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SHORT COMMUNICATION

Chromosomal promoter replacement in *Saccharomyces cerevisiae*: Construction of conditional lethal strains for the cloning of glycosyltransferases from various organisms

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Heterologous complementation in yeast has been a successful tool for cloning and characterisation of genes from various organisms. Therefore we constructed conditionally lethal *Saccharomyces cerevisiae* strains by replacing the endogenous promoter from the genes of interest (glycosyltransferases) by the stringently regulated GAL1-promoter, by a technique called chromosomal promoter replacement. Such yeast strains were constructed for the genes Alg 1, Alg7, Sec59, Wbp1 involved in N-Glycosylation, the genes Gpi2, Gpi3/Spt14, Gaal, Pis1, involved in GPI-anchor biosynthesis and Dpm involved in both pathways. All strains show the expected conditionally lethal phenotype on glucose-containing medium when expression of the respective gene is turned off.

Keywords: **heterologous complementation cloning, yeast promoter replacement, homologous recombination, glycosylation, GPI-anchors**

Introduction

The yeast *Saccharomyces cerevisiae* provides a powerful model system for studying molecular biology of the eucaryotic cell. Heterologous complementation of conditional lethal mutants of yeast has been successfully used to isolate functional homologues from various species. Based on the high conservation of gene function in cells from different eucaryotic species, many of the known or unknown essential genes from yeast are potential targets for heterologous complementation screens, which depend on the availability of conditional lethal mutants of the gene of interest. So far these mutants were mainly temperature sensitive mutants, which are often difficult to handle in complementation screens and tend to revert with a certain, sometimes high frequency.

We decided to generate conditional lethal mutants of essential genes by the well-known method of replacing the natural promoter with the stringently regulated, glucose-repressed GAL-1 promoter [1]. Such strains are viable on galactose medium and stop growing on medium containing glucose as only carbohydrate source when the GAL1-promoter is turned off. As an example for this approach we wanted to generate several conditional lethal mutants of genes involved in protein glycosylation (N- and O-glycosylation and GPI-anchor biosynthesis) by Chromosomal Promoter Replacement (CPR).

N-glycosylation plays an important role in protein folding and quality control in the ER and is crucial for biosynthetic trafficking of proteins beyond the ER and throughout the secretory pathway as reviewed in [2,3]. Glycosyl-Phosphati-

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dylinositol membrane anchors, a principle of anchoring protein in plasma membranes which first has been described in *Trypanosoma brucei* has meanwhile been shown to be, like N-glycosylation, ubiquitous among eucaryotes. There is a growing body of evidence that these GPI-anchors exhibit a variety of functions other than the mere anchoring of membrane proteins [for review see 4,5,6]. Since glycosylation is a hallmark of most surface proteins these biosynthetic pathways play a particularly interesting role in the biology of parasites since surface antigens and their carbohydrate structures have to interact with the host's immune system. Through recombinant DNA technology it should be possible to clone and express these enzymes which are not readily accessible due to the often limited availability of parasite material. Determination of key biochemical parameters for these enzymes might have a profound impact on the development of vaccines and new antiparasitic drugs based on specific inhibitors for the parasite enzymes. We therefore decided to clone and characterise enzymes involved in N-glycosylation and GPI-anchor biosynthesis by using the approach of heterologous complementation in yeast. We have previously cloned the gene for the Dolicholphosphat-Mannose Synthase from *Trypanosoma b. brucei* [7], using the temperature-sensitive yeast strain DPM 1–6 [8]. To circumvent the problems of a high number of false positives often encountered with ts-strains we decided to construct synthetically lethal yeast strains by replacing the endogenous promoters of these genes by the stringent regulatable GAL1 promoter via homologous recombination, by transfecting yeast with a His-Gal cassette PCR fragment. The construction of such strains and their phenotypic characterisation will be described and their usefulness for the cloning of glycosyltransferases will be discussed.

Materials and methods

Materials

Zymolyase 20000 was purchased from Seikagaku Corp. Tokyo. Restriction endonucleases and other DNA modifying enzymes used in recombinant DNA experiments were from Boehringer-Mannheim, New England Biolabs or Stratagene and were used in accordance with the manufacturer's instructions.

