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Research Article

Combined use of two transcriptional reporters improves signalling assays for G protein-coupled receptors in fission yeast

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Abstract

The biochemical and genetic tractability of yeasts make them ideal hosts for the analysis of signalling from G protein-coupled receptors (GPCRs). Selected modifications to the strains allow the introduction of non-yeast components, while signal-dependent expression of reporter genes provides growth selection or enzyme read-out as assays for signalling. One issue with such systems is reporter expression in the absence of stimulation, usually because of spontaneous activation of intracellular signalling components and/or incomplete repression of the signal-dependent promoter. This limits the difference between reporter activity in the presence and absence of stimulation, often referred to as the signal : background ratio. In an effort to extend the applicability of the yeast system, we generated a *Schizosaccharomyces pombe* strain containing pheromone-dependent reporters for both growth selection and β-galactosidase production. Simultaneous use of the two reporters provided several advantages over strains expressing only one reporter, particularly when coupled to the use of a competitive inhibitor of the nutritional reporter. For example, the β-galactosidase signal : background ratio following stimulation with 10^{-6} M P-factor increased from 35 for a strain containing a single *lacZ* reporter to almost 2500 for the double reporter. The sensitivity of the system was also improved, with higher signal : background ratios allowing detection of lower concentrations of P-factor. Although we have used *Sz. pombe* and focused on GPCR-based induction of β-galactosidase, the principles described can be applied to other yeasts, different signalling pathways and alternative reporters. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: ***Schizosaccharomyces pombe*; yeast; GPCR; screen; double reporter; *ura4*; *lacZ***

Introduction

G protein-coupled receptors (GPCRs) are a diverse family of membrane proteins that enable cells to respond to a wide variety of extracellular stimuli. The receptors act via heterotrimeric G proteins composed of Gα, Gβ and Gγ subunits. In unstimulated cells the Gα subunit is bound to GDP and associated with the Gβγ dimer, but an activated receptor stimulates the exchange of GDP for GTP and Gα-GTP is released from the Gβγ dimer. The dissociated subunits then regulate the activity of effector proteins to bring about changes in cell behaviour. GPCRs influence all of the major physiological responses in most eukaryotes and defects in the signalling pathways can have dramatic consequences. It is not surprising, therefore, that they are of major interest to the academic, clinical and pharmaceutical communities, and a wide variety of approaches have been developed to study these pathways.

Both the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces*

pombe have become popular hosts for analysing GPCR signalling (Ladds *et al*., 2005). Each contains only two endogenous GPCRs, which respond to mating pheromones and glucose, are amenable to genetic and biochemical manipulation and can be easily adapted for high-throughput screening. Studies in yeast have made several significant contributions to our understanding of GPCR signalling, including the ability of G$\beta\gamma$ subunits to activate signalling cascades (Whiteway *et al*., 1989), the identification and analysis of RGS (regulators of G protein signalling) proteins (Dietzel and Kurjan, 1987; Dohlman and Thorner, 1997) and AGS (activators of G protein signalling) proteins (Cismowski *et al*., 1999; Takesono *et al*., 1999). Furthermore, with only minor modifications to the strains, many of the yeast components can be replaced by their mammalian counterparts, allowing the mammalian proteins to be analysed in a simpler experimental background (Dowell and Brown, 2002; Ladds *et al*., 2005).

