MX6* and *kanMX* markers. The 0·9 kb GFP reporters consist of wild-type GFP or GFP-S65T coding sequences, lacking the ATG, fused to the *S. cerevisiae ADH1* terminator. PCR-synthesized 2·4 kb-long double modules flanked by 40–45 bp-long guide sequences were successfully targeted to the carboxy-terminus of a number of *S. cerevisiae* genes. We could estimate that only about 10% of the transformants carried inactivating mutations in the GFP reporter. © 1997 John Wiley & Sons, Ltd.

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INTRODUCTION

Modification of *Saccharomyces cerevisiae* genomic DNA by one-step targeted integration with PCR products (PCR-targeting) has become a standard technique in yeast molecular biology (Amberg *et al.*, 1995; Baudin *et al.*, 1993; Eberhardt and Hohmann, 1995; Güldener *et al.*, 1996; Horton, 1995; Laengle-Ronault and Jacobs, 1995; Lorenz *et al.*, 1995; Maftahi *et al.*, 1996; Mallet and Jacquet, 1996; Manivasakam *et al.*, 1995; McElver and Weber, 1992; Wach, 1996; Wach *et al.*, 1994). As little as 30 bp of homologous DNA on each flank of the transforming linear DNA molecule is sufficient for sequence-directed targeting in *S. cerevisiae* (Manivasakam *et al.*, 1995). Two important improvements were achieved with the introduction of *kanMX* (Wach *et al.*, 1994), a completely heterologous marker for efficient

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expression of geneticin-resistance driven by a fungal promotor (Steiner and Philippsen, 1994). First, it was no longer required to work with *S. cerevisiae* strains carrying specific auxotrophic markers, and second, the unavoidable background of gene conversions of auxotrophic mutations observed in PCR-targeting with *S. cerevisiae* genes as selectable markers was eliminated, increasing the yield of correctly targeted transformants to 98% (Brachat, 1997).

For experimental demands aiming at PCR-targeting of more than one gene locus, additional selection markers lacking homology to *S. cerevisiae* genes are desirable. One solution would be to use heterologous genes complementing auxotrophic markers common in *S. cerevisiae* strains, like *leu2* or *ura3*. We have tried the *Ashbya gossypii LEU2* gene (Wach *et al.*, 1994), which is able to complement a *S. cerevisiae leu2* mutation (Mohr, 1995). Unfortunately, the expression level was low and Leu^+ transformants took 1 week to form small colonies. PCR-targeting using the *Kluyveromyces lactis URA3* gene has also been reported (Laengle-Ronault and Jacobs, 1995). With this selection marker a rather high level of gene conversion of the *ura3* marker was observed, except with *S. cerevisiae* strains carrying a complete deletion of the *URA3* locus. Another frequently used auxotrophic marker is *his3*. Two homologs of the *S. cerevisiae HIS3* gene have been cloned: the *S. kluyveri HIS3* gene (Weinstock and Strathern, 1993) and the *Schizosaccharomyces pombe his5* gene (Erickson and Hannig, 1995). Both are able to complement a *S. cerevisiae his3* mutation. This ability, together with open reading frame (ORF) sizes of only 0·7 kb and over 30% sequence divergence to the *S. cerevisiae HIS3* gene, encouraged us to test these heterologous genes as selection markers for PCR-targeting.

Targeted fusions of reporter genes to promoters or ORFs are also important tools for functional analysis of *S. cerevisiae* genes. Our first set of pFA-kanMX plasmids (Wach *et al.*, 1994) contained *Escherichia coli lacZ* reporter constructs. The use of these *lacZMT-kanMX* double modules in PCR-targeting would require amplification of DNA fragments of more than 5 kb. Although it is possible to produce by PCR even longer DNA fragments (Barnes, 1994), the risk of introducing inactivating mutations into the reporter gene is high and directly correlates with its length. The coding sequence of the light excitable green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is only 0·71 kb (Chalfie *et al.*, 1994). Double modules with GFP as reporter and *kanMX* or heterologous *HIS3* as selection marker would consist of slightly more than 2 kb and would therefore be well-suited for PCR targeting.

In this paper, we describe the construction and testing of new pFA plasmids carrying a heterologous hybrid *HIS3* marker called *HIS3MX* as well as GFP reporter modules with *HIS3MX* or with *kanMX* as the selection marker. We provide evidence that *HIS3MX* works as efficiently as *kanMX* in *S. cerevisiae* transformations using short flanking homology (SFH) PCR products and that the GFP reporter modules perform very well in promotor fusions, in construction of strains with GFP-labeled organelles, as well as in subcellular localization analyses of gene products.