Strains and Media:

The following *Saccharomyces cerevisiae* and *E.coli* strains were used in this work. YPH 499 [Mat *a*; ura3-52; lys2-801^{amber}; ade2–101^{ochre} trpl-D63; his3-D200; leu2-D1] (Stratagene) was used for the construction of the synthetically lethal strains. *S. cerevisiae* wild type strains were grown in YPAD medium (1% Bacto yeast extract, 2% Bactopeptone, 2% dextrose, 4 mg/l adenine) or SD medium (2% dextrose, 0.17% Bacto yeast nitrogen base) and the nutritional supplements necessary to complement strain auxotrophies. Recombinant strains were plated on SGR-His after transformation (4% galactose, 2% raffinose, 1.5% agar for plates, 0.17% Bacto yeast nitrogen base, 0.5% ammonium sulfate and the nutritional supplements necessary to complement strain auxotrophies.)

Recombinant DNA techniques

Standard recombinant DNA techniques were performed essentially as described by Sambrook et al [9]. Plasmid isolation from yeast followed the protocol of Hoffman and Winston [10]. Transformation of yeast was performed according to [11].

PCR reactions were performed using the Hotwax Optistart Kit for PCR Optimisation from Invitrogen in accordance with the manufacturer's instructions. 10 ng Template (pGAL1/HIS3) and 50 ng of each primer (70 bp) were used for each PCR. Whole cell PCR was performed using YPH499 as wild type control and the corresponding promoter mutants for verification of the correct insertion of the His Gal cassette. The His GAL1 cassette was constructed by cloning of the GAL1-promoter-fragment from p416GAL1 into pRS17 [12,13].

Results and Discussion

Strategy of Chromosomal Promoter Replacement (CPR)

The aim of this work was to use a well-known, quick and reliable procedure to generate conditional lethal yeast mutants involving various genes essential in glycan assembly. Such mutants are suitable for use in heterologous complementation screens. We decided to generate variants of several essential genes, by bringing their expression under the control of the stringently regulated GAL1-promoter. This promoter is induced in the presence of galactose and the absence of glucose but is tightly repressed in the presence of glucose [1]. This should allow to turn off the expression of any open reading frame brought under control of this promoter.

We achieved our aim by using the technique of Chromosomal Promoter Replacement (CPR) in which the wild type promoter of an essential gene of interest is exchanged by the GAL1-promoter (Fig. 1). The promoter replacement is achieved by a classical one step replacement, in which the target promoter is exchanged by a selection marker/promoter HIS3/GAL1-cassette [14,29]. The vector pGAL1/HIS3 serves as a template for the PCR-reaction generating the GAL1-promoter/HIS3-selection marker cassette flanked by short segments for the homologous recombination. Target sequences for the homologous recombination were chosen 200 bp upstream of the corresponding ATG-start codon in the promoter region and at the ATG-start codon of the coding region. Because homologous recombination in yeast is very efficient the flanking regions for recombination can reduced to 50 bp, which allows to generate the HIS3/GAL1-replacement