Most yeast systems for studying GPCR signalling use reporter constructs to provide readouts for transcription from signal-dependent promoters. Bacterial β-galactosidase (encoded by the *Escherichia coli lacZ* gene) is a common reporter that is suitable for both quantitative liquid assays and qualitative assays on agar plates where a change in the colour of a colony can provide a useful indicator of signalling. Alternative reporters include enzymes that allow cell growth in the absence of a particular nutrient, such as uracil or histidine. Again, these can be used in both liquid- and plate-based assays. One issue with transcriptional reporter systems is that all of the GPCR-dependent promoters currently available have a low level of expression in the absence of stimulation, usually due to spontaneous activity of the signalling components and/or incomplete repression of the signal-dependent promoter. This affects the ratio between the response in the presence of stimulation and that in the absence of stimulation (the signal : background ratio). For most studies, maximum stimulation generates a response that is only about 10–20-fold higher than that in the absence of stimulation (see e.g. Erickson *et al*., 1998; Erlenbach *et al*. 2001a, 2001b; Arias *et al*., 2003; Floyd *et al*., 2003) and few have signal : background ratios above this (Didmon *et al*., 2002). Inefficient coupling between receptors and other signalling components means that these ratios are usually even lower in strains expressing non-yeast receptors (Ladds *et al*., 2003).

Several approaches have been tried to increase the signal : background ratio. Fluorogenic substrates extend the dynamic range of β-galactosidase assays (Wise *et al*., 2003) but are more expensive than traditional substrates. For growth assays, compounds such as 3-aminotriazole (3-AT, for *HIS3* selection in *S. cerevisiae*) or 6-azauracil (6-AU, for *URA3* selection in *S. cerevisiae, ura4* in *Sz. pombe*) can competitively inhibit the low-level activity produced in the absence of signalling, and help emphasize the effects of signalling. Alternatively, the yeast strains can be genetically modified to reduce the effects of negative regulators such as RGS proteins (Price *et al*., 1995; Erickson *et al*., 1998; Chambers *et al*., 2000; Sommers *et al*., 2000; Watson *et al*., 1999) and phosphatases (Zhan *et al*., 1997; Didmon *et al*., 2002), or increase the effects of positive regulators such as Ras1 (Nielsen *et al*., 1992). Unfortunately, although many of these approaches increase the sensitivity of the strains, they also increase reporter production in the absence of stimulation and generally produce only about a two-fold improvement in the signal : background ratio.

In an effort to improve the signalling ratio of *Sz. pombe* reporter strains for GPCRs, we have investigated the effects of combining growth selection with β-galactosidase production. Several reports describe the use of *S. cerevisiae* strains containing both reporters (Olesnicky *et al*., 1999; Floyd *et al*., 2003; Wise *et al*., 2003) and the benefits of combining the two reporters have been reviewed briefly (Dowell and Brown, 2002). However, no direct comparison of double and single reporter strains has been reported, and the approach has not been described in fission yeast. We show that *Sz. pombe* strains expressing both *lacZ* and *ura4* under the control of the pheromone-dependent *sxa2* promoter exhibit much higher signal : background ratios for β-galactosidase production than strains expressing either reporter on its own. Incorporating the Ura4 competitive inhibitor 6-AU into the growth medium led to further improvements, producing a signal in stimulated cells that was almost 2500-fold greater than that observed in the absence of stimulation.

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Materials and methods

General methods

General yeast procedures were performed as described previously (Davey *et al*., 1995; Ladds *et al*., 1996). Culture media used were yeast extract (YE; for routine cell growth), a defined minimal medium (DMM; for selective growth and all assays) and amino acid medium (AA; for plate-based assays) (Davey *et al*., 1995). Cell concentrations were determined using a Coulter Channelyser (Beckman Coulter, Luton, UK). DNA manipulations were performed by standard methods. Oligonucleotides were synthesized by TAG Newcastle Ltd (Gateshead, UK). Amplification by the polymerase chain reaction (PCR) used *Pwo* DNA polymerase according to the supplier's instructions (Boehringer-Mannheim Biochemicals, Lewes, East Sussex, UK). All constructs generated by PCR were confirmed by sequencing.

