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Short communications

Application of the β-glucuronidase gene fusion system to *Saccharomyces cerevisiae*

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Summary. Bacterial β-glucuronidase (GUS) has been described as a useful reporter enzyme for gene fusion studies in bacteria and plants. Here we report the expression of GUS in yeast to illustrate further applications of this enzyme as a quantitative tool for measuring gene activity, as a colour selection marker and as a versatile system for protein targeting studies. There is no intrinsic GUS activity in any yeast strain tested. GUS was expressed in transgenic yeast on a multiple-copy vector under the control of the alcohol dehydrogenase 1 (*ADH1*) promoter. The enzyme is stable in yeast and its activity may be monitored by very sensitive colorimetric or fluorometric methods in extracts, or by the histochemical reagent 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) on plates. To test the efficacy of GUS as a reporter for targeting proteins into different subcellular compartments in vivo, we fused the presequence of the mitochondrial tryptophanyl-tRNA-synthetase gene (MSW) to the amino terminus of GUS. The activity of the fusion protein is not substantially impaired and it is imported efficiently into yeast mitochondria.

Key words: Reporter gene - Transformation - Protein targeting - Chimeric genes

Introduction

The use of gene fusion has been a powerful tool for analysis of gene structure and function. This approach is largely predicated on the availability of reporter or responder genes that encode enzymes with suitable properties, and with numerous simple and sensitive assays. Sensitivity, in turn, is determined both by the available assays for the enzyme and the absence of background or interfering activity in the system being studied.

The GUS gene fusion system has been developed as a useful tool for molecular biology in bacteria, animals and plants (Jefferson et al. 1986, 1987; Kavanagh et al. 1988). GUS (β-glucuronidase, E.C. 3.2.1.31) is encoded by the *gusA* (formerly *uidA*) locus of *E. coli*, and has numerous assays, and excellent properties for gene fusion analysis.

To further expand the utility of the GUS system we have investigated the use of GUS gene fusions in the baker's yeast, *Saccharomyces cerevisiae.* Concern has been expressed whether yeast cells producing GUS are viable, since glucuronidases are included in compounds used for the removal of yeast cell walls.

Here we show that the synthesis of GUS does not have deleterious effects on *Saccharomyces cerevisiae* and that its growth rate is not significantly reduced. Furthermore, untransformed yeast cells do not contain measurable GUS activity. This opens new perspectives for the application of GUS as a colour selection marker and as a reporter enzyme for intracellular protein targeting in yeast.

Materials and methods

Yeast strains and transformation. Yeast strain DC04 was a kind gift of Dr. L. Breeden, Seattle, and has been described by Carle and Olson (1985). Strain AH216 was received from Prof. Dr. Schatz, Basel and has been described by Yaffe and Schatz (1984). Yeasts were transformed using the lithium acetate method of Ito et al. (1983). Transformants were selected for a $LEU2^{+}$ phenotype on minimal plates (0.67% yeast nitrogen base, DIFCO, 2% glucose, 2% agar) containing 20 μg/ml histidine. For the colorimetric detection of GUS activity these plates were supplemented with 50 μg/ml 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc, Molecular Probes Inc., USA). Individual transformants were grown in lactate medium (Daum et al. 1982) after preselection in minimal medium.

Vector construction. The bacterial β-glucuronidase gene was placed under the control of the promoter and terminator regions of the alcohol dehydrogenase I (*ADH1*) gene (Bennetzen and Hall 1982) in the multiple-copy expression vector pAAH5 (Ammerer 1983). The GUS gene was excised using *Sal*I and *Eco*RI from vector pRAJ 275 (Jefferson 1987), the ends were filled in with Klenow enzyme to generate blunt ends and synthetic linkers were added to clone the gene into the unique *Hin*dIII site of pAAH5. The construction of the