MATERIALS AND METHODS

Strains, media and plasmids

The *E. coli* strain XL1-blue (Bullock *et al.*, 1987) served as plasmid host. For selective growth, the bacteria were grown on 2 × YT (10 g yeast extract, 16 g tryptone, 5 g NaCl) containing either 100 mg/l ampicillin or 50 mg/l kanamycin (Fluka AG, Buchs, Switzerland). Three *S. cerevisiae* strains were used: FY1679 (**a**/α *ura3-52/ura3-52 leu2Δ1/+ trp1Δ63/+ his3Δ200/+*) constructed in B. Dujon's laboratory by crossing FY23 with FY73 (Winston *et al.*, 1995), CEN.PK2 (**a**/α *ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-289/trp1-289 his3Δ1/his3Δ1*; K.-D. Entian and P. Kötter, personal communication), and W303 (**a**/α ura3-*1*/*ura3-1 leu2-3*,112/*leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100*; Thomas and Rothstein, 1989). The *S. kluyveri* strain CBS3082 was used to isolate genomic DNA of this yeast species. The plasmid Ep478 (Erickson and Hannig, 1995) was used to extract the *Sz. pombe his5* cDNA. Several pFA plasmids were used for cloning (Wach *et al.*, 1994; Wach, 1996). Yeast was grown in 2% yeast extract, 1% peptone, and 2% glucose (YPD) or on complete synthetic media, containing per liter: 7 g of yeast nitrogen base (Difco Laboratories), 1·1 g of dropout mix, supplemented with all amino acids (Treco, 1989) except those used for selection, and 2% glucose (SD). Solid media contained, in addition, 2% agar (Difco Laboratories). G418 resistant strains were grown on YPD plates containing 200 mg/l of G418 (geneticin; Gibco BRL, Gathersburg, MD).

Thermostable DNA polymerases

Thermostable DNA polymerase from *Thermus aquaticus* (Taq DNA polymerase) was purchased from Boehringer, Mannheim, Germany. Thermostable DNA polymerase from *Thermococcus litoralis* (Vent DNA polymerase) was obtained from New England Biolabs, Beverly, MA, USA. For PCR a mixture of Taq and Vent DNA polymerases of 10:1 (U:U) was used.

All DNA manipulations were performed according to standard protocols (Sambrook *et al*., 1989). If not otherwise stated, PCR amplification of DNA fragments was done in 25 µl reaction volume containing 1 × Vent buffer (as specified by the supplier) and either 200 ng of plasmid DNA or 1 µg genomic DNA as template, 1 µM of each primer and 200 µM dNTPs. The PCR was performed in a PCR machine with a heated lid. After an initial denaturation period of 2 min at 92°C, the PCR was started by the addition of 2 U of Taq/Vent DNA polymerase mix. Each cycle consisted of 30 s at 92°C, 30 s at 55°C, and 60 s/kb of amplification product at 72°C. The number of cycles was 12 for plasmid template, 20 for genomic DNA template, 18 for SFH-PCR, or 30 for yeast cells (treated in a microwave for 1 min). Before cloning, PCR products were treated with phenol/chloroform, ethanol precipitated, and digested with restriction enzymes if required. Restriction-enzyme-cleaved DNA fragments were further purified by agarose gel electrophoresis and eluted from gel slices by centrifugation (Heery *et al*., 1990). These eluates were directly used in DNA ligation reactions.

Cloning of S. kluyveri HIS3 *into pFA6a*

The *S. kluyveri HIS3* gene (AC Z14125; Weinstock and Strathern, 1993) was PCR amplified with genomic DNA of the strain CBS3082 as template and four different oligonucleotides as primers: SkH3U (5′-AGAAGATCTGCTTGC TTTTTCTTTTTTTTTTTC-3′), SkH2R (5′-AA ACGACTCGAGAACTGTATATATTAAG-3′), SkH2cU (5′-TAGCCATGGCAGAACCAGCCC AAAAAAAGC-3′) and SkH3cR (5′-ACTTCAC ATCAAAACACCTTTGGTTG-3′). The following primer combinations were used to amplify DNA fragments of the *S. kluyveri HIS3* gene: (a) SkH3U and SkH3R (0·94 kb, promotor, coding sequence, and terminator); (b) SkH3cU and SkH3cR (0·70 kb, coding sequence only). The resulting PCR products were cleaved by either *Bgl*II (a) or *Nco*I (b) and ligated into pFA6a-kanMX6 digested with *Bgl*II, *Pme*I (a) nor *Nco*I, partial (b). The resulting plasmids were named pFA6a-HIS3$_k$PT6 (a) and pFA6a-HIS3$_k$MX6 (b).

Cloning of Sz. pombe his5 *coding sequence into pFA6a*

The plasmid Ep478, containing the *Sz. pombe his5* cDNA, was used as template in a PCR with the primers Sph5U (5′-GCACCATGGGTAG GAGGGCTTTTGTAGAAAG-3′) and Sph5R (5′-ATCCTTTACAACACTCCCTTCGTGC-3′) to amplify the *Sz. pombe his5* coding sequences (ACU07831; Erickson and Hannig, 1995). The resulting 0·65 kb PCR product was cut with *Nco*I and ligated with the *Nco*I, partial *Sca*I-digested pFA6a-kanMX6 plasmid. The resulting plasmid was called pFA6a-HIS3MX6.