Yeast strains

The yeast strains used in this study are listed in Table 1. Construction of JY546 (contains the *sxa2>lacZ* reporter) and JY603 (*sxa2>ura4*) have been described previously (Didmon *et al*., 2002). JY994 was created by transforming JY546 with a construct that integrates the ura4$^+$ cassette (Grimm *et al*., 1988) into the *Sna*BI restriction site that lies just downstream of the *Sz. pombe krp1* gene. The construction and use of this construct has been described previously (Davis *et al*., 1999). To create JY1310, a *sxa2>ura4* construct containing ~1200 bp upstream of the *sxa2* initiator codon and ~500 bp downstream of the stop codon was amplified by PCR, using genomic DNA from JY603 as template, and cloned into the *Sna*BI restriction site of the *Sz. pombe krp1*. Digestion with *Pvu*II released a fragment containing the *sxa2>ura4* reporter flanked by regions from *krp1* and this was used to transform JY994. Ura$^-$ transformants were plated on medium lacking uracil but containing 10^{-6} M P-factor to identify isolates containing the *sxa2>ura4* construct. All gene replacements were confirmed by Southern blot analysis. By convention, *Sz. pombe* genes are shown in lower case and italicized; their corresponding proteins are not italicized and the initial letter is in upper case.

Assay of β-galactosidase activity

Assays were performed using a method modified from Didmon *et al*. (2002). Cells were cultured to a density of $\sim 5 \times 10^5$ cells/ml in DMM and 500 µl aliquots were transferred to 2 ml Safe-Lock tubes (Eppendorf, Fisher Scientific, Loughborough, UK) containing 5 µl P-factor in HPLC-grade methanol. The tubes were incubated at 29 °C on a rotating wheel and, at appropriate times, 50 µl samples were transferred to 750 µl Z-buffer containing 2.25 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The reactions were stopped after 90 min by adding 200 µl 2 M Na_2CO_3, and β-galactosidase activity was calculated as optical density at 420 nm (OD_{420}) and expressed per 10^6 cells or per ml culture medium.

Results and discussion

We have reported previously the production and use of *Sz. pombe* strains that express reporter constructs following activation of the pheromone response pathway (Didmon *et al*., 2002; Ladds *et al*., 2003). These contain either the *E. coli lacZ* gene (encodes β-galactosidase) or the *Sz. pombe ura4* gene (encodes orotidine 5′-monophosphate decarboxylase, an enzyme involved in uracil biosynthesis) under the control of the pheromone-dependent *sxa2* promoter. The *sxa2* gene is expressed only

Table 1. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype	
JY546	*mat1-M* Δ*mat2/3 ura4-D18 cyr1-D51 sxa2>lacZ*	Didmon *et al*. (2002)
JY603	*mat1-M* Δ*mat2/3 ura4-D18 cyr1-D51 sxa2>ura4*	Didmon *et al*. (2002)
JY994	*mat1-M* Δ*mat2/3 ura4-D18 cyr1-D51 sxa2>lacZ ura4*$^+$	
JY1310	*mat1-M* Δ*mat2/3 ura4-D18 cyr1-D51 sxa2>lacZ sxa2>ura4*	

The terms *sxa2>lacZ* and *sxa2>ura4* are used to indicate constructs in which the *lacZ* or *ura4* open reading frames are under the transcriptional control of the pheromone-dependent *sxa2* promoter.

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in M-cells (cells of mating type minus) following stimulation with P-factor (the peptide mating pheromone released by plus cells) and encodes a carboxypeptidase that inactivates P-factor by removing the C-terminal leucine residue (Imai and Yamamoto, 1992; Ladds *et al*., 1996; Ladds and Davey, 2000). Other modifications made to the strains include removing information at the *mat2* and *mat3* mating loci to ensure that the strains are unable to switch mating type (Klar and Miglio, 1986) and deleting the *cyr1* gene (encodes adenylate cyclase) to enable them to respond to P-factor under conditions that support mitotic growth (Davey and Nielsen, 1992; Imai and Yamamoto, 1994). The properties of these strains are illustrated in Figure 1.