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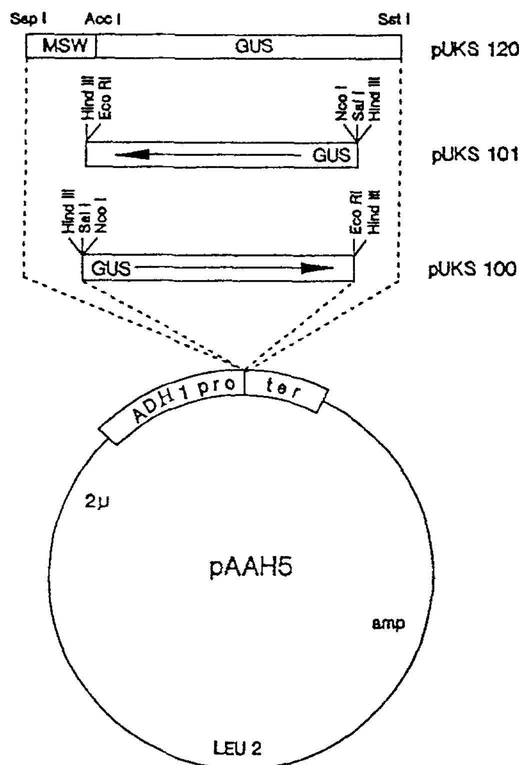

Fig. 1. Vector construction used for the expression of GUS in yeast. The GUS gene from *E. coli* was end modified as described in "Materials and methods" and cloned into a *Hin*dIII site between the promoter (pro) and terminator (ter) regions of the yeast alcohol dehydrogenase gene (*ADH1*). In vector pUKS120 the NH_2-terminal region of the MSW gene has been fused with GUS as described in Schmitz and Lonsdale (1989)

fusion gene harbouring the complete presequence of the mitochondrial tryptophanyl-tRNA synthetase gene from yeast (Myers and Tzagoloff 1985) in front of GUS has been described elsewhere (Schmitz and Lonsdale 1989). *Hin*dIII linkers were added to the gene construct after modifying the termini with T4 polymerase and it was cloned into pAAH5.

Isolation of yeast mitochondria. Yeast transformants were grown overnight in lactate medium (see above) and the mitochondria were isolated essentially as described by Daum et al. (1982). Yeast cells were converted to spheroplasts with Zymolyase 20,000 (Seikagaku Kogyo, Japan) and in all subsequent preparation steps the buffers used contained 1 mM PMSF.

Measurement of GUS activity. Yeast cells were washed several times and resuspended in "GUS extraction buffer" (Jefferson 1987) containing 1 mM PMSF. They were lysed by sonication or by osmotic shock after the cell wall had been removed enzymatically (see above). The homogenate was cleared from cell debris by a short centrifugation (3 min, 15,000 g) and at timepoint 0 the solution was adjusted to 1 mM 4-methylumbelliferone glucuronide (MUG, Sigma). The solutions were incubated at 37°C in the wells of a microtiter plate and the production of 4-methyl-umbelliferone (MU) was monitored with a Fluoroscan II (Flow Laboratories). Activity was measured at three different timepoints after stopping the reactions by adding 9 volumes of a sodium carbonate solution (0.2 M). Kinetics of the reactions were calculated using the programme "Plates" (Wolfe and Jefferson, in preparation). The protein content in each sample was determined according to the method of Bradford (1976) using a BioRad kit. All data were normalized to yield values expressed in nanomoles of product (MU)/min/mg protein.

Table 1. Measurement of GUS activity in untransformed and transformed yeast strains. The activity has been determined in independent transformants and is given in nmoles MU/min/mg protein

Yeast strains	Plasmids			
	–	pUKS100	pUKS101	pUKS120
DC04	0.002	0.60	0.003	0.76
	0.003	0.72	0.004	0.69
AH216	0.001	0.45	0.002	0.62
	0.002	0.57	0.003	0.68

Protein isolation and Western blot. Yeast cells were disrupted by sonication and Vortex mixing with glass beads in a buffer containing 50 mM Tris/HCl (pH 7.5), 0.5% SDS, 0.1 M 2-mercaptoethanol and 1 mM PMSF. The protein extracts were clarified by centrifugation. Proteins were separated on SDS/polyacrylamide gels (Laemmli 1970) and blotted onto nitrocellulose sheets (BA 85, Schleicher and Schull) according to Towbin et al. (1979). The filters were incubated with antibodies raised against bacterial β-glucuronidase and antigen-antibody complexes were identified using ^{125}I-labelled protein A (Amersham, UK). Radioactive bands were visualized by 2–3 days exposure to X-ray film.

