RGS Proteins and G Protein Signalling

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Declaration

I hereby declare that the work described in this thesis was conducted by myself under the supervision of Professor John Davey, with the exception of those instances where contributions of others have been specifically acknowledged.

None of the information herein has been used in any previous application for a degree.

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Some of the work presented in this thesis has been published in:

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Abstract

The work within this thesis is concerned with the creation of a temperature-sensitive *Schizosaccharomyces pombe* marker protein, and the regulation of the pheromone communication system of *Sz. pombe* reporter strains by RGS proteins.

There are a limited number of marker proteins available for use in the genetic manipulation of *Sz. pombe*, and the generation of a temperature-sensitive Ura4p was envisaged to expand the scope of carrying out sequential gene disruptions in the fission yeast. PCR-based mutagenesis was used to introduce mutations in the *ura4* cassette, and a leucine to proline mutation identified at residue 261 in the *ura4* open reading frame conferred a temperature-sensitive requirement for uracil. To demonstrate the use of the Ura4^{ts}p marker in gene disruption, the *Sz. pombe irp1* gene was disrupted with the *ura4*^{ts} cassette, and subsequently, the *prk1* gene was disrupted with the wild-type *ura4* cassette.

RGS proteins are a recently discovered family of proteins that negatively regulate G protein-coupled signalling pathways. This thesis describes the ability of mammalian RGS proteins to regulate the pheromone communication system of *Sz. pombe* reporter strains. Human RGS1 and human RGS4 displayed the greatest ability to negatively regulate the *Sz. pombe* pheromone signalling pathway when expressed from multicopy expression vectors. Human RGS2, human RGS3, human RGS9-2 and murine RGS2 displayed lesser, varying abilities. Expression of human RGS1 from single copy reduced signalling at low pheromone concentrations. Expression of human RGS4 from single copy was incapable of reducing pheromone-independent and pheromone-dependent signalling.

This thesis also describes the search for gain-of-function RGS proteins. Two potential gain-of-function szRgs1p mutants were previously identified, and these mutants were recreated. The two mutations identified (histidine to arginine at szRgs1p residue 171 and valine to isoleucine at szRgs1p residue 305) conferred gain-of-function szRgs1p phenotypes in an *sxa2*::*ura4* reporter strain. Hydroxylamine treatment of the human RGS4 open reading frame resulted in the identification of a potential gain-of-function RGS4 mutant. The lysine to arginine mutation at huRGS4p residue 20 conferred a gain-of-function huRGS4p phenotype in an *sxa2*::*ura4* reporter strain.

Abbreviations

ATP	adenosine triphosphate
βAR	β-adrenergic receptor
bp	base pair
cAMP	cyclic adenosine 3,5-monophosphate
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytidylate triphosphate
ddA	dideoxy adenosine triphosphate
DMM	defined minimal media
E. coli	*Escherichia coli*
EDTA	diaminoethane tetra-acetic acid sodium salt
FOA	5-fluoro-orotic acid
GAP	GTPase-activating protein
GTP	guanosine triphosphate
GDP	guanosine diphosphate
G protein	guanosine nucleotide-binding protein
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
LMP	low melting point
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen–activated protein kinase kinase kinase
mRNA	messenger RNA
ONPG	*o*-nitrophenyl-β-D-galactoside
ORF	open reading frame
PCR	polymerase chain reaction
PKA	cAMP dependent protein kinase A

PMSF	phenylmethylsulphonyl fluoride
RGS	regulators of G protein signalling
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
Sz. pombe	*Schizosaccharomyces pombe*
TCLK	N-TOSYL-$_L$-Phenylalanine chloromethyl ketone
Tris	tris (hydroxymethyl) aminomethane
v/v	volume for volume
w/v	weight for volume
YE	yeast extract

Chapter 1. General Introduction

1.1. Cell communication

1.1.1. Intercellular and intracellular communication

Both unicellular and multicellular organisms are constantly being challenged by a changing environment, and in order for them to react favourably to changing circumstances, cells within unicellular or multicellular communities must be able to influence and communicate with each other.

Unicellular organisms communicate with each other for reproduction and survival. For example, yeast cells secrete pheromones that induce mating responses in target cells. Multicellular organisms, besides communicating with each other, have circulatory systems that enable cells to communicate with cells at distant physiological sites.

Signals received at the cell surface are transmitted to intracellular targets via intracellular signal transduction pathways. Signalling cascades within cells transmit and amplify signals received at the cell surface to intracellular effector molecules. This results in a change in cell physiology and/or biochemistry. Regardless of the nature of stimulant, target cells recognise agonists via cell surface receptors, and the huge diversity in the chemical structure of agonists requires a similarly diverse array of receptors. Diversity among receptors and intracellular signalling pathways means that different cells can respond differently to the same agonist.