Sz. pombe requires expression of *ura4* to grow in the absence of uracil and the *sxa2>ura4* reporter strain (JY603) did not form colonies on plates containing less than 10^{-8} M P-factor. Addition of at least 10^{-8} M P-factor induced sufficient Ura4 production to support colony formation. Ura4 also converts 5-fluoro-orotic acid (FOA) into a toxic product that causes cell death (Boeke *et al*., 1984), and the *sxa2>ura4* reporter was unable to form colonies on plates containing FOA and at least 10^{-8} M P-factor. Such plate-based assays depend upon the production of a threshold level of Ura4 that supports growth in the absence of uracil, or death in the presence of FOA. Exposing the *sxa2>lacZ* reporter strain (JY546) to P-factor resulted in the pheromone- and time-dependent production of β-galactosidase (Didmon *et al*., 2002). Reporter activity reached a plateau 10 h after stimulation and 10^{-6} M P-factor produced a signal : background ratio of 34, which is typical for GPCR-signalling assays in yeast. The low level expression of the *sxa2>lacZ* reporter in the absence of P-factor is due largely to the spontaneous activation of the Gα subunit (Obara *et al*., 1991; Ladds *et al*., 2003). We presume that this also happens with the *sxa2>ura4* reporter but that the level of Ura4 produced is below that required to support growth.

Construction of a double reporter strain

We used the *sxa2>lacZ* reporter (JY546) as the starting point for creating a strain containing both reporters. To reduce variability between expression of the reporters, we sought to integrate the *sxa2>ura4* reporter construct into the yeast chromosome. Deletion analysis suggests that a clone containing 1158 bp upstream of the *sxa2* open reading frame possesses those regions required for regulated expression (Imai and Yamamoto, 1992) and we therefore prepared a construct in which this region was fused to the *ura4* ORF. The *sxa2>ura4* construct also contained ~500 bp downstream of the *sxa2* ORF, and was flanked by sequences homologous to a region immediately downstream of the *krp1* locus (Davey *et al*., 1994). This was integrated into the *Sz. pombe* genome by homologous recombination and confirmed by Southern blotting. We have previously integrated other constructs at this site with no observable effects on cell behaviour, especially the pheromone response (Davis *et al*., 1999). The resulting strain, JY1310 (*sxa2>lacZ, sxa2>ura4*), was compared to the *sxa2>lacZ* reporter strains JY546 (Ura4$^-$, contains no *ura4* gene) and JY994 (Ura4$^+$, contains a functional ura4$^+$ cassette integrated at the *Sna*BI site just downstream of the *krp1* gene) (Figure 2). To incorporate differences in cell growth, we calculated β-galactosidase production per ml of culture, rather than per 10^6 cells as previously.

The slower growth and lower final density of JY994 (*sxa2>lacZ*, Ura4$^+$) in the presence of P-factor compared to the absence of P-factor is due to the transient arrest of the cell cycle caused by the pheromone (Davey and Nielsen, 1994). The low-level expression of the *sxa2>lacZ* reporter in the absence of P-factor was greatly increased following stimulation with P-factor, despite the presence of fewer cells. In contrast, JY546 (*sxa2>lacZ*, Ura4$^-$) showed very limited growth and no increase in the production of β-galactosidase in response to P-factor, confirming that Ura4 production is required for cell growth and β-galactosidase production. To enable comparison between different strains, we used the β-galactosidase values produced after 72 h as there was no further increase in cell number or β-galactosidase activity after this time. Thus, JY994 (*sxa2>lacZ*, Ura4$^+$) produced 985 units/ml β-galactosidase in the presence of 10^{-6} M P-factor and 28 units/ml in the absence of P-factor, representing a signal : background ratio of ~35.