Results

We have expressed bacterial β-glucuronidase in different yeast strains using the multi-copy plasmid pAAH5 which contains the control regions of the alcohol dehydrogenase gene, *ADH1* (Ammerer 1983). Figure 1 illustrates the construction of vectors pUKS100 and pUKS101 in which the GUS gene was inserted in normal and reverse orientation between the promoter and terminator of the *ADH1* gene in pAAH5. In the pAAH5 derivative, pUKS120, the complete presequence of the tryptophanyl-tRNA synthetase gene, fused in frame to the 5′ end of the GUS gene (Schmitz and Lonsdale 1989), was inserted.

Expression of the GUS gene at the translational level in yeast cells was monitored by immunodetection techniques and by measurements of enzyme activity. As shown in Table 1 there is no significant GUS activity in the untransformed yeast strains analysed. The transformants exhibit a GUS activity level which is several hundred fold higher than the minimum detectable limits determined with fluorometric methods. The level of GUS activity may differ when the pUKS plasmids are introduced into different yeast strains. The growth rate of yeast expressing GUS may be reduced in comparison to untransformed cells but transformants having a GUS activity level corresponding to the production of 0.5–0.8 nmoles MU/mg protein/min or less show a nearly normal growth rate (Fig. 2). The GUS enzyme of all transformants is stably synthesized and has the same size as the purified bacterial enzyme (Fig. 3). Specific antibodies raised against GUS only detect the enzyme in transformants which have the gene in the correct orientation with respect to the *ADH1* promoter.

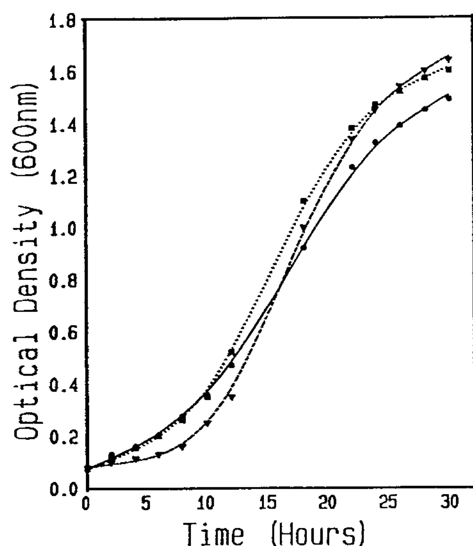

Fig. 2. Growth curves for yeast strain DC04 at 30°C in minimal medium. The cells were transformed with plasmid pAAH5 (■) and the pAAH5 derivatives pUKS100 (▲) and pUKS101 (●)

Yeast cells transformed with plasmid pUKS100 turn blue within a few days when grown on minimal plates supplemented with X-Gluc. The colour reaction is enhanced by growing the cells in suspension culture where the coloured compound may appear within hours. Possibly, under these conditions more oxygen, necessary for the oxidation of the indogenic precursor, is available; alternatively, the uptake of the substrate into cells could be facilitated.

To determine whether GUS is a useful passenger protein for targeting experiments in yeast we have fused a DNA fragment encoding the first 102 amino acids of mitochondrial tryptophanyl-tRNA synthetase with the GUS gene (Fig. 1).

When protein preparations from yeast cells transformed with pUKS120 were fractionated by SDS/PAGE, a single band of about 76 kd was detectable with a specific antibody (Fig. 4). This band is only found in the mitochondrial fraction and is not present in the supernatant representing the cytoplasmic fraction of lysed yeast cells (Fig. 4). While the GUS activity of the mitochondrial fraction is resistant to externally added proteinase K (0.2 mg/ml), mitochondria lysed with 0.1% Triton prior to a proteinase K treatment lack any detectable GUS protein. As the complete precursor protein retaining the mitochondrial presequence would have a molecular weight of about 80 kd it seems likely that the fusion protein has been processed during translocation across the mitochondrial membranes. These data suggest that the fusion protein is imported very efficiently into yeast mitochondria and processed upon translocation across the mitochondrial membranes.