1.1.2. Adaptation in signal transduction pathways

The presence of an agonist rarely remains at the same concentration, and in order for a cell to adapt to the removal of an agonist or its continued presence, mechanisms exist that switch off signalling through a pathway, and reset the cell for future stimulation. Adaptation mechanisms exist at all stages in signalling pathways. The agonist may be inactivated or internalised even before it binds to a receptor. Likewise, the receptor can be inactivated or internalised. Downstream effector molecules are also subject to adaptation mechanisms.

Adaptation mechanisms are activated by agonist-induced signal transduction, and provide negative feedback mechanisms that turn off signalling.

1.1.3. G protein signalling pathways

The largest receptor family is the G protein-coupled receptor (GPCR) family, whose members possess seven trans-membrane domains. GPCRs couple to heterotrimeric G proteins within the cell, which are activated upon ligand binding to receptors. Activation of the cognate G protein in turn modulates the activity of downstream second messenger molecules, resulting in a physiological response. Signal amplification arises from the ability of a single receptor to activate many G proteins, and the resulting stimulation of many cycles of the effector.

1.2. G protein-coupled signalling pathways

1.2.1. Overview

Signal transduction pathways that are coupled to heterotrimeric G proteins have undergone intense research in the last decade. Heptahelical receptors represent the largest family of cell surface receptors, and it has been estimated that over half of pharmaceutical therapeutic drugs are directed towards G protein-coupled signalling pathways (Milligan and Rees, 1999; Marchese *et al.*, 1999).

G protein signalling pathways are ubiquitous throughout eukaryotic systems, and represent one of a number of signalling pathway families responsible for transducing signals from the extracellular environment to the intracellular machinery. G protein signalling pathways consist of a member of the seven transmembrane receptor family, a heterotrimeric guanine nucleotide-binding protein (G protein) and downstream effectors (including adenylyl cyclases, phosphodiesterases and phospholipase isoforms) (Figure 1). Agonist binding causes conformational changes in the cell surface heptahelical receptor, which are

