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One-step PCR mediated strategy for the construction of conditionally expressed and epitope tagged yeast proteins

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ABSTRACT

With the availability of the complete yeast genomic sequence, techniques which allow the rapid functional analysis of genes of interest are of increasing importance. Here we report a technique which allows the initial characterisation of genes of interest, through the construction of conditionally expressed mutations for functional analyses and the generation of epitope-tagged fusion proteins for immuno-localisation and immuno-purification, entirely by PCR.

A PCR-based technique for the creation of chromosomal gene disruptions has been described (1). We extend this technique to allow the rapid creation of conditionally expressed alleles (*GAL* mutants) and the synthesis of proteins fused to epitope tags. The technique relies on the PCR amplification of HIS3-p*GAL* or *HIS3*-p*GAL*-TAG cassettes using two primers containing flanking sequences specific to the target gene followed by the transformation of the PCR product into a *his3*$^-$ strain.

Four vectors have been designed and tested (Fig. 1). In these the *HIS3* marker is flanked either only by the *GAL10* promoter (vector pTL26) or by the *GAL10* promoter fused to different epitope tags. The epitope tag sequences are 2× Protein A, 3× c-myc and His_8 (vectors pTL27, pTL28 and pTL32 respectively; see legend of Figure 1 for full description). As an example of the use of these vectors, the construction of a *GAL*-regulated, ProtA::Ssb1p fusion is outlined in Figure 2. The *SSB1* flanking sequences present on the 5′ and 3′ primers target the chromosomal integration of the PCR construct upstream of, and in frame with, the initiator AUG of *SSB1* (Fig. 2).

In order to test the strategy, fusions constructs were made for the genes *SSB1*, *RRP3* (C. L. O'Day, F. Chavanikamannil and J. Ableson, submitted for publication) and *RRP41* (Tables 1 and 2). To avoid ectopic integration at the *HIS3* and/or *GAL1-10* locus, recipient strains carrying both the *his3-Δ200* and a *GAL1-10* deletion were used (strains YDL401 and YDL402; Table 3). The purified PCR fragment (250–500 ng) was used for transformation with the LiAc technique (2). Five to ten transformants were typically obtained per transformation (Table 2). Integration at the correct chromosomal locus was verified by PCR amplification on DNA from yeast colonies, using primers flanking the sites of integration (data not shown). Different constructs gave frequencies of correct integration ranging from 40 to 100% (Table 2). Expression of the tagged alleles was checked by Western blotting (shown for the strain expressing the ProtA::Ssb1p fusion in Fig. 3).

SSB1 is a non-essential gene and transformants were directly plated on 2% glucose minimal medium lacking histidine (SD–his). For the essential genes *RRP3* (C. L. O'Day, F. Chavanikamannil and J. Ableson, submitted for publication) and *RRP41* (P. Mitchell, D. Lafontaine and D. Tollervey, unpublished), transformants were plated under permissive conditions

Table 1. Sequences of the PCR primers

5′ primers:	
SSB1-HIS3	5′-TAATCATTCTGCAAATTTAAAGGAAGTTACTGAGTTATCACTACACTCTTGGCCTCCTCTAGT-3′
RRP3-HIS3	5′-TTATTATTAAACATACTACTCACTCAAACCCAGATATCGAACTAACTCTTGGCCTCCTCTAG-3′
RRP41-HIS3	5′-TGAATGAATGACATTGCGAACCATTGTGCAATGAACATCATCCAACTCTTGGCCTCCTCTAG-3′
3′ primers:	
SSB1-ProtA	5′-CAAGTTGTTTACGGCATTAGTAGCTTCTTCAATTTCAGCAGACATATTCGCGTCTACTTTCGG-3′
SSB1-His_8	5′-CAAGTTGTTTACGGCATTAGTAGCTTCTTCAATTTCAGCAGACATATGGTGATGGTGATGGTGATGGTGAGA-3′
RRP3-GAL	5′-CTCATTGATTAATTTTAAATTTTTCATTTTTATCGCCTTAGACGACGAATTCCTTGAATTTTCAAA-3′
RRP3-ProtA	5′-ATGTACAATTGCTAAATGCTCATTGATTAATTTTAAATTTTTCATATTCGCGTCTACTTTCGG-3′
RRP41-GAL	5′-GTATATTTCTAGTCTTGACATATGATGATCCTGCTGTAGTTATGGCGAATTCCTTGAATTTTCAAA-3′
RRP41-ProtA	5′-ATCGAGACGTAGCCCTTCTGGCGAGTATATTTCTAGTCTTGACATATTCGCGTCTACTTTCGG-3′
RRP41-c-myc	5′-ATCGAGACGTAGCCCTTCTGGCGAGTATATTTCTAGTCTTGACATGGATCCGTTCAAGTCTTC-3′