Cloning of the Aequorea victoria *GFP coding sequence into pFA6*

The plasmids pGFP (Clontech, Palo Alto, CA, USA) and pRSET-S65T containing a mutated GFP allele (Heim *et al*., 1995) were used as templates. The primers GFP-U (5′-CGCTTAAT TAAC AGT AAA GGA GAA GAA CTT TTC AC-3′) and GFP-R (5′-ATAGGCGCGCC CTA TTT GTA TAG TTC ATC CAT GC-3′) were designed in such a way that only the GFP coding sequence (codon 2 to stop codon) and a *Pac*I site (plus additional C) upstream of codon 2 and an *Asc*I site downstream of the stop codon were amplified. The PCR product was digested with *Pac*I and *Asc*I and ligated with *Pac*I, *Asc*I-cut pFA6a-lacZMT-kanMX3 giving pFA6a-GFPMT-kanMX3. Other GFP-containing plasmids were constructed by insertion of the *Pac*I-*Bgl*II fragment (GFP plus *ADH1* terminator) from pFA6a-GFPMT-kanMX3 into pFA6a-kanMX6, or pFA6a-HIS3MX6 (*Sz. pombe*). Corresponding pFA6 plasmids with frame b or c were then constructed by transferring the *Pac*I, *Pme*I-extracted reporter-marker double modules into *Pac*I, *Pme*I-cut pFA6b or pFA6c. Correct frames were checked by dideoxy nucleotide sequencing.

Transformation of yeast (Gietz and Woods, 1994) and verification of G418^r transformants by PCR according to Huxley *et al*. (1990) was done as described earlier (Wach *et al*., 1994; Wach, 1996).

For microscopy a Zeiss Axioplan 2 Microscope (Zeiss, Jena, Germany) equipped with a filter wheel with shutter and controller (MAC2000, Ludl

Electronic Products Ltd, Hawthorne, NY, USA), and a VI-470 RGB remote head microscope video camera with image processor (Optronics, Goleta, CA, USA) was used. GFP fluorescence in living cells was imaged at room temperature with Zeiss filter set 10 (FITC) and a 75 W Xenon light source. Excitation filters were removed from the filter cube and placed in the filter wheel. Cut-off filters and emission filters were left in the filter cube. Image acquisition and processing was performed on a PowerMac 7600/120 computer using the public domain NIH image 1·60 program (developed by Wayne Rasband at the US National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GEI). Custom macros were used to control the microscope, the video camera, the MAC2000, and an LG-3 frame grabber (Scion Corp., Frederick, MD, USA). For microscopic analysis, yeast was grown overnight in 5 ml of liquid YPD at 23°C until mid-exponential phase (2×10^7 cells/ml). Five µl of cell suspension were mounted on a microscope slide, sealed with a cover slip, and immediately inspected.

Requests for pFA plasmids

Please send your plasmid request to Peter Philippsen (fax: int-41-61-2672118 or E-mail: Sekretariat4@UbaClu.UniBas.ch). The following plasmids (see Figure 4 for details) are available for scientific research: pFA6a-HIS3MX6, pFA6a-GFPMT-kanMX3, pFA6a-$GFPS_{65}$T-kanMX3, pFA6a-GFPMT-kanMX6, pFA6a-$GFPS_{65}$T-kanMX6, pFA6a-GFPMT-HIS3MX6, pFA6a-$GFPS_{65}$T-HIS3MX6 (all GFP-containing plasmids are also available in frames b or frames c). Investigators who plan to use one or more of the plasmids for commercial purposes should please state this fact in their request.

For plasmids containing the GFPS65T variant, a Howard Hughes Medical Institute material transfer agreement has first to 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 at fax int(1)619-534 5270 and mention that you want to use the pFA plasmids with GFPS65T registered on A. Wach and P. Philippsen. We are unable to ship plasmids before you send us a copy of your Howard Hughes Medical Institute material transfer agreement.

RESULTS AND DISCUSSION

Testing of heterologous his3 *complementing genes in* S. cerevisiae

The genes encoding the histidine biosynthetic enzyme imidazole glycerol phosphate (IGP) dehydratase have been named *HIS3* in *S. cerevisiae* (Struhl *et al.*, 1976), *k-HIS3* in *S. kluyveri* (Weinstock and Strathern, 1993) and *his5*$^+$ in *Sz. pombe* (Erickson and Hannig, 1995). On the DNA level, the heterologous IGP-dehydratase ORFs have an overall identity of 70·4% (*S. kluyveri*) and 58·6% (*Sz. pombe*) to the *S. cerevisiae HIS3* ORF. An alignment of the *HIS3* ORFs of *S. kluyveri* and *S. cerevisiae* revealed six clusters of 30 to 60 nucleotide pairs with 80 to 90% sequence identity (black bars in Figure 1). The alignment of *Sz. pombe his5*$^+$ with *S. cerevisiae HIS3* did not show clusters with that high level of identity; only three clusters with 70 to 76% identical nucleotides were found (grey bars in Figure 1).