The properties of the double reporter strain JY1310 (*sxa2>lacZ, sxa2>ura4*) in the presence of P-factor were very similar to those for the single reporter JY994 (*sxa2>lacZ*, Ura4$^+$). Despite expressing Ura4 from different constructs (JY994

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Figure 1. Signalling pathway for P-factor mating pheromone in *Sz. pombe*. Binding of P-factor to its receptor (Mam2) on the surface of M-cells leads to GDP : GTP exchange on the Gα subunit (Gpa1) and activation of a MAP kinase cascade. Phosphorylation of the Ste11 transcription factor by Spk1 (Kjaerulff *et al.*, 2005) induces expression of genes that possess a pheromone response element (PRE). These genes, which include *sxa2*, are required for mating (reviewed in Davey, 1998). Replacing the *sxa2* open reading frame with reporter genes such as the *Sz. pombe ura4* (encodes orotidine 5′-monophosphate decarboxylase, an enzyme required for uracil biosynthesis but which also converts 5-fluoro-orotic acid [FOA] into a toxic compound) or *E. coli lacZ* (encodes β-galactosidase) provide simple assays for signalling through the response pathway (Didmon *et al.*, 2002). (Left) JY603 (contains the *sxa2>ura4* reporter) was spread on plates containing increasing concentrations of P-factor and assayed for Ura4 production by the ability to form colonies in the absence of uracil (upper series of plates) or the inability to grow in the presence of FOA (lower series of plates). Approximately 2000 cells were spread on each plate and incubated at 29 °C for 3 days. (Right) JY546 (*sxa2>lacZ*) was exposed to P-factor and β-galactosidase production assayed using ONPG as substrate. Activity is expressed as OD_{420} units per 10^6 cells and the values shown are the means of three independent isolates. In the upper panel, cells were assayed at hourly intervals following exposure to P-factor. In the lower panel, cells were exposed to P-factor for 16 h before being assayed; the inset table compares the amount of β-galactosidase produced at each P-factor concentration with that produced in the absence of P-factor