The regions which are complementary to the pTL vectors are underlined. The EMBL accession numbers of genes *SSB1*, *RRP3* and *RRP41* are M17244, YHRO65c and X82775 respectively.

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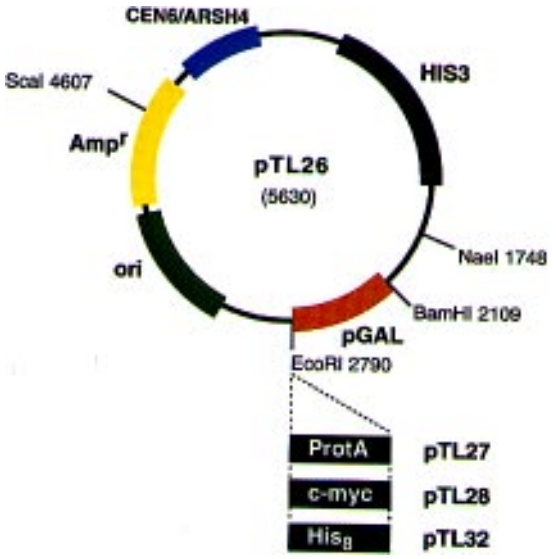

Figure 1. Structure of the pTL vectors used as PCR templates. The pTL vectors were constructed as follows. In plasmid pTL26, the *GAL1–10* promoter region of plasmid pDL503 (4), isolated by *Eco*RI–*Bam*HI digestion, was subcloned into plasmid pRS313 (6) in order to be fused to a HIS3 marker. The 2× ProtA cassette, which contains the two IgG binding domains of the *S.aureus* Protein A, was amplified by PCR from plasmid p28NZZtrc (7) using two primers which create flanking *Nco*I sites (primer 1: 5′-CCCATGGCAGGCCTTGCGCAACAC-3′ and primer 2: 5′-CTTTCCCATGGCATTCGCGTCTACTTTCGGCGC-3′). The PCR product was digested by *Nco*I and subcloned into a *Nco*I containing vector. The 2× ProtA cassette was isolated from this plasmid by *Nco*I digestion, filled with Klenow DNA polymerase and inserted at the filled *Eco*RI site of plasmid pTL26 to yield vector pTL27. The 3× c-myc cassette which contains three human c-myc epitopes bridged by glycine residues was amplified by PCR from plasmid pUC119-myc-tag3 using two primers which create flanking *Nco*I sites (primer 1: 5′-AATTATACCATGGGTACCCGGGGATCCTCTAGA-3′ and primer 2: 5′-CTTTCCCATGGCTCTAGAGGATCCGTTCAAGTC-3′). The PCR product was digested by *Nco*I and subcloned into a *Nco*I containing vector. The 3× c-myc cassette was isolated from this plasmid by *Nco*I digestion, filled with the Klenow enzyme and inserted at the filled *Eco*RI site of plasmid pTL26 to yield vector pTL28. The His_8 tag was created by annealing the following oligonucleotides: (i) EcoRI-8His-F 5′-AATTCATGAGAGGTTCT-CACCATCACCATCACCATCACCATC-3′; (ii) XhoI-8His-R 5′-TCGAGAT-GGTGATGGTGATGGTGATGGTGAGAACCTCTCATG-3′. The linker was digested by *Eco*RI–*Xho*I and subcloned in pTL26. The resulting plasmid was called pTL32. pTL32 can be used for the in-frame fusion of 6 or 8 histidines residues. All constructions were checked by sequencing. PCR reactions were performed using 30 ng of the appropriate pTL vector and 100 pmol of each primer in buffer containing 10 mM Tris–HCl (pH 8.3 at 20°C), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 U *Taq* DNA polymerase (Boehringer) in a final volume of 100 µl. Following an initial denaturation step, 5 min, 94°C, *Taq* DNA polymerase was added and amplification was performed 30 times (1 min, 45°C; 4 min, 72°C; 30 s, 94°C), followed by incubation for 10 min, 72°C. The sequences of PCR primers used for these amplifications are given in Table 1. For each target gene the 5′ primer is common for all templates. The PCR products were digested with *Xmn*I and gel purified using a QIA quick kit (QIAGEN). The optional *Xmn*I digestion step cuts the pTL vector template and avoids problems with contamination of the PCR product by intact vector. The lengths of the PCR products are 2584 bp (HIS3-GAL), 2973 bp (HIS3-GAL-2× ProtA), 2721 bp (HIS3-GAL-3× c-myc), 2613 bp (HIS3-GAL-His_6) and 2619 bp (HIS3-GAL-HiS_8)