We constructed three heterologous *HIS3* modules in plasmid pFA6a: *k-HIS3* with the cognate *S. kluyveri HIS3* gene carrying 110 bp upstream of the ATG, *k-HIS3MX6* consisting of *A. gossypii TEF*-promotor/terminator sequences flanking the *S. kluyveri HIS3* ORF, and *p-HIS3MX6* consisting of *A. gossypii TEF*-promotor/terminator sequences flanking the *Sz. pombe his5*$^+$ gene (see Materials and Methods for details and Figure 1 for maps). These three modules were targeted to the *S. cerevisiae* gene YNL040w in the diploid tester strain CEN.PK2 using short flanking homology PCR products as outlined in Figure 2. This multiple marked strain is histidine auxotroph due to homozygous *his3Δ1* mutant alleles in which 190 bp (27%) have been removed from the middle of the *HIS3* ORF (Scherer and Davis, 1979).

Both hybrid *HIS3* modules (*k-HIS3MX6*, *p-HIS3MX6*) complemented the *his3* mutation in CEN.PK2 very well. After 2 days, 50–100 fast-growing His$^+$ transformants were obtained with 1 µg PCR product. In contrast, the construct with the cognate *S. kluyveri HIS3* gene (*k-HIS3*) only yielded one to two His$^+$ transformants with 3 µg PCR product. Weinstock and Strathern (1993)

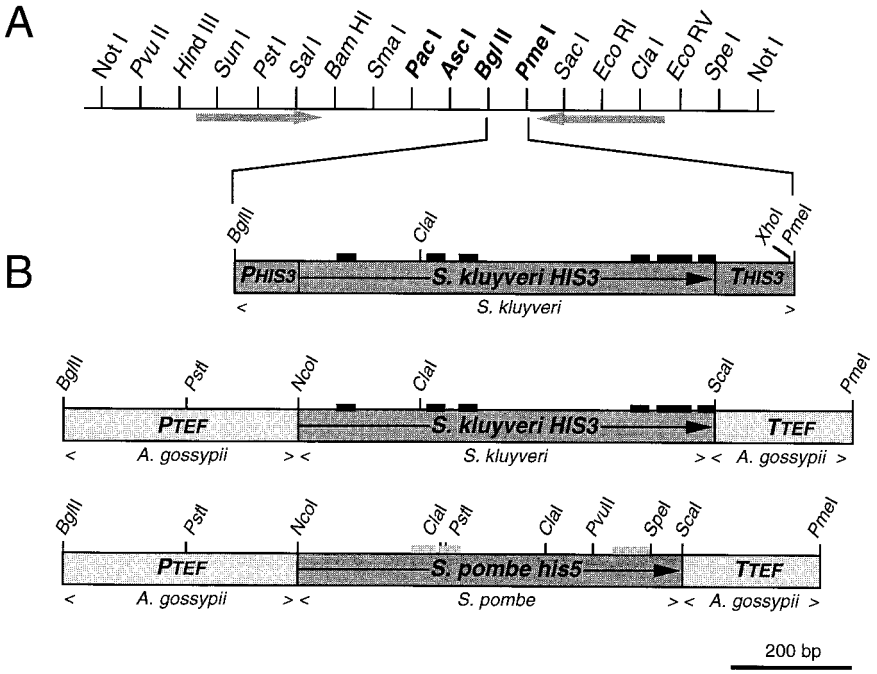

Figure 1. *HIS3* heterologous marker modules for histidine prototrophic selection in *S. cerevisiae*. (A) Restriction enzyme recognition sequences in the multiple cloning site (MCS) of pFA6a (Wach *et al*., 1994). Sites used for cloning of the marker modules (and for GFP reporters, see below) are shown in bold. Sequences of the MCS, underlined by grey arrows, representing the 3′-part of chimeric disruption primers (see also Figure 2) are indicated as small black bars. (B) Maps of the heterologous *HIS3* marker modules constructed in this work. Top panel: wild-type *S. kluyveri HIS3* gene (Weinstock and Strathern, 1993). Middle panel: chimeric gene consisting of the *S. kluyveri HIS3* ORF flanked by the *A. gossypii TEF* gene promotor (P_{TEF}) and terminator (T_{TEF}) elements (Steiner and Philippsen, 1994). Bottom panel: chimeric gene consisting of the *Sz. pombe his5*$^+$ ORF (Erickson and Hannig, 1995) flanked by *A. gossypii TEF* gene regulatory elements. Only those restriction enzyme sites are indicated which are also present in the MCS. Brackets are used to delimit elements that originate either from *S. kluyveri*, *Sz. pombe* or *A. gossypii*. The GCG program *Compare* (Wisconsin University) was used to identify regions of 30 or more base pairs with highest sequence identity to the *S. cerevisiae HIS3* ORF. Regions with 80–90% identical nucleotides are shown as black bars and those with 70–76% identical nucleotides as grey bars.

reported that a *S. kluyveri HIS3* fragment with 110 bp upstream of the start codon and cloned in a CEN/ARS, *URA3* plasmid could complement a *his3* mutation is *S. cerevisiae*. However, cells transformed with this plasmid were first selected on medium lacking only uracil before they were tested on medium lacking histidine. Our data indicate that the *S. kluyveri HIS3* gene under control of its native promoter is not well suited for direct selection of integrative transformants in *S. cerevisiae*.