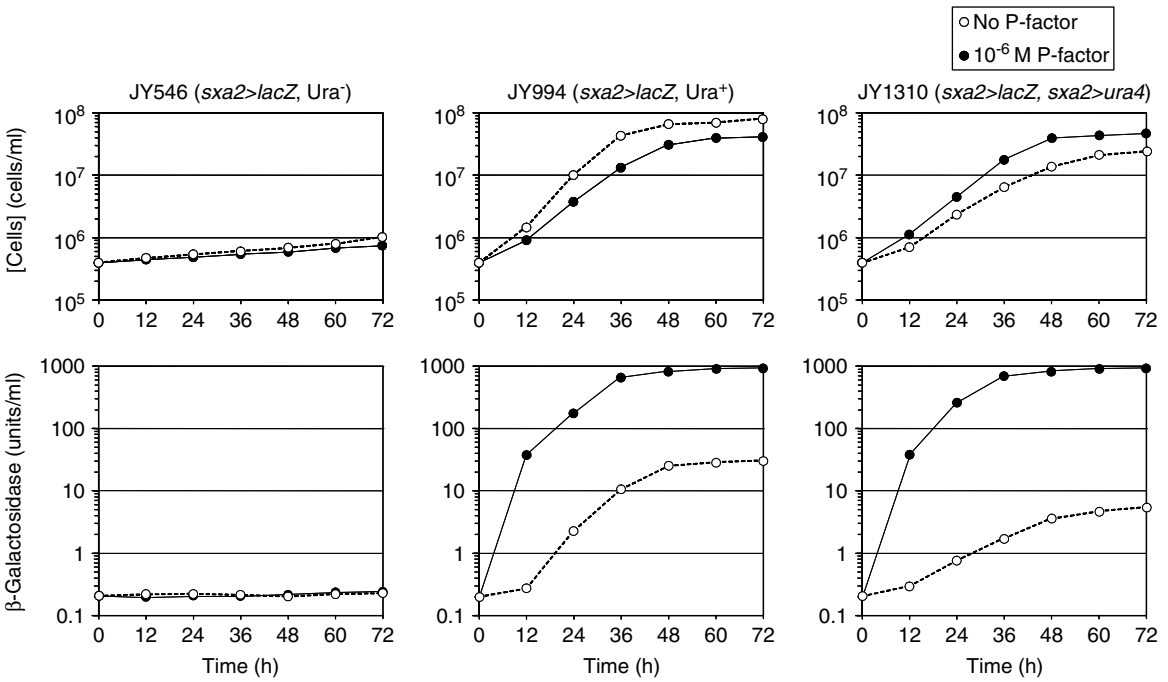

Figure 2. Growth and β-galactosidase production in *Sz. pombe* reporter strains. Strains were cultured to mid-exponential phase in medium containing uracil (DMM + uracil), washed in medium lacking uracil (DMM — uracil), and resuspended at $\sim 4 \times 10^5$ cells/ml in DMM — uracil supplemented with either no P-factor or 10^{-6} M P-factor. Cultures were incubated at 29 °C and cell concentration and β-galactosidase activity were determined every 12 h. Note, in contrast to Figure 1, β-galactosidase activity is expressed as OD_{420} units/ml culture. The values shown are the means of three independent isolates

has a functional ura4$^+$ cassette that is expressed constitutively, JY1310 contains the pheromone-dependent *sxa2>ura4*), the rate and extent of growth were similar for the two strains. Furthermore, the rate and extent of β-galactosidase production in the two strains were almost identical following stimulation with P-factor, perhaps not surprising as both contain the same *sxa2>lacZ* reporter. In contrast, the two strains behaved differently in the absence of P-factor; the growth of the double reporter JY1310 was markedly reduced, leading to a reduction in β-galactosidase production. This reduction in cell growth appears to be due to insufficient production of Ura4 from the *sxa2>ura4* reporter, as adding uracil to the medium recovered growth and β-galactosidase production to the levels for JY994 (*sxa2>lacZ*, Ura4$^+$; not shown). There was clearly some expression from the *sxa2>ura4* reporter in the absence of P-factor, as cell growth is higher than for the Ura$^-$ strain (JY546), but it appears to be insufficient and limits cell growth. Although both JY994 and JY1310 produce similar levels of β-galactosidase in the presence of P-factor, the fact that the double reporter strain produces less β-galactosidase in the absence of P-factor increases the signal : background ratio at 72 h from $\sim$35 to $\sim$190.

Use of 6-AU

6-Azauracil (6-AU) is a competitive *in vivo* inhibitor of orotidine decarboxylase, the *ura4* gene product (Grimm *et al.*, 1988), and we investigated whether it would be a useful tool to further manipulate the behaviour of JY1310. However 6-AU also inhibits inosine-5′-monophosphate (IMP) dehydrogenase, the enzyme that catalyses the NAD-dependent reduction of IMP into xanthosine 5-phosphate. This is the rate-limiting reaction of *de novo* GTP biosynthesis and the reduction in nucleotide levels following treatment with 6-AU can reduce RNA polymerase activity (reviewed in Wind and Reines, 2000). Treating *Sz. pombe* with 6-AU therefore has both Ura4-specific effects

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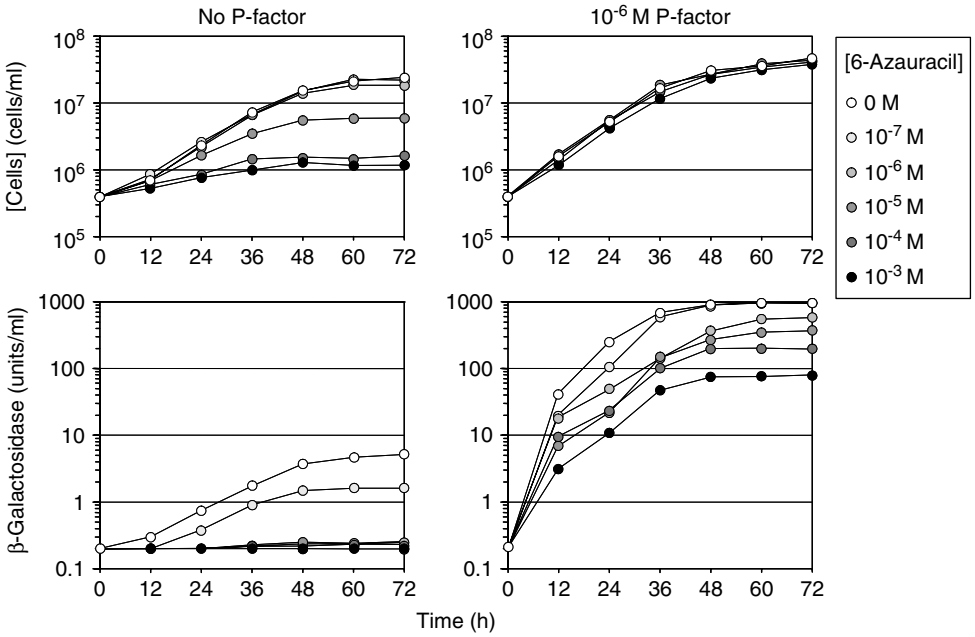