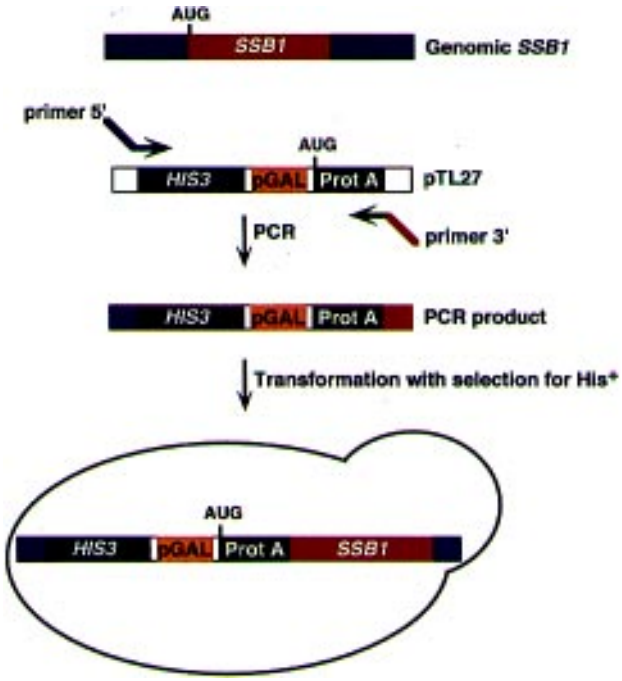

Figure 2. Construction and integration of fusions. In the example illustrated, PCR amplifications were performed on the pTL27 vector (pGAL-ProtA) using 5′ and 3′ primers. Each primer contains a sequence required for amplification on the template DNA and a sequence from *SSB1* required to specifically target the integration. The 5′ primer includes 45 nt of the *SSB1* promoter region. The 3′ primer includes the sequence complementary to the first 45 nt of the *SSB1* ORF. PCR products were transformed into strain YDL401 and transformants selected for histidine prototrophy. Homologous recombination leads to the integration of the PCR cassette generating a chromosomal *GAL-ProtA::SSB1* allele.

