BENCHMARKS

Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

Shuttle-Vector System for *Saccharomyces cerevisiae* Designed to Produce C-Terminal-Myc-Tagged Fusion Proteins

BioTechniques 25:936-938 (December 1998)

While the completion of whole genome sequence analysis of *Saccharomyces cerevisiae* has been achieved, the function of its 2300 genes are still unknown, and the genetic and biochemical analysis on these genes are ongoing (5). Determining the subcellular localization, co-immunoprecipitation analysis and the disruption or overexpression of unknown gene products provides us the basic information to understand the function of these genes. The addition of a commonly used epitope such as the decapeptide of the *myc* oncogene product, -EQKLISEEDL (Myc-tag) recognized by mouse monoclonal antibody (MAb) 9E10 (Oncogene Science, Cambridge, MA, USA), facilitates this process (2).

Here, we describe the new shuttle-vector system that produces a C-terminal-Myc-tagged fusion protein from a polymerase chain reaction (PCR)-amplified gene of interest. Figure 1A shows a schematic of vectors pAMT20 and pGMT10. The starting plasmid is pGBT9 (CLONTECH Laboratoies, Palo Alto, CA, USA), a 2-μm ori-type shuttle vector with a *TRP*1 selection marker. After disrupting the *Sph*I site at the hind part of the multiple cloning site (MCS), the *GAL*4 open reading frame (ORF) of pGBT9 was removed, and the alternative MCS and the Myc-tag sequence (Figure 1B) from pPADHmyc-Ch, a derivative of pKT10 (7), were added to generate pAMT20. To generate pAMT10, the *Sca*I-*Sph*I DNA fragment containing the 2-μm ori of pAMT20 was replaced with the *Sca*I-*Sph*I fragment of the *CEN*6/*ARSH*4 cassette from pCEN6-118 containing a *Sca*I-*Alu*I fragment of pRS413 (6). The *Sca*I-*Eco*RI (blunt-end) fragment of the *GAL*1 promoter from pYX223 (R&D Systems Europe, Oxon, England, UK) was cloned into the *Hinc*II site of pUC118 (pGAL1-118). The *Sph*I-*Eco*RI fragment of the *ADH*1 promoter

Table 1. Characteristics of the Vectors

Marker	Name[a]	Size (kb)	Usable Sites[b]
*TRP*1	pAMT10	4.3	5
	pAMT20	5.1	5
	pGMT10	4.6	5
	pGMT20	5.4	5
*HIS*3	pAMH10	4.5	6
	pAMH20	5.3	6
	pGMH10	4.8	6
	pGMH20	5.6	6
*LEU*2	pAML10	5.1	6
	pAML20	5.9	6
	pGML10	5.4	6
	pGML20	6.2	6
*URA*3	pAMU10	4.6	6
	pAMU20	5.4	6
	pGMU10	4.9	6
	pGMU20	5.7	6

[a]All plasmids are obtainable from Riken DNA Bank (**http://www.rtc.riken.go.jp/DNA/HTML/engsearch.html**).

[b]Unique restriction site in the MCS in each vector was indicated.

Table 2. Protocol for Immunopecipitates' Preparation from Cell Lysates with Rabbit Polyclonal Anti-Myc-Tag Antibody

1. After a 6–9-h induction in SG (0.67% Bacto-yeast nitrogen base without aa, 20–400 mg/mL aa mixture and adenine sulfate and uracil, 2% galactose), 5 mL culture of each strain carrying a Myc-tagging vector were washed once with distilled water.
2. An equal volume of zirconium beads were added to the cell pellets and then resuspended with 500 µL of ice-cold sample buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet® P-40 (NP40), 0.1% sodium dodecyl sulfate (SDS), 0.1% deoxycholic acid, 1 mM benzamidine).
3. Cell suspension was crushed with Mini-BeadBeater® (Biospec Products, Bartlesville, OK, USA) for 5 min at 4°C.
4. The crushed-cell mixture was centrifuged at 12 000× *g* for 10 min at 4°C, and then the supernatant (cell lysate) was transferred to a newly prepared tube.
5. Fifty micrograms of rabbit polyclonal antibody against Myc-tag (MBL) conjugated with protein-A Sepharose® (Sigma-Aldrich Chemical, Tokyo, Japan), were added to each cell lysate and incubated for overnight at 4°C.
6. The Sepharose beads were washed 3× with ice-cold sample buffer.
7. The precipitates were resuspended with 10 µL of 1× SDS-polyacrylamide gel electrophoresis (PAGE) loading solution [65 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% phenol red, 42 mM dithiothreitol (DTT)] and boiled for 3 min.
8. The prepared samples were loaded on 12% SDS-PAGE gel, run for the appropriate separation time and then transferred to Hybond™ ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, Bucks, England, UK).
9. Detection of the immunoprecipitated protein was done with ECL™ Western Blotting Detection Reagents (Amersham Pharmacia Biotech) using mouse MAb 9E10 or mouse anti-Gts1p polyclonal antibody as the primary antibody and peroxidase-conjugated rabbit anti-mouse IgG as the secondary antibody, respectively.
10. The photo images of X-ray films in Figure 2 were processed with Adobe® Photoshop® Version 3.0 (Adobe Systems, San Jose, CA, USA).