Thirty-six randomly picked CEN.PK2 His$^+$ clones each from the *k-HIS3MX6* and the *p-HIS3MX6* transformation experiments were analysed by PCR for correct integration of the marker module as outlined in Figure 2. We found that 32 of the 36 *k-HIS3MX6* transformants and all 36 *p-HIS3MX6* transformants carried correctly targeted marker modules. Since the hybrid module with the *Sz. pombe HIS3* homolog gave the best result (no false positives), we exclusively used this module for further constructions and experiments and, for reasons of simplicity, called it *HIS3MX6*.

In order to test whether gene conversion can occur between *HIS3MX6* and the genomic *his3Δ1* allele, CEN.PK2 cells were transformed with *HIS3MX6* PCR fragments carrying or lacking

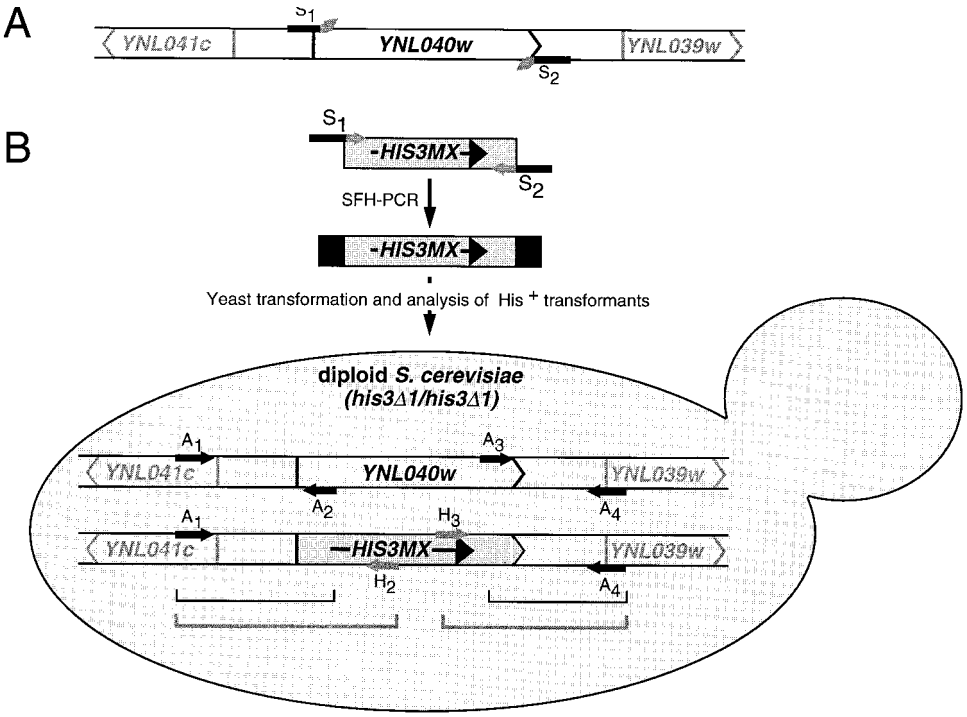

Figure 2. Transformation of *S. cerevisiae* with SFH-PCR products carrying heterologous *HIS3* modules. (A) Selection of chimeric primer sequences for targeted deletion of ORF YNL040w. Black bars indicate ORF-specific and grey arrows pFA6a-MCS-specific nucleotides. The sequence of S_1 is: 5′-GC ACA CAA CAG CGT CAA AAA TTG ATT AAA AGG TAA GTT ATC **ATG** CGTACG CTGCAGGTCGAC-3′ (44 nucleotides upstream of the target ORF, start codon shown in bold, plus 18 nucleotides of pFA6a-MCS carrying *Sun*I, *Pst*I, *Sal*I sites, underlined sequence). The sequence of S_2 is: 5′-TT ATG TAT ACA ACA AAT CTG CGT ATA CAA ATG GCA CAT TTC **TCA**C ATCGAT GAATTC GAGC TC G-3′ (45 nucleotides downstream of the target ORF, triplet complementary to the stop codon shown in bold, plus 19 nucleotides of pFA6a-MCS carrying *Cla*I, *Eco*RI, *Sac*I sites, underlined sequence). (B) Synthesis by PCR of YNL040w-specific disruption cassettes and genotype of a correctly targeted *S. cerevisiae* cell. ORF replacements are identified by PCR with the help of diagnostic primers which bind outside of the target ORF (A_1 and A_4), within the target ORF (A_2 and A_3), and within the selection marker (H_2 and H_3). With primers A_1, A_2, and H_2 in one PCR and primers A_3, A_4, and H_3 in a second PCR, DNA fragments are synthesized with sizes characteristic for the wild type and the targeted locus as indicated by black and grey brackets, respectively.

flanking guide sequences. Sixty His^+ transformants were obtained with 1 µg SFH-PCR product (45 bp flanking homology to YNL040w) and two His^+ transformants with 3 µg PCR-made *HIS3MX6* lacking guide sequences. Analytical PCR with diagnostic primers binding upstream and downstream of the natural *HIS3* locus on chromosome XV showed that these two His^+ transformants still carried both *his3Δ1* mutant alleles. Obviously, they became His^+ by non-homologous integration of the PCR fragment and not by gene conversion. These data allow the conclusion that the targeting fidelity of SFH-PCR fragments with *HIS3MX6* as selection marker is over 95%.