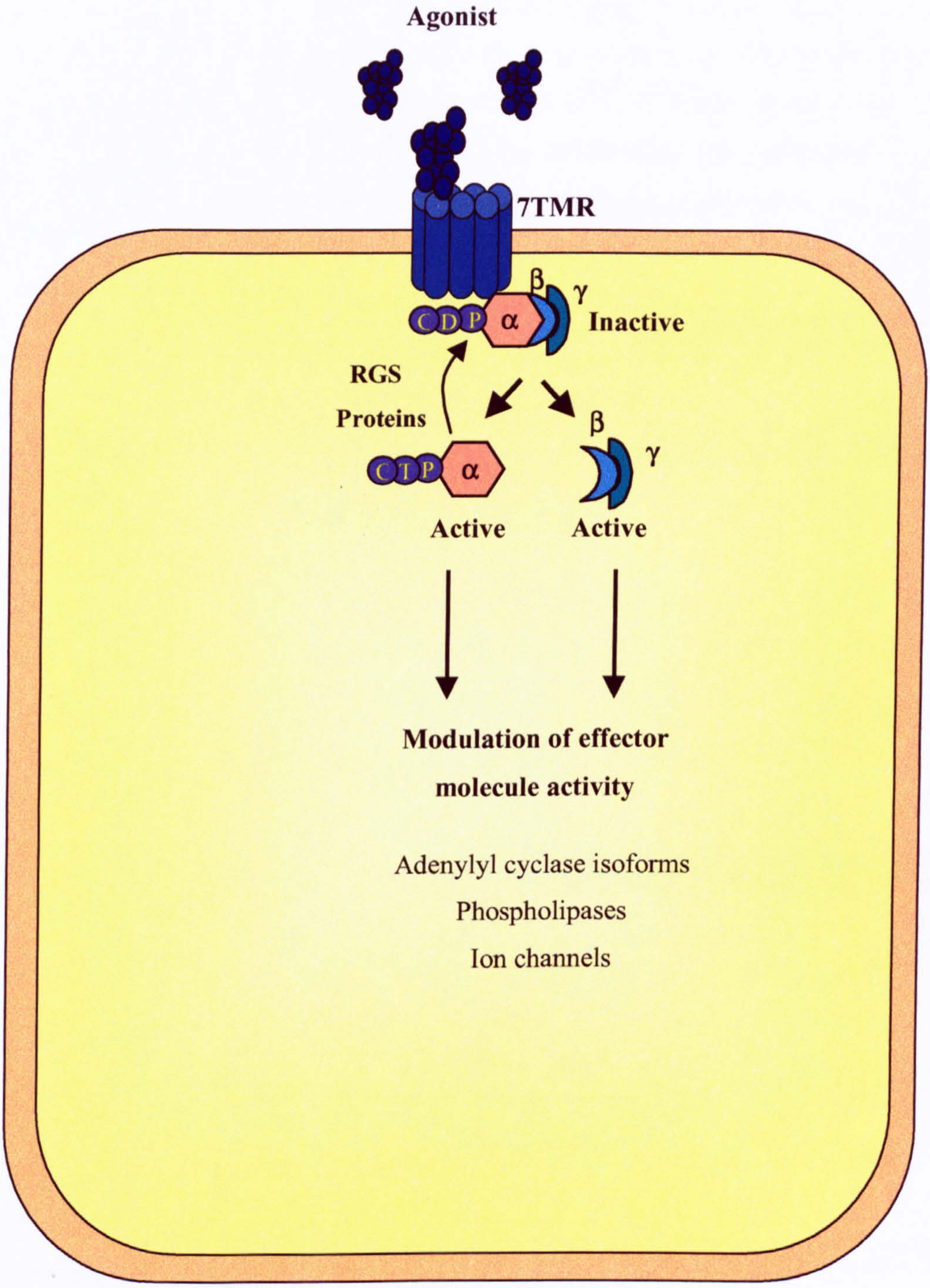


Figure 1. G protein signalling pathways

Agonist binding to a seven-transmembrane receptor results in a conformational change being transmitted to an inactive heterotrimeric G protein. Activation of the cognate G protein causes the dissociation of GDP from the G_{α} subunit, which is followed by the binding of GTP to the G_{α} subunit. The binding of GTP to the G_{α} subunit causes the dissociation of the $G_{\alpha\text{-GTP}}$ and $G_{\beta\gamma}$ subunits, which are then available to modulate the activity of second messenger molecules within the cell.

sterically transmitted to a coupled heterotrimeric G protein on the cytoplasmic face of the plasma membrane. The inactive heterotrimeric G protein is comprised of three subunits (α, β, γ). In the inactive heterotrimeric G protein, GDP is bound to the G_α subunit. Activation of the G protein results in the dissociation of G_α-bound GDP and the subsequent binding of GTP. The G_α conformational change following GTP binding results in the liberation of the G_α-GTP complex from the $G_{\beta\gamma}$ complex. The $G_{\beta\gamma}$ subunit exists as a tightly bound heterodimer, and has low affinity for the GTP-bound G_α subunit. One or both of these liberated complexes are then free to modulate the activity of downstream effectors in the pathway, or act as effectors themselves. Thus, in G protein signalling pathways, agonist binding to a cell surface heptahelical receptor is coupled to downstream events via guanine nucleotide binding regulatory proteins.

G proteins may stimulate or inhibit signal transduction pathways, via the modulation of second messenger activity. The G_α subunit has an intrinsic GTPase activity, which slowly hydrolyses the bound GTP to GDP. The high affinity of G_α-GDP for the $G_{\beta\gamma}$ subunit causes the G_α and $G_{\beta\gamma}$ subunits to reassociate, reforming the inactive heterotrimeric G protein.

1.2.2. G protein-coupled receptors

G protein-coupled receptors share a common seven-helix trans-membrane structure, and provide the link between the extracellular and intracellular environments (reviewed in Dohlman *et al.*, 1991; Inglese *et al.*, 1993). A diverse number of agonists are detected by GPCRs, such as photons, odorants, peptide hormones and neurotransmitters (Dohlman *et al.*, 1991), and it is the primary function of GPCRs to discriminate between the hundreds of ligands in the extracellular environment in order for the cell to carry out an appropriate response. The specificity with which the receptor can interact with G proteins defines the range of responses a cell can make. Receptors are highly specific for their cognate ligand, and a ligand that gives a specific response in one cell may cause a pleiotropic response in another.

Ligand binding induces conformational changes in the tertiary structure of the receptor (reviewed in Hulme *et al.*, 1999), which are transmitted to an inactive, GDP-bound heterotrimeric G protein. Evidence suggests that the second and third cytoplasmic loops are the interaction sites with G proteins in several GPCRs (Liu *et al.*, 1995; Eason and Liggett, 1996; Yamashita *et al.*, 2000). Experimental evidence suggests that structural constraints keep GPCRs in an inactive conformation that prevents an effective interaction between peptide sequences in the intracellular loops of the GPCR and the G protein. Agonist binding or mutations in constitutively active mutant (CAM) GPCRs are believed to relieve the constraints and to cause the GPCR to adopt an active conformation (reviewed in Leurs *et al.*, 1998). It has been hypothesised that particular sequences in the cytoplasmic domain of heptahelical receptors become accessible to epitopes on the G protein as a consequence of ligand-induced activation. One crucial interaction is with the C-terminus of the G_α subunit, which is important in dictating receptor specificity.