Table 2. Frequencies of correct integration and expression

Target gene	Type of fusion	His$^+$ transformants	Correct integrants as tested by:	
			PCR	Western
SSB1	GAL-ProtA	3	3/3	1/2
SSB1	GAL-His_8	7	5/5	2/2
RRP3	GAL	8	5/5	NT
RRP3	GAL-ProtA	10	5/5	2/2
RRP41	GAL	10	3/5	NT
RRP41	GAL-ProtA	8	2/5	2/2
RRP41	GAL-c-myc	5	2/5	2/2

The table shows the number of His$^+$ transformants which were recovered following transformation of the PCR constructs into strain YDL401. In each case five transformants were analysed by PCR to determine whether the construct was integrated at the correct genomic locus, except for *GAL-ProtA::SSB1*, for which only three transformants were recovered. Correct integration frequencies ranged from 40 to 100%. For several constructs, two strains in which integration was at the correct locus were further analysed by Western blotting to determine whether the expected fusion protein was being synthesised. In all strains this was found to be the case with the exception of one of the *GAL-ProtA::SSB1* strains. This may arise if a mutation is introduced in the epitope tag sequence during PCR amplification. NT = not tested.

for *GAL* transcription; minimal medium lacking histidine and containing 2% sucrose, 2% raffinose and 2% galactose. Strains were then streaked on SD–his to check for the effects of depletion.

The expression level of the ProtA::Ssb1p fusion was tested on medium lacking histidine and containing 4% sucrose + 2% galactose (Fig. 3, lane 4), 2% sucrose + 2% raffinose + 2% galactose (Fig. 3, lane 5) and 4% raffinose + 2% galactose (Fig. 3, lane 6). Galactose functions as a non metabolizable inducer in strains YDL401 and YDL402 since they carry the *gal*Δ*108* mutation (3) (Table 3). These strains also carry a mutation in the galactose permease gene (*gal2*). The effects of galactose addition to medium containing 2% sucrose

Table 3. Yeast strains used in this study

Name	Genotype	reference
FY1679-28C	*a; ura3-52; trp1Δ63; leu2-Δ1; his3-Δ200; GAL2⁺*	(1)
YNN72	*α; ura3-52; trp1-289; gal2; galΔ108*	(3)
YDL400	*a/α; ura3-52/ura3-52; trp1-Δ63/trp1-289; leu2-Δ1/LEU2; his3-Δ200/HIS3; GAL2⁺/gal2; GAL1,7,10⁺/galΔ108*	FY1679-28C x YNN72
YDL401	*a; ura3-52; trp⁻ (trp1-Δ63 or trp1-289); leu2-Δ1; his3-Δ200; gal2; galΔ108*	segregant from YDL400
YDL402	*α; ura3-52; trp⁻ (trp1Δ63 or trp1-289); leu2-Δ1; his3-Δ200; gal2; galΔ108*	segregant from YDL400
ProtA::nop1	*α; nop1::ura3* (pUN100-ProtA::NOP1)	R. Jansen and E. Hurt

Strains construction: strains FY1679-28C and YNN72 were mated and the resulting diploid strain (YDL400) was isolated on galactose minimal medium minus leucine. Strain YDL400 was sporulated and dissected; strains YDL401 and YDL402 were isolated from a complete tetrad showing a 2:2 segregation pattern for Gal.