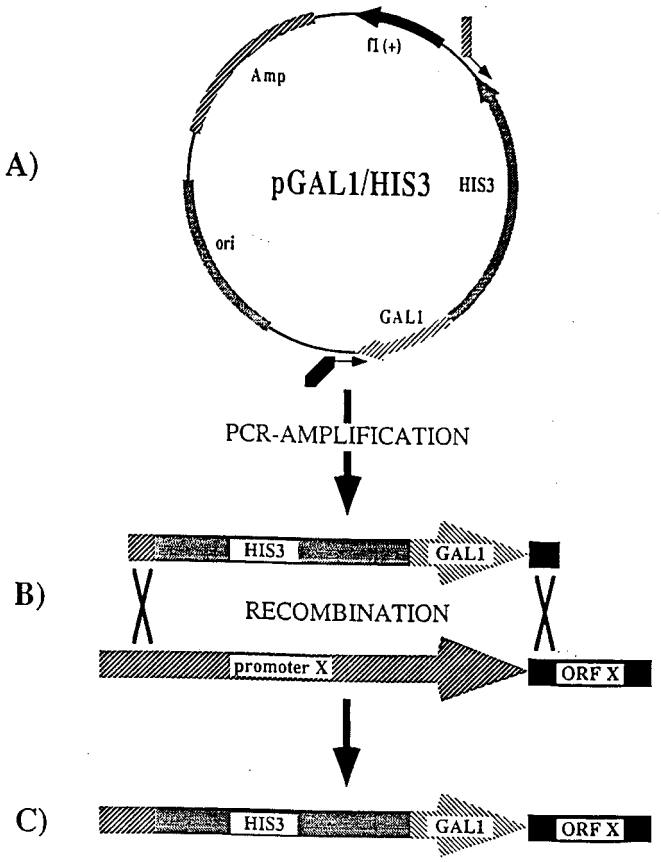

Figure 1. Schematic outline of the chromosomal CPR recombination. A: the vector pGAL1/HIS3 containing the GAL1/HIS3 cassette with the positions of the PCR primers being indicated. B: Postulated recombination event between the amplified GAL1/HIS3 cassette and the promoter region of the gene in question. C: Scheme of the cassette integrated into the yeast chromosomal DNA immediately upstream the targeted open reading frame.

cassette by the polymerase chain reaction [11,15,16,28]. For the amplification of the HIS3/GAL1 cassette and replacement of the endogenous promoter the primers used have to fulfil two criteria: One part of the primer (19-mer) is needed for the amplification of the His3/Gal1 cassette in the pGAL1/HIS3 vector. The second part of the primer (a 50-mer overhang) must be complementary to the putative promoter region, approximately 200 bp upstream the gene of interest or to sequences including the start-codon respectively. Thus the endogenous promoter of the gene of interest should be replaced by the Gal1-promotor via homologous recombination after transformation with the amplified His-Gal fragment as depicted in Figure 1. The PCR-generated CPR-cassette can then be transformed into yeast and transformants can be selected on minimal medium lacking histidine and containing galactose. Strains carrying the correct integration can be verified by southern blot or PCR and should show the expected conditional lethal phenotype when shifted to glucose-containing medium.

Replacement cassettes of this type were generated for promoters of the following genes: Dolichol-phosphate-mannose Synthase (DPM) [8]; Dolichol-cycle N-acetylglucosamine-1-phosphate Transferase (Alg7) [17,18]; Dolichol-cycle Mannosyl-I- transferase (Alg1) [19]; Dolichol-Kinase (sec59) [20]; Phosphatidylinositol-synthase [21]; N-oligosaccharyl-transferase-subunit (Wbp1) [22]; GPI-N-acetylglucosaminyl-transferase 2 (Gpi2) [23]; GPI-N-acetylglucosaminyltransferase 3 (Gpi3/Spt14) [23,24]; GPI-Protein-Transamidase (Gaal) [25]. Sequences of the oligonucleotides used and their locations in the corresponding promoters relative to the ATG-start codon are shown in Table 1.

Transformation of 1 µg of the respective PCR-fragment into the haploid strain YPH499 yielded routinely 5–25 transformants on galactose containing plates lacking histidine. Over 90% of the transformants showed the expected conditional lethal phenotype when plated under non permissive conditions on glucose medium (Fig. 2). Depending on the modified gene some strains showed a residual slow growth for 10–30 generations on glucose plates, probably due to differences in mRNA- and/or protein-stability of the corresponding proteins.

The correct insertion of the His-Gal fragment into genomic DNA was confirmed by whole cell PCR using primers 5′ and 3′ adjacent to the integration sites. The sizes of the PCR fragments were verified by agarose gels with corresponding fragments from YPH 499 as control (Fig. 3). To finally confirm that the lethality was due to repression of the gene in question, the strains were transformed with plasmids harbouring the corresponding wild type yeast genes. For all strains rescue of the conditional lethal phenotype was observed (data not shown).