GPCRs can spontaneously couple to signal transduction pathways (Leurs *et al.*, 1998), and can signal through associations with intracellular partners other than G proteins, including polyproline binding proteins, arrestins, GRKs, small GTP binding proteins, SH2 domain-containing proteins and PDZ domain-containing proteins (reviewed in Hall *et al.*, 1999; Marinissen and Gutkind, 2001). This presumably allows receptors to initiate multiple intracellular signalling pathways, with each subtype of receptor likely coupled to a relatively unique set of effectors.

The biological outcome of GPCR activation probably results from the integration of a complex network of biochemical responses, which are highly dependent on receptor expression levels, coupling specificity and the repertoire of signalling molecules expressed in each cellular system.

1.2.3. Heterotrimeric G proteins

Heterotrimeric G proteins are comprised of three subunits, and it is the G_α subunit that is responsible for the activation state of the G protein. When the G_α subunit is in its GDP-bound state the G_α subunit is associated with both the intracellular carboxy-terminal tail of a seven-transmembrane receptor and a weakly bound $G_{\beta\gamma}$ dimer, and both G protein moieties are unable to modulate the activity of

downstream effector molecules. Upon activation by its cognate receptor, GDP dissociates from the G_α subunit (probably as a result of conformational changes [Bourne, 1997]), and GTP is consequently bound by the G_α subunit. The binding of GTP results in the dissociation of G_α-GTP from the $G_{\beta\gamma}$ subunits (Lambright *et al.*, 1994). The GTP-dependent conformational switch disrupts the extensive switch interface by rearranging many of the residues on the G_α subunit that are directly involved in $G_{\beta\gamma}$ contact (reviewed in Lambright *et al.*, 1996).

G_α subunits are composed of two domains. A GTPase domain contains the guanine nucleotide binding pocket, as well as sites for binding receptors, effectors and the $G_{\beta\gamma}$ subunit (reviewed in Neer, 1995). The function of the second domain is not clear, but this helical domain is thought to play a role in defining the specificity of receptor interactions in some G proteins. G proteins are classified according to their G_α subunits. There are 23 distinct members of the G_α family encoded by 17 genes, and they are divided into four sub-families based on amino acid sequences (G_α subunits share ~45-95% amino acid similarity) and shared intracellular effector molecules (Strathmann *et al.*, 1989; Strathmann and Simon, 1991). The $G_{\alpha s}$ class (αs, αolf) stimulate adenylate cyclase isoforms. There are numerous members of the $G_{\alpha i/o/t}$ class (Giα1-3, αz, αo1/2, αt, αgust), which function to down-regulate a number of G protein-coupled signal transduction pathways. The $G_{\alpha q}$ class of G_α subunits (αq, α11, α14, α15/16) activate the β isoforms of PLC and non-receptor tryrosine kinases of the Btk family. The fourth class of G_α subunits, the $G_{\alpha 12/13}$ subunits, regulate low molecular weight G proteins of the Rho family and the Na^+H^+ exchanger (Freissmuth *et al.*, 1999). Thus, G_α subunits can exhibit opposing activities upon effectors (adenylate cyclase is stimulated by $G_{\alpha s}$ and inhibited by $G_{\alpha i}$).

The G_β and G_γ subunits also exist in multiple forms (reviewed in Clapham and Neer, 1993). Six highly conserved G_β subunits and twelve more divergent G_γ subunits can associate to form multiple tightly bound heterodimers (though they do not pair indiscriminately), providing further specificity. The G_γ moieties are more divergent than G_β subunits (Cali *et al.*, 1992), and are thought to have significant influence on the specificity of $G_{\beta\gamma}$ interactions with receptor molecules within the

heterotrimeric G protein. $G_{\beta\gamma}$ subunits have also been implicated in the recruitment of GRKs to the membrane (Touhara *et al.*, 1994). The G_{β} N-terminal region is thought to form an amphipathic alpha-helix (Lupas *et al.*, 1992) followed by seven repeating units of ~43 amino acids each (Simon *et al.*, 1991). These are an example of the WD repeats found in a family of proteins involved in signal transduction, cell division, and transcription. The G_{γ} subunit is predicted to be largely alpha-helical (Lupas *et al.*, 1992).

Many G protein subunits are expressed ubiquitously (Strathmann and Simon, 1991; Kaziro *et al.*, 1991), while others have differential spatial and/or temporal expression patterns. G proteins can exist at cellular locations distinct from the plasma membrane, where they may be functional and involved in important cellular processes (reviewed in Willard and Crouch, 2000). There is emerging evidence that serpentine receptors exist in the nucleus and are coupled to G proteins (Rubins *et al.*, 1990; Crouch, 1991). There is also experimental evidence demonstrating direct association of G proteins with the cytoskeleton (Rasenick and Wang, 1988) and Golgi membranes (Stow *et al.*, 1991; Maier *et al.*, 1995). Heterotrimeric G proteins are reviewed in Neer (1995), Hamm and Gilchrist (1996) and Offermans and Simon (1996).