The *HIS3MX6* module was also successfully used for ORF replacements in the diploid strain W303 carrying two *his3-11,15* alleles (Thomas and Rothstein, 1989) and the haploid strain FY73, a S288C derivative carrying the *his3-Δ200* mutation (Winston *et al.*, 1995). Successive SFH-PCR-targeting using *HIS3MX6* and *kanMX4* or *vice versa* should allow efficient generation of any double gene inactivations at homologous or heterologous loci. Positive double disruptants can easily be identified by selecting transformants

which are geneticin-resistant and histidine prototroph. However, it should be noted that geneticin selection only works on yeast full medium and not on high-salt SD minimal medium.

GFP-HIS3MX *and* GFP-kanMX *reporter/marker modules*

We wanted to test the GFP from *A. victoria* as heterologous reporter in PCR-targeting experiments. Cloned fusion genes with GFP have been successfully used as self-fluorescent protein reporters in a variety of different hosts, including *S. cerevisiae* (Chalfie *et al.*, 1994; Halme *et al.*, 1996; Kahana *et al.*, 1995; Niedenthal *et al.*, 1996; Searns, 1995; Waddle *et al.*, 1996). Several reports have described GFP variants with improved spectral properties (Delagrave *et al.*, 1995; Ehrig *et al.*, 1995; Heim *et al.*, 1994, 1995; Heim and Tsien, 1996). In this work, two GFP alleles, the wild-type coding sequence and the GFPS65T mutant variant, were cloned in the multiple cloning site of pFA6a-HIS3MX6 or pFA6a-kanMX6 plasmids and used as templates for PCR as outlined in Figure 3. For more details see Materials and Methods and Figure 4. Since *GFP-HIS3MX6* and *GFP-kanMX6* are only 2·3–2·4 kb long, SFH-PCR products could be synthesized using standard PCR conditions. All PCR products showed similar efficiencies in transformation experiments.

When we compared the fluorescence intensity of transformants in which wild-type GFP or GFTS65T was expressed under the control of the strong *S. cerevisiae TEF1* promotor, the fluorescence intensity, located in the cytoplasm, was approximately five times higher with the mutant GFP (data not shown). Examples of *in vivo* fluorescence of live *S. cerevisiae* cells in which the mutant GFP reporter sequences had been targeted to the 3′-end of differently expressed genes are shown in Figure 3. In the first example, the non-essential nuclear pore membrane protein Pom152 (Wozniak *et al.*, 1994) was targeted with *GFPS65T-HIS3MX6* (Figure 3C). In His^+ transformants, the nuclear membrane fluorescence could only be observed by accumulation of 100 video frames. In contrast, with cells expressing a histone H4-GFP fusion protein (Figure 3D) uniformly green fluorescent nuclei could be detected without video frame accumulation. In these transformations different stages of the cell cycle can easily be distinguished. In tubulin-GFP (*TUB1*) fusions, nuclear as well as cytoplasmic microtubules became visible when 25 video frames were accumulated (Figure 3E). Depending on the cell cycle stage a short interphase spindle or mitotic spindles of different length were observed. This demonstrates the ease of using GFPS65T reporter modules to create, by PCR-targeting, cells expressing self-fluorescent fusion proteins which can then be studied in live cells.

The presently available GFP modules (Figure 4) have been targeted to the 3′-end of ten novel yeast genes. Localized green fluorescence could be observed with half of the tested carboxy-terminal fusions. Modified modules allowing PCR-targeting to 5′-ends of novel genes are under construction. Recently, *CEN*/*ARS*-based *S. cerevisiae* plasmids were described which allow, after cloning of ORFs, the expression of N-terminal or C-terminal GFP fusion proteins (Niedenthal *et al.*, 1996). Because of the inherent genetic instability of plasmids, targeted integration of the reporter to a genomic locus may be favourable if, in the absence of selection pressure, a genetically stable GFP labelling, *e.g.* of organelles, is desired.

PCR-introduced reporter-inactivating mutations

We tried to determine the fraction of transformants carrying inactivating mutations in the GFP reporter introduced by PCR. Ten SFH-PCR with the same primer pair and with *GFPS65T-kanMX6* as template were performed in parallel. Primers were designed for targeted in-frame integration of the PCR products at the last codon of the histone H4 gene YNL030w. The 2·4 kb PCR products of the ten reactions were pooled and aliquots were used for 40 independent transformations of CEN.PK2. Two geneticin-resistant colonies from each transformation were restreaked on selective medium and grown for 48 h at 23°C to allow isolation of one single colony each. Cells of these colonies were inspected by fluorescence microscopy. Seventy-two of the 80 independent clones (90%) showed a strong homogeneous fluorescence of the nucleus. The remaining clones were either non-fluorescent (4) or only weakly fluorescent (4). This low percentage of inactivated GFP reporters is probably due to a reduced PCR-error rate because we used a *Taq/Vent* DNA polymerase mixture. With such a small number of clones carrying inactivating GFP mutations, characterization of GFP fusion proteins of unknown localization can be done with confidence by inspection of only a small number of independent clones.