Heterologous complementation of CPR mutants

To finally confirm the usefulness of these synthetically lethal yeast strains for heterologous complementation screens, the strain YPH-GAL1-DPM was transformed with the previously cloned Dol-P-Man synthase from *T. b. brucei* [7] and was tested for the ability to grow on glucose only. Transformants harbouring the recombinant *T. b. brucei* gene showed the expected phenotypic conversion (Fig. 4) thus confirming that CPR strains can be used for the cloning of genes by heterologous complementation

In addition the yeast strain YPH-GAL1-ALG7 was transformed with a human cDNA library constructed from human lung fibroblasts. 250 000 transformants were plated on SD/His-medium, yielding 18 colonies growing under these conditions. After a second screening step for loss of plasmid by plating on FOA-medium [26], one clone could be identified as the human N-acetylglucosamine-1-phosphate Transferase [27]. These results confirm the main objective of the cloning strategy presented here; cloning of genes by functional complementation in yeast with only a few false positives to be eliminated in a subsequent screening step.

Table 1. Compilation of the oligonucleotides used for the PCR-amplification of the HIS3/GAL1 promoter replacement cassettes. The sequences are written in the conventional 5′-3′ direction. Sequences homologous to the vector are printed in italics. The 5′-primer is complementary to the putative promoter region, approximately 200 bp upstream the gene of interest and the 3′-Primer is complementary to the start of the open reading frame, beginning with the start codon ATG (see complementary TAC in each sequence next to the italicised vector sequence, underlined).

DPM	5′	TGTAATACAATATCATTCTATAGCACTCGCATCGCGTATTTTTTTTTTATTTT *GGGCGAATTGGAGCTCCAC*
	3′	ATGTTCAGCTTTTCATGGTAAGCGGGAACGATAACAGAGTATTCGATGCTCAT *GGGGATCCACTAGTTCTAG*
Sec59	5′	TCTAGATGTATGGGTGTGCGGGAATGCTAAGATACGTTATTGAGGGCTGTCCG *GGGCGAATTGGAGCTCCAC*
	3′	TTCTGGGTTAGTTTAATTGTGGTAAAGGAAGCATGAGGTATTATAGCGACCAT *GGGGATCCACTAGTTCTAG*
Alg7	5′	GTTACGAATACAAACACAGAGGTTGATGCATGAATTTTTTCTAGCTACTACCA *GGGCGAATTGGAGCTCCAC*
	3′	TTGGAATAGTAGATTAAGCATGTGATAAGTGCCAGTGAAAAAAGTCGCAACAT *GGGGATCCACTAGTTCTAG*
Pis1	5′	TGAGATGAGATGAGATGAGCTCTAAATTTCCATAAAATTTTTTCATTTTATAA *GGGCGAATTGGAGCTCCAC*
	3′	TACCACAGAACGTGTTCTGCAGTAACCTTTTCTGGTGTTGAATTCGAACTCAT *GGGGATCCACTAGTTCTAG*
Gpi2	5′	TAAACCAATTTAAAGGTACAATCACACTTTAAGTATTGTTGTTAGACGGT *GGGCGAATTGGAGCTCCAC*
	3′	CTGGGTACTCCTGTTTCAACCATAGTAGGCGCTTCCAGGGAGATCTTGTCAT *GGGGATCCACTAGTTCTAG*
Spt14	5′	CCTTTGTATACTCATATGCGAACTTGTCTAATACAATGTATACCAAAAATAA *GGGCGAATTGGAGCTCCAC*
	3′	ATGTATTTGGAACACATGCTGTGAGCTAGACATACGCTATATTGAAGCCCAT *GGGGATCCACTAGTTCTAG*
Gaa1	5′	CCTAGAAAGGTTGAAGCTTATAAAACCGTGCCAACAGCTTTATAGTGGGG *GGGCGAATTGGAGCTCCAC*
	3′	GGGACAAGCCCCATATCAACAATCCTTCGATGCAACTTCTCCAATAAGGCCAT *GGGGATCCACTAGTTCTAGC*
Alg1	5′	TCAGGTTCTACTTATGTTTGCTTTGTCGATTTTTGCATGCTTTGACTGTGACT *GGGCGAATTGGAGCTCCAC*
	3′	GAAAGGTATAATATTATTAAGGCAAGTAACCACCGAGGAATTTCCAAAAACAT *GGGGATCCACTAGTTCTAG*
Wbp1	5′	GGGGCTCTCATTGTTTTATAGATACATATTAGTATACTACAATTAAAGATATC *GGGCGAATTGGAGCTCCAC*
	3′	ACGAAAATGGCCTGCAGAAGGATACAAAAGAAAAAATTCCAATCGGTCCGCAT *GGGGATCCACTAGTTCTAGC*