There is evidence that G proteins can be activated independently of receptor stimulation (Cismowski *et al.*, 2001). AGS proteins (Activators of G protein Signalling) activate G protein signalling in the absence of a typical receptor, and appear to do so by a number of different mechanisms.

1.2.3.1. GTPase activity of the Gα subunit

G proteins act like molecular switches – signalling is activated when GDP is exchanged for GTP on the G_{α} subunit, which is catalysed by agonist binding to the cognate receptor. The G_{α} and/or $G_{\beta\gamma}$ subunits are then able to modulate the activity of downstream effector molecules. Signalling is turned off when the bound GTP is hydrolysed to GDP.

The slow intrinsic GTPase activity of G_{α} subunits hydrolyses the bound GTP to GDP, with the consequence that the G_{α} and $G_{\beta\gamma}$ subunits reassociate, re-forming

the inactive heterotrimer (Gilman, 1987). Thus, the lifetime of activation depends upon the rate at which GTP is hydrolysed to GDP.

Upon G_α activation, a structural change in the heterotrimer affects the guanine nucleotide affinity of the G_α subunit, where it preferentially binds GTP and Mg^{2+} over GDP. The nucleotide-binding site of G_α is composed of three distinct 'switch' regions, which undergo considerable conformational change upon GTP hydrolysis (Wall *et al.*, 1995; Lambright *et al.*, 1996). As a result of the formation of the G_α-GTP-Mg^{2+} complex, modifications in the structure of the three switch regions facilitate the dissociation of G_α from $G_{\beta\gamma}$. Termination of the signal results when the process is reversed by the hydrolysis of the bound GTP molecule. Reassociation of G_α with $G_{\beta\gamma}$ then occurs, which results in the inactivation of the G protein. The duration and strength of the G protein signal is therefore dependent on the GTPase activity of the G_α protein.

Different G_α subunits have different intrinsic rates of GTP hydrolysis (Carty *et al.*, 1990; Linder *et al.*, 1990), which can affect which pathway predominates if a GPCR couples to more than one G protein.

The slow rate of GTP hydrolysis by G_α subunits *in vitro* is inconsistent with signal inactivation rates *in vivo* (Kurachi, 1995; Arshavsky *et al.*, 1998), and this inconsistency has been resolved by the recent discovery of proteins that accelerate G_α GTP hydrolysis. RGS proteins (Regulators of G protein Signalling) act as GTPase-activating proteins (GAPs) for G_α subunits (Section 1.3.).

1.2.3.2. G_α and $G_{\beta\gamma}$ as signal propagators

Both the G_α-GTP and $G_{\beta\gamma}$ moieties of the activated G protein can go on to modulate the activity of downstream effector molecules (Logothetis *et al.*, 1987). G_α-GTP and $G_{\beta\gamma}$ transmit signals via interactions with downstream effector proteins, and their activities persist until the intrinsic GTPase activity of G_α returns the G_α subunit to the GDP-bound state. Reassociation of $G_{\beta\gamma}$ with $G_{\alpha\text{-GDP}}$ obscures critical effector contact sites, thereby terminating effector activation (Gilman, 1987; Conklin and Bourne, 1993).

The GTP-bound G_α subunit is known to interact with and regulate a wide variety of second messenger enzymes and ionic channels (reviewed in Birnbaumer, 1992; Clapham and Neer, 1993; Gautam *et al.*, 1998).

The $G_{\beta\gamma}$ subunit (reviewed in Clapham and Neer, 1993; Gautam *et al.*, 1998) was originally thought to be the inactive membrane-anchoring partner in the G protein, negatively regulating the activity of the G_α subunit. Recently however, the importance of $G_{\beta\gamma}$ subunits in transmitting signals to downstream effectors has become acknowledged (Gautam *et al.*, 1998; Vanderbeld and Kelly, 2000), and they are no longer viewed as mere negative regulators of the G_α subunit. The first evidence of this was the finding that $G_{\beta\gamma}$ can activate the muscarinic K^+ channel (Logothetis *et al.*, 1987). It has subsequently been shown that $G_{\beta\gamma}$ dimers act as regulators of a large number of effectors, including adenylate cyclase, PLCβ2, β-ARK and GIRK1, as well as voltage-gated Ca^{2+} channels, and brain Na^+ and K^+ channels (Jing *et al.*, 1999). A recently discovered function of the $G_{\beta5}$ subunit is its ability to bind to G_γ-like (GGL) domains in proteins, including RGS proteins (Kovoor *et al.*, 2000).