Figure 3. Effect of 6-azauracil on a strain containing both *lacZ* and *ura4* reporters. JY1310 (*sxa2>lacZ, sxa2>ura4*) was cultured to mid-exponential phase in DMM + uracil, washed in DMM — uracil, and resuspended at $\sim 4 \times 10^5$ cells/ml in DMM — uracil containing different amounts of 6-AU. The cultures were supplemented with either no P-factor or 10^{-6} M P-factor, and incubated at 29 °C. Cell concentration (cells/ml) and β-galactosidase activity (OD_{420} units/ml) were determined every 12 h. The values shown are the means of three independent isolates

and more general effects on gene transcription. However, the growth of wild-type strains is not markedly affected until concentrations of 6-AU exceed 10^{-3} M (Wind and Reines, 2000) and we limited our investigation to concentrations below this (Figure 3).

Growth of JY1310 in the absence of P-factor was reduced progressively by increasing concentrations of 6-AU. At 10^{-6} M and below, 6-AU had no noticeable effect on cell growth. However, growth was partially inhibited at 10^{-5} M 6-AU and almost completely inhibited at 10^{-4} M 6-AU, suggesting that this concentration is sufficient to inhibit almost all of the Ura4 produced from the *sxa2>ura4* reporter in the absence of stimulation. The reduced growth is likely to be a combination of two 6-AU effects, a reduction in expression of the *sxa2>ura4* reporter (as a consequence of reduced RNA polymerase activity) and an inhibition of the Ura4 produced. However, it does not appear to be due to a generalized toxic effect of 6-AU on cell growth. Not only are the concentrations used in the study lower than those that affect growth of wild-type cells (Wind and Reines, 2000) but the effects were abolished when JY1310 was grown in medium containing uracil (not shown). 6-AU also reduced the production of β-galactosidase from the *sxa2>lacZ* reporter, although its effects were evident at concentrations much lower than those required to affect cell growth; β-galactosidase production was partially inhibited at 10^{-7} M 6-AU and completely inhibited at 10^{-6} M 6-AU (a concentration that had no effect on cell growth). As 6-AU has no direct effect on β-galactosidase, the reduction in activity presumably reflects reduced expression from the *sxa2>lacZ* reporter.

6-AU had little effect on the growth of JY1310 in the presence of 10^{-6} M P-factor (Figure 3). Clearly, the amount of Ura4 produced from the *sxa2>ura4* construct following stimulation is more than that required for growth. It is certainly enough to overcome the effects of 6-AU on both gene transcription and Ura4 activity and still not be growth-limiting. Pheromone-dependent production of β-galactosidase in JY1310 was reduced progressively in response to increasing concentrations of 6-AU. The effect is due entirely to inhibition of transcription by 6-AU, as there was no effect

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Table 2. Signalling characteristics of a strain containing both *lacZ* and *ura4* reporters