and the MCS of pAMT10 or pAMT20 were replaced with the *Sph*I-*Eco*RI fragment of the *GAL*1 promoter and the MCS of pGAL1-118 to generate pGMT10 or pGMT20. Five restriction sites of the MCS are usable in the pA/GMT series, and the *Xba*I site is added in other series (Figure 1B). For construction of other series having *HIS*3, *LEU*2 and *URA*3, DNA fragments with selection markers were cloned at the *Pvu*II-*Hin*dIII site of the pA/GMT vectors. Table 1 summarizes the characteristics of each vector. Numbers 10 and 20 at the end of each vector's name indicates the type of replication origin as *CEN*6/*ARS*H4 and 2-µm ori, respectively.

Using this vector system, the structural analysis was performed on the two gene products involved in flocculation machinery in *S. cerevisiae*. The primary structure of the *GTS*1/*LSR*1 (1,4) gene product has been controversial, because it has been reported that the ORF of *LSR*1 is equal to that of *GTS*1, except for the single T deletion 990 bases from the start codon of its ORF (1). Therefore, we assessed the primary structure of Gts1p (or Lsr1p) again. GDU2168, BJ2168 (9) with *gts*1::*URA*3, was transformed with pGML20-GTS1 and pGML20-LSR1. Both were designed to attach the Myc-tag in their C terminus if the DNA sequence of the ORF is correct, and the whole-cell lysate from each

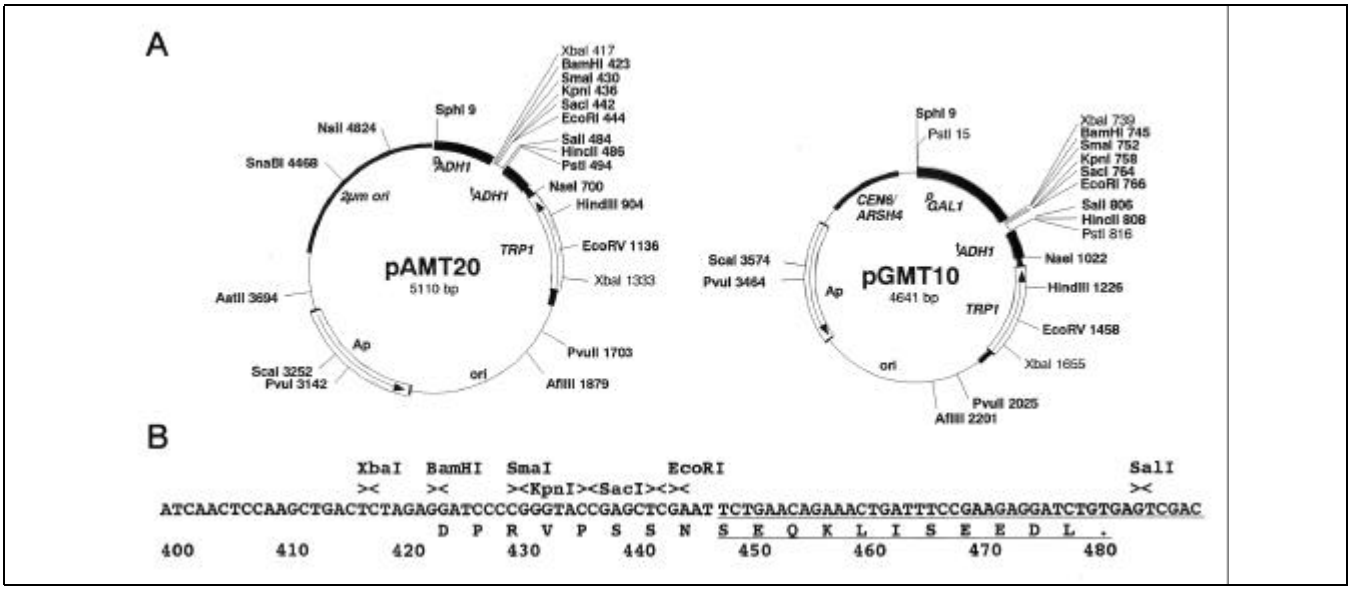

Figure 1. (A) Schematic representation of the pAMT20 and pGMT10 vectors. Bold characters indicate unique restriction sites. (B) The relevant DNA and aa sequence around MCS of pAMT20 is shown. The Myc-tag sequences are indicated with underline.

