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 promoter 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 geneticin 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; Guldener et al., 1996; Horton, 1995; Laengle-Ronault and Jacobs, 1995; Lorenz et al., 1995; Maitah 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
expression of geneticin-resistance driven by a fungal promotor (Steiner and Philippens, 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 Klyueromyces 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. kluveri HIS3 gene (W einstock 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 lacZM T-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 HIS3M X as well as GFP reporter modules with HIS3M X or with kanMX as the selection marker. We provide evidence that HIS3M X 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 promoter 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 (α/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 (α/α ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-289/trp1-289 his3Δ1/his3Δ1; K.-D. Entian and P. Kött er, personal communication), and W303 (α/α ura3-1/ura3-1 leu2-3,112/eu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100; Thomas and Rothstein, 1989). The S. kluveri strain CBS3082 was used to isolate genomic DNA of this yeast species. The plasmid Ep478 (Erickson and Hannig, 1995) was used to extract the S. 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 g of dropout mix, supplemented with all amino acids (Trecro, 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, Gaithersburg, M. D.)
Thermostable DNA polymerases

Thermostable DNA polymerase from Thermus aquaticus (Taq DNA polymerase) was purchased from Boehringer, Mannheim, Germany. Thermostable DNA polymerase from Thermus aquaticus (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 polymerases 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. kluveri HIS3 into pFA6a

The S. kluveri 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′-AGAAAGATCTGCTTGC TTTTTTCTTTTTTCTT-3′), SkH2cU (5′-AGAAAGATCTGCTTGC TTTTTTCTTTTTTCTT-3′), SkH2cU (5′-TAGCCATGGCAGAACCCGCC AAAAAAAGC-3′) and SkH3cR (5′-ACTTCCAC ATCAAAAAACACCTTTGGTGG-3′). The following primer combinations were used to amplify DNA fragments of the S. kluveri HIS3 gene: (a) SkH3U and SkH3R (0-94 kb, promoter, coding sequence, and terminator); (b) SkH3cU and SkH3cR (0-70 kb, coding sequence only). The resulting PCR products were cleaved by either BglII (a) or NcoI (b) and ligated into pFA6a-kanMX6 digested with BglII, Pmel (a) nor NcoI, partial (b). The resulting plasmids were named pFA6a-HIS3PT6 (a) and pFA6a-HIS3MX6 (b).

Cloning of Sz. pombe his5 coding sequence into pFA6a

The plasmid Ep478, containing the Sz. pombe his5 DNA, was used as template in a PCR with the primers Sph5U (5′-GACCATGGGTAG GAGGCTTTTGTAGAAAG-3′) and Sph5R (5′-ATCCTTTACACACCTCCTTCTGGC-3′) to amplify the Sz. pombe his5 coding sequences (ACU07831; Erickson and Hannig, 1995). The resulting 0-65 kb PCR product was cut with NcoI and ligated with the NcoI, partial Scal-digested pFA6a-kanMX6 plasmid. The resulting plasmid was called pFA6a-HIS3M X6.

Cloning of the Aequorea victoria GFP coding sequence into pFA6

The plasmids pGFP (Clontech, Palo Alto, CA, USA) and pRSET-56ST containing a mutated GFP allele (Heim et al., 1995) were used as templates. The primers GFP-U (5′-CGCTTAAATT AACA GTAAAAAGA GAA CATTTTAC-3′) and GFP-R (5′-ATAGGGCGCGCC 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 PacI site (plus additional C) upstream of codon 2 and an Ascl site downstream of the stop codon were amplified. The PCR product was digested with PacI and Ascl and ligated with PacI, Ascl-cut pFA6a-lacZM T-kanM X 3 giving pFA6a-GFPMT-kanMX3. Other GFP-containing plasmids were constructed by insertion of the PacI-BglII fragment (GFP plus ADH1 terminator) from pFA6a-GFPMT-kanMX3 into pFA6a-kanMX6, or pFA6a-HIS3M X 6 (Sz. pombe). Corresponding pFA6 plasmids with frame b or c were then constructed by transferring the PacI, Pmel-extracted reporter-marker double modules into PacI, Pmel-cut pFA6b or pFA6c. Correct frames were checked by dideoxy nucleotide sequencing.