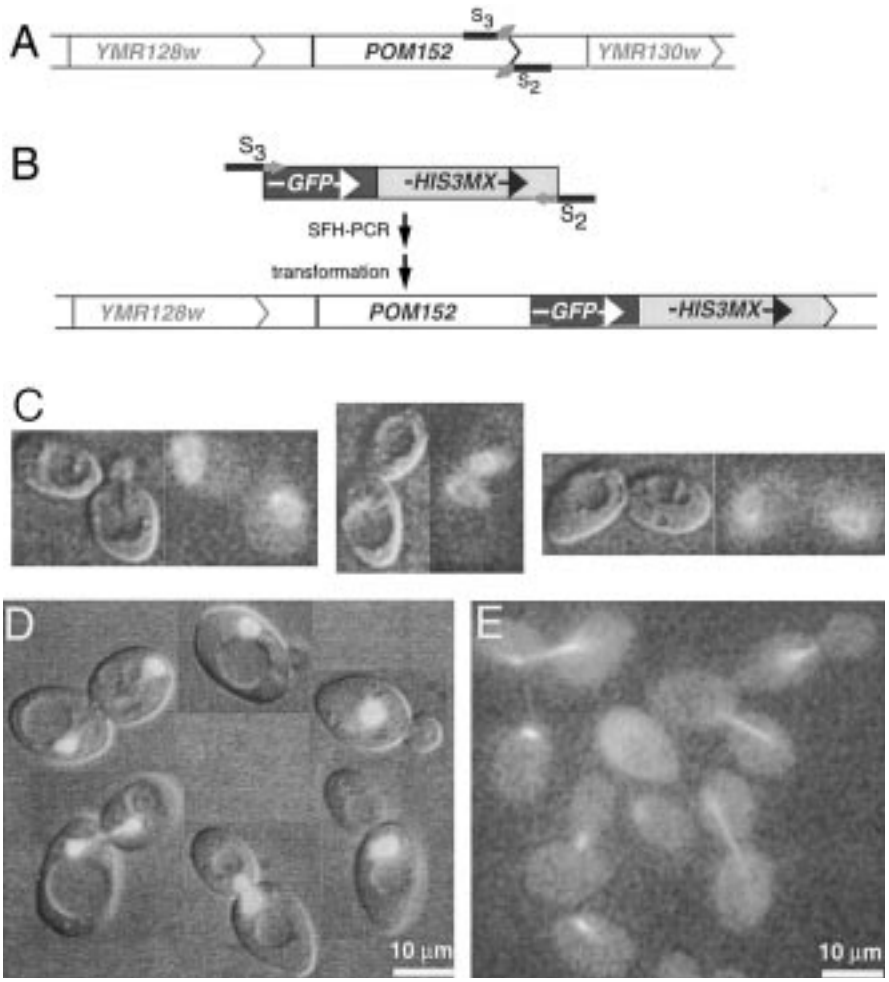

Figure 3. Examples of PCR-targeting of GFP reporter cassettes. (A) Origin of chimeric primer sequences used for targeted in-frame carboxy-terminal fusions to *POM152*. Target homoloy sequences are shown as black bars and marker homology sequences as grey arrows. The sequence of S_3 is: 5′-GAT GCT TAT TGT TTT GCC AAA AAT GAT CTT TTT TTC AAT AAC G GTCGACGGATCCCCGGG-3′ (14 codons of the 3′ end of *POM152*, plus G, plus 17 underlined nucleotides of pFA6a carrying *Sal*I, *Bam*HI, *Sma*I). The sequence of S_2 is: 5′-CT GAT GTA CAG AGA TAT ATT ATA CAT TAC AAT TGT ACA AAC ATCGAT GAATTC GAGCTCG-3′ (41 nucleotides complementary to nucleotides 45 to 85 downstream of the stop codon plus 19 nucleotides of pFA6a-MCS carrying *Sac*I, *Eco*RI, *Cla*I sites, underlined sequence). (B) Synthesis by PCR of *POM152*-specific GFP reporter cassette and genomic map of correctly targeted *GFPS65T-HIS3MX6*. Transformants were verified as described in Figure 2. (C) Heterozygous CEN.PK2 His^+ transformants expressing a Pom152-GFP fusion protein observed with differential interference contrast (DIC) and with accumulated fluorescence images of 100 frames. (D) CEN.PK2 cells containing one copy of *GFPS65T-kanMX6* targeted to the 3′ end of the histone H4 ORF (YNL030w). The sequences of S_3 was in this case: 5′-GCT TTG AAG AGA CAA GGT AGA ACC TTA TAT GGT TTC GGT GGT G GTCGACGGATCCCCGGG-3′ (14 codons of the 3′-end of YNL030w excluding the stop codon, plus G, plus 17 underlined nucleotides of pFA6a carrying *Sal*I, *Bam*HI, *Sma*I). Six cells at different stages of the cell cycle are shown. The fluorescence is very intense allowing simultaneous recording of DIC and fluorescence (life image). (E) Accumulated fluorescence image of 25 frames of FY1679 cells with one copy of *GFPS65T-kanMX6* targeted to the 3′-end of the *TUB1* ORF (YML085c). The sequence of S_3 was in this case: 5′-C GAA GTG GGT GCC GAC TCA TAC GCT GAG GAA GAG GAA TTT CGTACGCTGCAGGTCGAC-3′ (13 codons of the 3′ end of YML085c excluding the stop codon plus 18 underlined nucleotides of pFA6b *Sun*I, *Pst*I, *Sal*I). In this case the double module was cloned in pFA6b (additional G in front of *Pac*I site) and not in pFA6a in order to allow in-frame fusion of the GFP coding sequence to the *TUB1* ORF. In the fusion protein, the tubulin sequence is separated by 13 amino acids from the GFP sequence and not by 9 amino acids as in the other two cases.