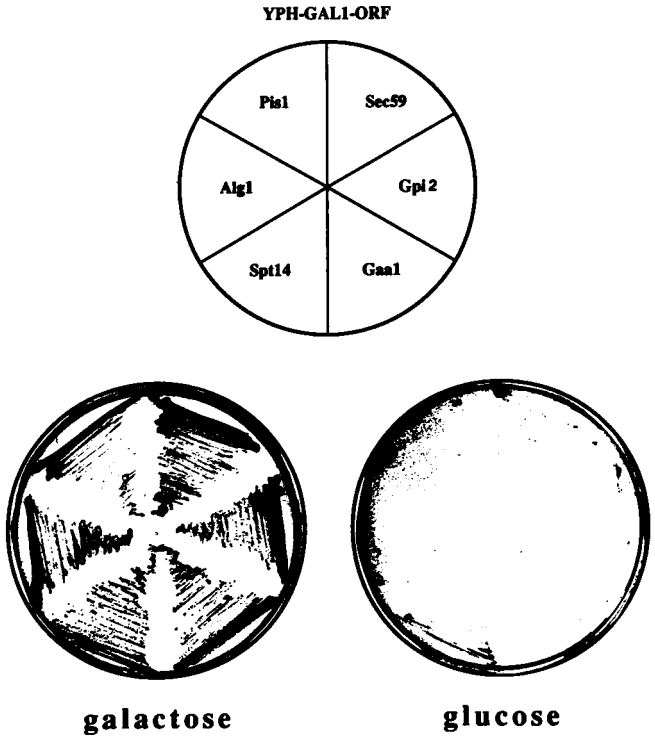

Figure 2. Comparison of growth properties of several CPR-strains on galactose and glucose respectively. The positions of the individual strains are indicated on top of the figure, the enzymes corresponding to the abbreviations are mentioned in the text. All CPR strains show clear growth arrest on medium containing glucose (SD) thus confirming the expected conditional lethal phenotype.

Conclusions

The data presented here confirm that synthetically lethal yeast strains can easily be constructed by promoter replacement via homologous recombination as long as the genes of interest are essential like those for N-glycosylation or GPI-anchor biosynthesis. By using the stringent regulated GAL1 promoter growth of the recombinant yeast strains can be controlled easily by simply plating them on Galactose/Raffinose or Glucose respectively. These strains can thus be used for the functional cloning of genes by complementation by plating them under non permissive conditions (SD) after transformation with expression cDNA libraries as could be demonstrated by the cloning of the human GlcNAc-I-P-transferase.

The CPR-strains described here are now being used to isolate the respective genes from cDNA libraries from humans and parasitic protozoans. CPR-strains dependent on the expression of cDNAs from other species can be applied directly to in vivo tests of already known drugs which inhibit those gene products. Furthermore they can be used to isolate new species specific drugs in high throughput screens. This strategy of using CPR-strains could in principle be adapted to every disease-relevant protein which can complement the function of an essential yeast gene.