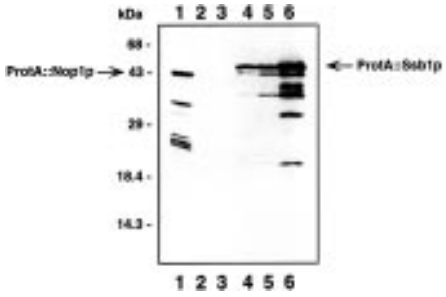

Figure 3. Western blot analysis of the *GAL-ProtA::SSB1* strain. Lane 1: strain expressing ProtA::Nop1p, Lane 2: negative control, strain YDL401. Both control strains were grown in YPD. Lanes 3–6: *GAL-ProtA::SSB1* strain (YDL500). Strain YDL500 was grown in minimal medium lacking histidine supplemented with 2% glucose (lane 3), 4% sucrose + 2% galactose (lane 4), 2% sucrose + 2% raffinose + 2% galactose (lane 5) or 4% raffinose + 2% galactose (lane 6). Expected sizes of ProtA::Nop1p and ProtA::Ssb1p are 49.177 and 47.530 kDa respectively. Both wild-type proteins migrate abnormally slowly, probably due to the presence of glycine- and arginine-rich domains (GAR domains); Nop1p (M_r 34.5 kDa) and Ssb1p (M_r 32.853 kDa) migrate with apparent sizes of 38 (8) and 43 kDa (9), respectively. Degradation products were detected for both ProtA::Nop1p and ProtA::Ssb1p. For protein extraction, cells equivalent to 5 OD_{600} units were harvested and resuspended in 100 µl of SDS loading buffer with 25 µl glass beads. Cells were vortexed for 1 min and incubated for 1 min at 95°C three times successively. Lysates were cleared by centrifugation for 10 min at 14 000 r.p.m. and supernatant equivalent to 0.375 OD_{600} units of cells was loaded per lane. Samples were run on 15% SDS–PAGE gels and blotted according to standard procedures. Western blots were decorated using appropriate antibodies and developed using the ECL detection kit (Amersham). Antibodies used to detect the tagged-proteins are: rabbit peroxidase-anti-peroxidase (PAP; Sigma, Cat. No. P2026) for ProtA fusions, Mouse Mab clone 9E10 (Cambridge Research Biochemicals, Cat. No. OM-11-908) for c-myc fusions and the ^{MRGS}·His antibody (QIAGEN, Cat. No. 34610) for poly-His fusions.

and 2% raffinose was tested for the GALHis$_8$::SSB1 strain. As expected, the presence of galactose in the medium was found to have little effect on the level of expression of the fusion protein (data not shown). A potential problem with the use of *GAL*-regulated constructs is that many proteins are heavily over-expressed when their genes are transcribed from induced GAL promoters. This, for example, can make the analysis of the sub-cellular localisation of the fusion protein unreliable. Ssb1p is an snoRNP protein and the level of ProtA::Ssb1p was compared with the level of expression of another snoRNP protein, ProtA::Nop1p, expressed under the control of its own promoter (Fig. 3, lane 1). In these strains the level of ProtA::Ssb1p expressed in medium containing 4% sucrose + 2% galactose is similar to that of ProtA::Nop1p, suggesting that its expression is in the same range as expression of endogenous Ssb1p. During growth on medium containing 2% glucose (Fig. 3, lane 2) the level of ProtA::Ssb1p was undetectable.

Many *GAL*-regulated mutants show incomplete growth inhibition on glucose medium due to residual transcription (4). The effects of the transcriptional repression can be enhanced at the translational level through modification of the context of the initiator AUG or by the introduction of an additional, out of frame upstream AUG sequence. In the system reported here, such mutants can simply be made by altering the sequence of the 3′ primer (primer 3′ in Fig. 2). The URA3 gene of *Kluyveromyces lactis* is functionally homologous to the *S.cerevisiae URA3* gene and fully complements *ura3*$^-$ strains, but has sufficient sequence divergence to prevent genetic recombination (5). To allow epitope-tagging of more than one protein in the same strain, we are currently constructing vectors based on the *K.lactis URA3* gene. Templates for the construction of C-terminal fusions are also in preparation.

The ease with which *GAL*-regulated and epitope-tagged alleles of genes of interest can be constructed using this strategy allows initial functional analyses of the effects of genetic depletion to be carried out using tagged alleles. This allows the degree of genetic depletion to be followed at the protein level in the absence of specific antibodies (Fig. 3). The construction of such alleles by conventional techniques typically involves several cloning steps and generally generates only plasmid-borne alleles. In the case of essential genes, these must be transformed into heterozygous diploid strains and suitable haploid progeny recovered after sporulation. In contrast, the technique reported here allows mutant alleles of essential genes such as *RRP3* and *RRP41*, to be simply constructed in haploid strains.

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