transformant was applied to immunoprecipitation and Western blot analysis. The anti-Gts1p antibody against both immunoprecipitates recognized the Lsr1p-type only (Figure 2A). Amino acid (aa) composition analysis, on the purified Gts1p expressed in *E. coli*, also indicated that the Lsr1p-type composition is more likely than that of the Gts1p-type (8). Thus, we concluded that the primary structure of the product from *GTS*1/*LSR*1 is the Lsr1p-type. Another protein involved in flocculation machinery, Sfl1p (3), was also tagged with Myc and detected by Myc-tag-specific 9E10 MAb (Figure 2B). However, the specific signal detected by 9E10 was much weaker than that of the anti-Gts1p antibody because of the low titration performance with this MAb or the labile nature of Sfl1p. Functional dissection of Gts1p (i.e., Lsr1p) has shown that the C-terminal half plays an essential role for flocculation (data not shown). Though the Myc-tagged Lsr1p showed fully functional flocculation property compared to that of intact Lsr1p, and the Myc-tagged Sfl1p showed the same results (data not shown). In this case, these two proteins were functional whether or not the Myc-tag is connected in their C terminus.

In this report, we describe the construction of a novel small epitope (Myc)-tagging *E. coli-S. cerevisiae* shuttle-vector system that is useful in the following ways: *(i)* relatively small-sized (4.3–6.2 kb) as a shuttle vector for yeast; *(ii)* five to six unique restriction enzyme sites in its MCS; *(iii)* four different selective markers (*TRP*1, *HIS*3, *LEU*2 and *URA*3); and *(iv)* two types of promoter (*ADH*1 and *GAL*1–10). It is hoped that this system will be a convenient tool for yeast researchers by eliminating the time-consuming antiserum generation.

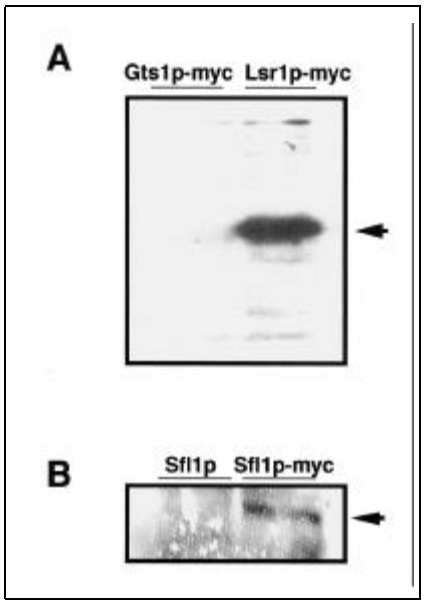

Figure 2. Re-evaluation of the primary structure of Gts1p and detection of Myc-tagged Sfl1p. (A) Western-blot analysis with polyclonal anti-Gts1p antibody. Arrowhead indicates polypeptides recognized with anti-Gts1p antibody (4). (B) Western-blot analysis with 9E10 MAb antibody against Myc-tag. Immunoprecipitates from cell lysates of GDU2168, carrying pGML20-GTS1 (Gts1p-Myc) and pGMT20-LSR1 (Lsr1p-Myc), and SDU2168, carrying YEp51-SFL1 (Sfl1p) and pGMT20-SFL1 (Sfl1p-Myc), were prepared with rabbit polyclonal anti-Myc-tag antibody (MBL, Tokyo, Japan) (see Table 2).

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We thank Akio Toh-e for providing plasmid pKT10, Tsutomu Ohta for providing yeast strain BJ2168 and So-ichi Yaguchi for technical support for the immunological experiment. *Address correspondence to Dr. Hidekatsu Iha, Department of Biochemistry, Yamanashi Medical University, 1110 Shimokato, Tamaho, Nakakoma, Yamanashi 409-3898, Japan. Internet: hiha@swallow.res.yamanashi-med.ac.jp*

Received 15 June 1998; accepted 24 August 1998.

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