1.2.4. Adaptation in G protein-coupled signalling pathways

Integral to all signal-response systems is the ability of a cell to recover from agonist stimulation in order for that cell to respond to subsequent stimulation. In the absence of a signal there is no requirement for the pathway to be activated, and indeed it can be detrimental to the cell. In the continued presence of agonist the cell becomes less responsive to agonist stimulation over time, and this phenomenon is termed desensitisation, or recovery. Desensitisation to therapeutic drugs is a major factor that limits their efficacy and duration of action. Cells have both short-term and long-term regulatory mechanisms that limit the duration of G protein signalling. Adaptation may exist at all levels; the receptor, G protein, downstream second messengers and targets themselves (Figure 2).

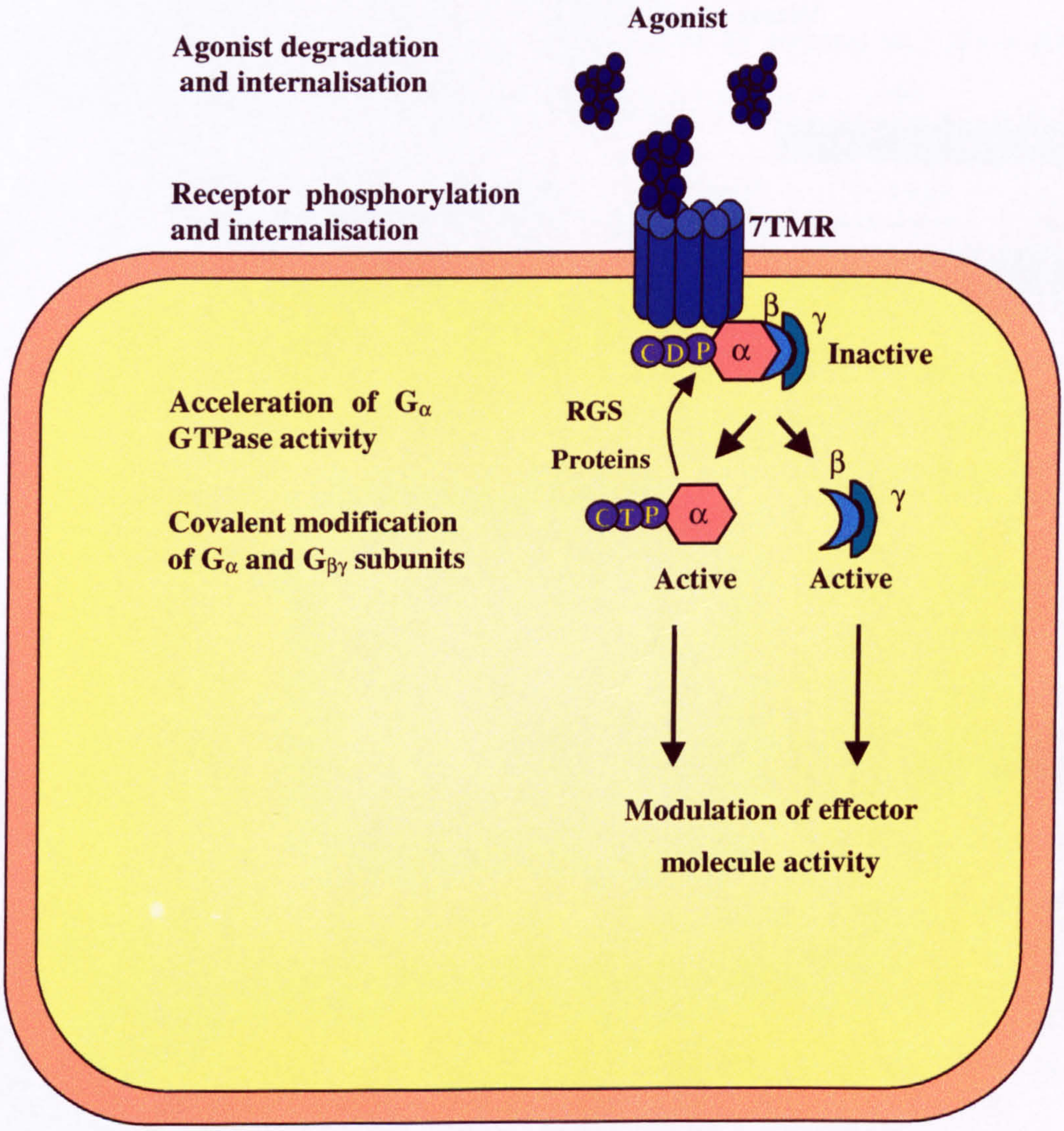


Figure 2. Adaptation in G protein signalling pathways

In order to adapt to persistent stimulation and reset cells for future stimulation, adaptation mechanisms exist at each stage in G protein-coupled signalling pathways. These mechanisms dampen signalling by inactivating or uncoupling signalling proteins.