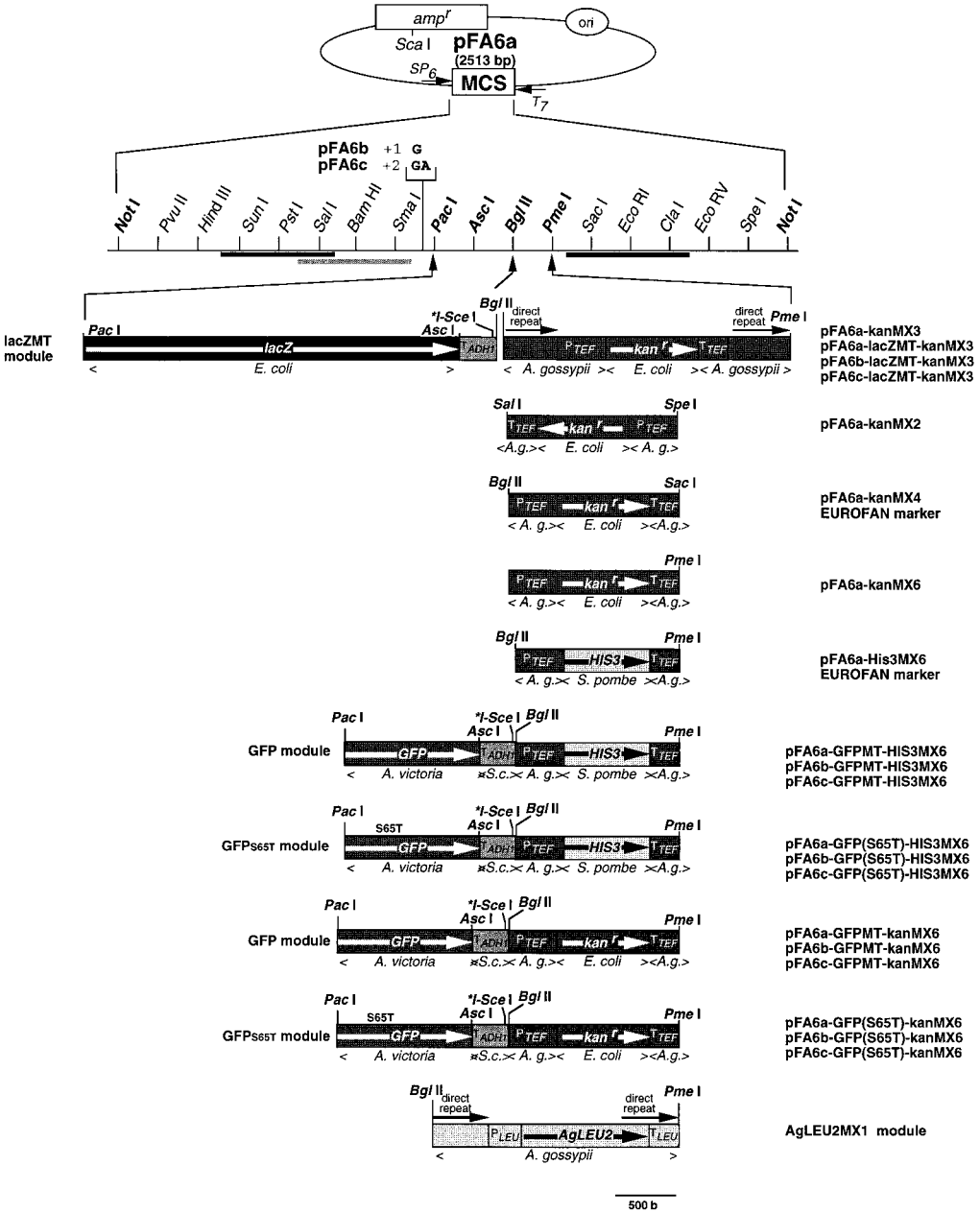

Figure 4. Map of heterologous markers and reporters cloned in pFA vectors. For further details of the pFA plasmid backbone and *kanMX* constructs see Wach *et al.* (1994) and Wach (1996).

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