[P-factor]	[6-Azauracil]											
	0 M		10^{-7} M		10^{-6} M		10^{-5} M		10^{-4} M		10^{-3} M	
0 M	**1.0**	*(5.19)*	**1.0**	*(1.48)*	**1.0**	*(0.26)*	**1.0**	*(0.22)*	**1.0**	*(0.21)*	**1.0**	*(0.22)*
10^{-9} M	**3.4**	*(17.7)*	**2.6**	*(3.84)*	**1.3**	*(0.33)*	**1.2**	*(0.27)*	**1.0**	*(0.21)*	**1.0**	*(0.22)*
10^{-8} M	**36**	*(187.2)*	**33**	*(78.3)*	**122**	*(31.7)*	**17**	*(3.74)*	**13**	*(2.66)*	**12**	*(2.69)*
10^{-7} M	**101**	*(525.9)*	**318**	*(470.8)*	**958**	*(249.2)*	**627**	*(137.9)*	**110**	*(23.3)*	**15**	*(3.23)*
10^{-6} M	**189**	*(985.4)*	**655**	*(970.2)*	**2424**	*(630.4)*	**1760**	*(387.1)*	**945**	*(198.6)*	**358**	*(78.8)*

JY1310 (*sxa2>lacZ, sxa2>ura4*) was cultured to mid-exponential phase in DMM + uracil, washed in DMM — uracil, and resuspended at $\sim 4 \times 10^5$ cells/ml in DMM — uracil containing different amounts of 6-AU. The cultures were supplemented with different concentrations of P-factor and incubated at 29 °C. β-galactosidase activity (OD_{420} units/ml) was determined after 72 h. The italicized numbers are the means for three independent isolates at each combination of P-factor and 6-AU. The emboldened numbers compare the amount of β-galactosidase produced at each P-factor concentration with that produced in the absence of P-factor.

on growth and all samples had approximately the same number of cells. The β-galactosidase activity in 10^{-6} M P-factor was compared to that in the absence of P-factor for each concentration of 6-AU (Table 2). The highest signal : background ratio (2424) was observed at 10^{-6} M 6-AU, primarily because this concentration almost completely inhibited β-galactosidase production in the absence of P-factor but had only minimal effect in the presence of P-factor.

The study was extended to include a range of P-factor concentrations and different concentrations of 6-AU (Table 2). Comparing the signalling ratios for JY1310 with those for the single reporter strains JY546 (*sxa2>lacZ*, Ura^-) (Figure 1) or JY994 (*sxa2>lacZ*, $Ura4^+$) (Figure 2) emphasizes the benefits of combining both reporters in one strain. For example, JY1310 exhibited much higher signal : background ratios; a 1.3-fold ratio at 10^{-8} M P-factor increased to 122-fold (in the presence of 10^{-6} M 6-AU) and a $\sim$35-fold ratio at 10^{-6} M P-factor increased to 2424-fold. Furthermore, combining the two reporters improved the sensitivity of the system; exposing JY1310 to 10^{-8} M P-factor caused a 36-fold increase in signalling compared to the absence of P-factor. Finally, combining the reporters extended the range over which signals could be detected; 10^{-9} M P-factor had no noticeable effect on β-galactosidase production in the single reporter strains but induced a 3.4-fold increase in JY1310. It is interesting to note how manipulating the assay conditions affected signalling ratios. For example, the highest signal : background ratio in the presence of high concentrations of P-factor (10^{-8} M and above) is obtained with 10^{-6} M 6-AU. However, for lower concentrations of P-factor, the best signal : background ratio is obtained in the absence of 6-AU. Such information should help establish the best assay conditions for different signalling scenarios, and will improve the use of *Sz. pombe* as a screening host for GPCRs.

Although we have used *Sz. pombe* and focused on GPCR-based induction of β-galactosidase and Ura4 reporters, the principles can be applied to other yeast and different signalling pathways. Other reporter combinations are also available. For example, the uracil-based system used for growth selection could be replaced by histidine, using the *HIS3* gene and 3-AT as inhibitor, and β-galactosidase could be replaced by a variety of other reporters, such as β-lactamase, luciferase, chloroamphenicol acetyl transferase or fluorescent proteins.

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