1.2.4.1. Adaptation at the signal level

Removal or inactivation of the agonist before it binds to its cognate receptor reduces the extracellular concentration of active agonist, and reduces the number of cell surface receptors activated. Agonists may be removed by internalisation via cell surface transporters (for example in the removal of neurotransmitters, reviewed in Atwell and Mobbs [1994]). Inactivation of the agonist by secreted enzymes is also a method of adaptation at the signal level (reviewed in Ladds *et al.*, 1996).

1.2.4.2. Adaptation at the receptor level

The second level of desensitisation in G protein signalling pathways lies at the level of G protein-coupled receptors (GPCRs). Mechanisms exist that desensitise GPCRs to stimulation both in the short term and long term, enabling cells to recover from both acute and chronic stimulation of G protein-coupled signalling pathways. Several mechanisms exist that regulate both the duration and strength of GPCR signals, depending on the receptor and cellular background.

In the short term, receptors may undergo either homologous desensitisation (reduced responsiveness to the original stimulus) or heterologous desensitisation (reduced responsiveness as a result of stimuli acting through other receptors). Both mechanisms are associated with receptor phosphorylation and a subsequent impairment of G protein coupling. Homologous desensitisation of GPCRs is initiated by agonist-dependent phosphorylation. G protein-coupled receptor kinases (GRKs) specifically phosphorylate agonist-activated receptors, leaving non-activated receptors unaffected (reviewed in Lefkowitz, 1993; Bünemann and Hosey, 1999). Second messenger-dependent kinases (Hausdorff *et al.*, 1990; Chuang *et al.*, 1996) carry out heterologous desensitisation of GPCRs, and activation of these kinases by any pathway is sufficient to cause GPCR phosphorylation.

GRK-dependent desensitisation has been investigated most thoroughly for the visual receptor rhodopsin (Kuhn, 1974) and the β2-adrenergic receptors (Parruti, 1993; Krupnick and Benovic, 1998; Lefkowitz, 1998). Termination of the active state for βAR and rhodopsin has been shown to be mediated by agonist- or light-dependent phosphorylation, respectively, of the receptor by GRKs.

Phosphorylation of GPCRs by GRKs promotes the binding of β-arrestin proteins (reviewed in Ferguson *et al.*, 1996), which uncouple the receptor from its cognate heterotrimeric G protein, preventing further interactions (Lohse *et al.*, 1990). The binding of β-arrestin also targets the receptor for endocytosis. The phosphorylated, arrestin-bound receptor is targeted for internalisation and endocytosis (Koenig and Edwardson, 1997; Zhang *et al.*, 1999), by virtue of the ability of β-arrestins to interact with clathrin (Goodman *et al.*, 1996; Zhang *et al.*, 1997). Arrestin- and clathrin-dependent internalisation is a dynamin-dependent event (Zhang *et al.*, 1996), but other pathways may involve arrestin-and/or clathrin-independent events (Lee *et al.*, 1998; Vogler *et al.*, 1998). Multiple pathways of GPCR internalisation exist, and the best understood is GRK and arrestin-dependent. The relative contributions of phosphorylation and β-arrestin binding to the desensitisation mechanism may vary in different systems (Richardson *et al.*, 1993).

Following internalisation and endocytosis, sequestered GPCRs are degraded or recycled back to the plasma membrane as fully functional receptors (Zhang *et al.*, 1997). The binding of β-arrestins is also required for dephosphorylation of receptors and subsequent resensitisation to their pre-ligand exposed state (Sibley *et al.*, 1986). Resensitisation to agonist is necessary to reset the cell for future stimulation, and the association of β-arrestin with GPCRs during clathrin-mediated endocytosis dictates the profile of receptor resensitisation (Oakley *et al.*, 1999). The interaction of β-arrestin with a specific motif in the GPCR C-terminal tail dictates the rate of receptor dephosphorylation, recycling and resensitisation (Oakley *et al.*, 1999). The bound β-arrestin protein may dissociate from the receptor at the plasma membrane (in the case of the β_2-adrenergic receptor) or be internalised with the receptor into endosomes (in the case of the vasopressin V2 receptor [Oakley *et al.*, 1999]). Thus, β-arrestins are required not only for desensitisation of receptors, but also for initiating processes responsible for receptor resensitisation.

Phosphorylation of GPCRs by other kinases does not necessarily lead to arrestin binding (Lohse *et al.*, 1992), and the cellular trafficking of β-arrestin proteins is differentially regulated by the activation of distinct GPCRs (Zhang *et al.*, 2000).

An emerging view is that phosphorylation and/or β-arrestins can also initiate a new set of signalling pathways in addition to blocking those mediated by G protein activation (Hall *et al.*, 1999). In this view, binding induces a switch in receptor signalling from classical second messenger-generating G protein mediated pathways to other pathways such as those involving Src and leading to the activation of MAP kinases (Pierce *et al.*, 2001).