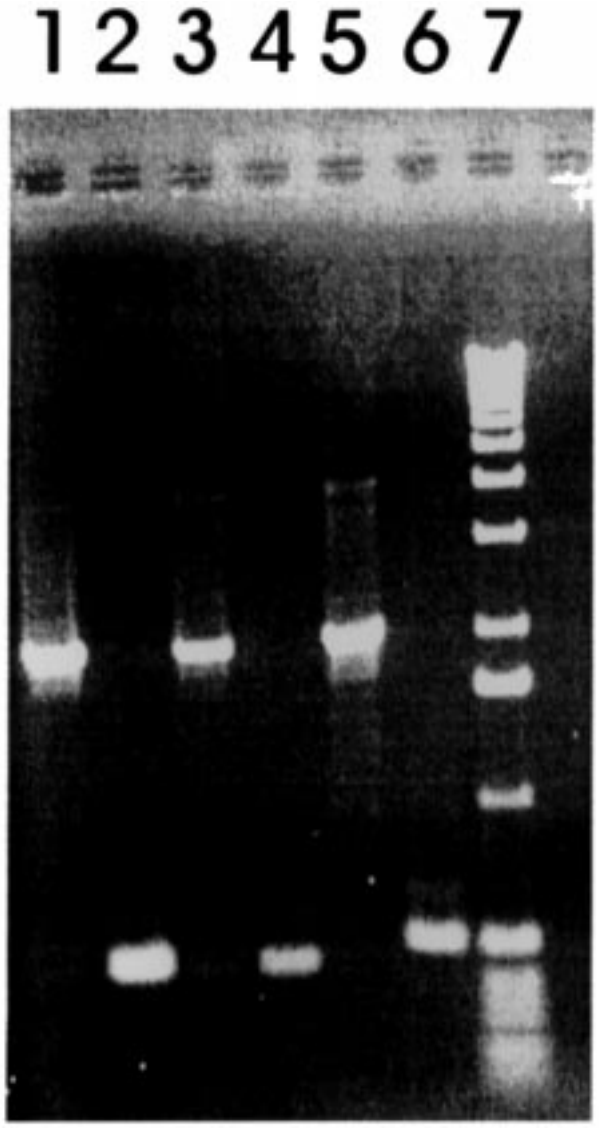

Figure 3. Example for whole cell PCR with three CPR-strains and the corresponding wild type controls. The strains used are: Lane 1: YPH-GAL1-DPM, Lane 3: YPH-GAL1-ALG1, Lane 5: YPH-GAL1-ALG7. Lanes 2, 4, and 6: Yeast strain YPH 499 serving as wild type control. Lane 7: Marker (1 kb marker; GibcoBRL). The PCR-analyses for the other CPR-strains revealed identical results.

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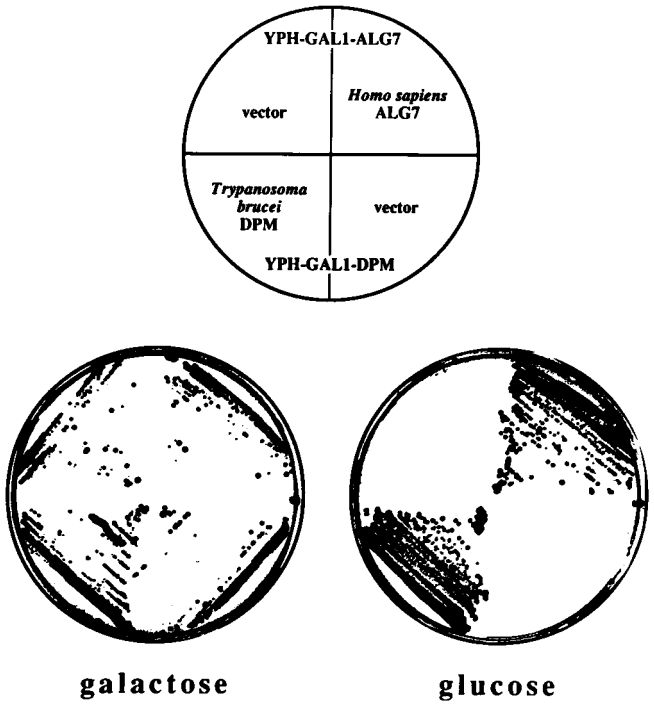

Figure 4. Rescue of the conditional lethal phenolype by heterologous complementation. The two CPR strains YPH-GAL1-ALG7 and YPH-GAL1-DPM were transformed with the human alg7 and the *T. brucei* DPM gene respectively and plated on either galactose or glucose plates. The same strains transformed with the vector pRS 426 Met25 (13) were used as controls.

ing yeast clones which were used as controls in this study and Dr. Louis Schofield for reading the manuscript.

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