Transformation of yeast (Gietz and Woods, 1994) and verification of G418r 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 (MAC 2000, Ludl)
Electronic Products Ltd, Hawthorne, N.Y., 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 X enon 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 PB95-500195G E1). Custom macros were used to control the microscope, the video camera, the MAC 2000, 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 × 10⁷ 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: Sekretariat@UbaClu.UniBas.ch). The following plasmids (see Figure 4 for details) are available for scientific research: pFA6a-HIS3MX6, pFA6a-GFP T-kanMX3, pFA6a-GFP P58T-kanMX3, pFA6a-GFP P58T-kanMX6, pFA6a-GFP P58T-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 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. kluuyveri (Weinstock and Strathern, 1993) and his5⁰ in Sz. pombe (Erickson and Hannig, 1995). On the DNA level, the heterologous IGP-dehydratase ORFs have an overall identity of 70-4% (S. kluuyveri) and 58-6% (Sz. pombe) to the S. cerevisiae HİS3 ORF. An alignment of the HIS3 ORFs of S. kluuyveri 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 his5⁰ 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. kluuyveri HIS3 gene carrying 110 bp upstream of the ATG, k-HIS3M X6 consisting of A. gossypii TEF-promotor/terminator sequences flanking the S. kluuyveri HIS3 ORF, and p-HIS3M X6 consisting of A. gossypii TEF-promotor/terminator sequences flanking the Sz. pombe his5⁰ 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-HIS3M X6, p-HIS3M X6) complemented the his3 mutation in CEN.PK2 very well. After 2 days, 50-100 fast-growing His⁰ transformants were obtained with 1 µg PCR product. In contrast, the construct with the cognate S. kluuyveri HIS3 gene (k-HIS3) only yielded one to two His⁰ transformants with 3 µg PCR product. Weinstock and Strathern (1993)
reported that a S. kluveri HIS3 fragment with 110bp upstream of the start codon and cloned in a CEN/ARS, URA3 plasmid could complement a his3 mutation in 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. kluveri 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-HIS3M X6 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-HIS3M X6 transformants and all 36 p-HIS3M X6 transformants carried correctly targeted marker modules. Since the hybrid module with the Sz. pombe HIS5 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 HIS3M X6 and the genomic his3Δ1 allele, CEN.PK2 cells were transformed with HIS3M X6 PCR fragments carrying or lacking
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-HIS3MX6 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 (Degaarde 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 GFPS65T was expressed under the control of the strong S. cerevisiae TEF1 promoter, 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 homology sequences are shown as black bars and marker homology sequences as grey arrows. The sequence of S3 is: 5\'-GAT GCT TAT TGT TTT GCC AAA AAT GAT CTT TTT TTC AAT AAC G GTCG ACC GAT CCC CCGG-3\' (14 codons of the 3\' end of POM152, plus G, plus 17 underlined nucleotides of pFA6a carrying Sall, BamHI, SmaI). The sequence of S2 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 Sacl, EcoRI, Clac 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.PK 2 His+ transformants expressing a Pom152-GFP fusion protein observed with differential interference contrast (DIC) and with accumulated fluorescence images of 100 frames. (D) CEN.PK 2 cells containing one copy of GFPS65T-kanMX6 targeted to the 3\' end of the histone H4 ORF (YNL030w). The sequences of S2 was in this case: 5\'-GCT TTT TGT AAG AGA CAA GGT AGA ACC TTA TAT GGT TTC GGT G GTGAC GGG ATCCC CCGG-3\' (14 codons of the 3\' end of YNL030w excluding the stop codon, plus G, plus 17 underlined nucleotides of pFA6a carrying Sall, BamHI, SmaI). 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 (YM L085c). The sequence of S2 was in this case: 5\'-C GAA GTG GGT GCC GAC TCA TAC GCT GAG GAA GAG AAT TTT GGT CAC GGT GAG GTCGC-3\' (13 codons of the 3\' end of YML085c excluding the stop codon plus 18 underlined nucleotides of pFA6b SunI, PstI, Sall). In this case the double module was cloned in pFA6b (additional G in front of PacI 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.
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