Discussion

We have expressed the β-glucuronidase gene of *E. coli* in *Saccharomyces cerevisiae* to evaluate its possible application as a gene fusion marker. We find that the GUS enzyme may be a useful tool for molecular genetic experiments in yeast:(i) as a colour selection marker (e.g. in cloning experiments), (ii) as a reporter for quantitation of chimeric gene activity, (iii) as a passenger for the study of protein transport and targeting.

All three applications rely on the absence of GUS, or very low intrinsic levels of it, in untransformed yeast cells. While GUS is found in the ER and its derivatives in mammalian cells (reviewed by Paigan 1979) the enzyme is not detectable with sensitive fluorometic methods in the yeast strains analysed. However, it could be shown that the GUS activity increases several hundred fold above detectable limits after transformation of yeast cells with the GUS gene. Although the growth rate of transformed yeasts may be slightly reduced, GUS is not apparently damaging or lethal. Furthermore, transformed yeast cells are capable of hydrolysing colourogenic glucuronides, such as X-Gluc, present in the medium. This allows for the design of colour selection systems which screen for blue or white colonies within a heterogenous yeast population. Similar to yeast cells expressing the β-galactosidase gene (Rose and Botstein 1983), yeast expressing GUS do not give satisfactory colour production on transformation selection plates. Colour production essentially depends on the pH of the medium (pH values above 6.5–7 give good results) and the presence of oxygen. Colour production is sufficient upon replica plating of transformed yeasts but it may also be enhanced by the application of an overlay (containing hydrogen superoxide in a solution buffered at

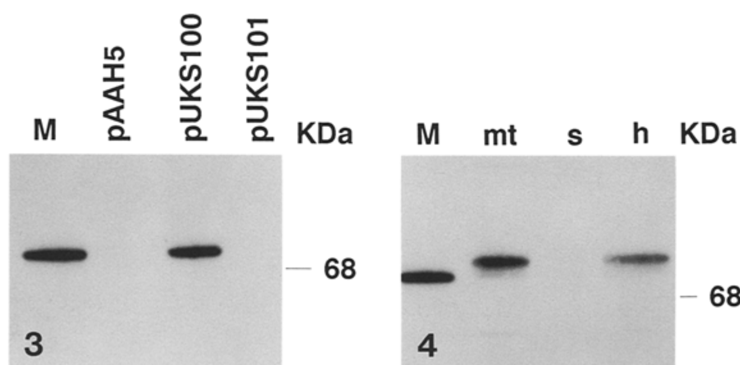

Fig. 3. Western blot of total protein isolated from yeast cells transformed with pAAH5, pUKS100 and pUKS101. Purified bacterial GUS was used as a marker (*M*). The blot was probed with antibodies raised against GUS

Fig. 4. Western blot of protein from yeast cells expressing an MSW-GUS fusion protein. Due to the presence of the mitochondrial presequence of the MSW gene, the chimeric protein is only found in yeast homogenate (*h*) and in the mitochondrial fraction (*m*), but not in the supernatant (*s*). The marker (*M*) and the antibodies used are the same as in Fig. 3

pH 8.0) on yeast colonies (K. Baker, personal communication).

Another interesting application of GUS gene fusion in yeast is the targeting of chimaeric proteins into different subcellular compartments using appropriate signal sequences. For example, it has been shown that an amphiphilic amino terminal stretch of amino acids is sufficient to direct a passenger protein into the mitochondrial compartment (reviewed by Hurt and van Loon 1986; Verner and Schatz 1988). Here we report that a fusion of the first 102 amino acids of the mitochondrial tryptophanyl-tRNA synthetase from yeast with GUS is imported into yeast mitochondria in vivo. The GUS protein obviously tolerates large amino terminal additions without substantial loss of activity (Jefferson et al. 1987). Thus, the subcellular location of the enzyme may be monitored be measurements of GUS activity in different subcellular fractions (Kavanagh et al. 1988; Schmitz and Lonsdale 1989). The GUS gene fusion system has been continuously improved during the last few years and a further step will be the expression of the bacterial glucuronide permease (Jefferson et al., in preparation) in yeast. The action of the permease would enhance the uptake of glucuronides and may open new possibilities for the application of the GUS reporter gene in yeast.

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