Long-term desensitisation, following the persistent elevation of agonist, is characterised by a reduction in the total number of cell surface receptors (Sleight *et al.*, 1995), as well as the attenuation of GPCR-G protein interactions. Following agonist stimulation, receptors are rapidly uncoupled from their cognate G protein and sequestered from the cell surface. There is decreased mRNA and protein synthesis as well as an increased rate of receptor degradation (Hausdorff *et al.*, 1990). In order for the number of cell surface receptors to be recovered, *de novo* protein synthesis is required.

1.2.4.3. Adaptation at the G protein level

Besides their intrinsic GTPase activity, several mechanisms suppress downstream signalling by G_α. Since $G_{\beta\gamma}$ inhibits guanine nucleotide dissociation, assembly of the inactive heterotrimeric protein inhibits G_α nucleotide exchange. The intrinsic GTPase activity of the G_α subunit of G proteins provides a slow mechanism for adaptation.

A recently discovered family of proteins has been identified that negatively regulate the activity of the active G_α subunit, and these proteins are collectively known as RGS proteins (Regulators of G protein Signalling). RGS proteins regulate G_α activity by accelerating the GTPase activity of the G_α subunit. This is achieved by stabilisation of the transition state conformation of the G_α subunit in the GTPase cycle, and effectively lowers the activation energy required for GTP hydrolysis (Tesmer *et al.*, 1997; Srinivasa *et al.*, 1998b). This family of proteins will be

discussed in Section 1.3. By turning off G_α subunits, RGS proteins also lead to reduced concentrations of $G_{\beta\gamma}$ dimers available for effector modulation. It has been routinely observed that the G_α-GTP GTPase activity *in vitro* is slower than that observed *in vivo*. The basis for this anomaly has been discovered recently to be due to the action of RGS proteins. The prototype RGS protein (SST2) was identified in the budding yeast *Saccharomyces cerevisiae* (Chan & Otte, 1982a). Following the isolation of SST2 the number of RGS protein family members has expanded to include members in fungi (FlbA in *Aspergillus nidulans*), nematodes (EGL-10 in *Caenorhabditis elegans*), mice, rats and humans. RGS proteins (reviewed in Ashavsky & Pugh, 1998; Kehrl, 1998) negatively regulate G protein signalling (Huang *et al.*, 1997), serving as GTPase-activating proteins (GAPs) for specific G_α sub-families (the $G_{i\alpha}$, $G_{q\alpha}$ and $G_{o\alpha}$ sub-families).

A third regulatory mechanism at the level of the G protein is covalent modification of the G_α and $G_{\beta\gamma}$ subunits (reviewed in Casey, 1994; Chen and Manning, 2001). G_α and $G_{\beta\gamma}$ subunits are subject to several kinds of co- and post-translational covalent modifications, consisting of lipid modifications and/or phosphorylation. It is lipid modifications such as palmitoylation, myristoylation and isoprenylation that are thought to be involved in conferring membrane localisation. In many cases phosphorylation of G_α subunits changes localisation or association with other proteins as opposed to changing activity (Koch *et al.*, 1995).

Myristoylation at an N-terminal glycine is a co-translational lipid modification for members of the $G_{\alpha i}$ family, and is necessary for membrane attachment and facilitates the binding of the $G_{\beta\gamma}$ subunit (Jones *et al.*, 1990). N-terminal myristoylation is irreversible, and apparently does not serve a regulatory role. Palmitoylation is a post-translational modification that occurs for these and most other G_α subunits. Palmitoylation is reversible, and this offers another level of regulation at the G protein level. One or both of these lipid modifications is required for plasma membrane targeting, and they contribute to regulating the strength of the G_α interaction with the $G_{\beta\gamma}$ dimer, effectors and RGS proteins. G_α subunits, including those with transforming activity, are often inactive when unable to be modified with lipids (Gallego *et al.*, 1992; Jones and Gutkind, 1998).

G_γ subunits are post-translationally modified by farnesyl or geranylgeranyl moieties (Fukada *et al.*, 1990). Lipid modifications are thought to target the proteins to membranes. G_γ subunits differ from each other in the prenyl group modifying the C-terminal cysteine (reviewed in Casey, 1994), and while prenylation is not necessary for dimer formation, it is necessary for membrane attachment of the $G_{\beta\gamma}$ dimer, and for association with G_α subunits (Iniguez-Lluhi *et al.*, 1992).

1.2.5. G protein signalling pathways and disease

Aberrant G protein signalling has been associated with a number of pathological conditions, including immune dysfunction, congestive heart failure (Milano *et al.*, 1994; Zou *et al.*, 1999), hypertension (Anand-Srivastava, 1996) and cancer. Deregulation of adaptation mechanisms plays an important role in many of these diseases.

A variety of diseases are ascribed to constitutive activity of GPCRs caused by naturally occurring mutations (Leurs *et al.*, 1998). The importance of the physiological roles of GPCRs is supported by studies using GPCR knockout animals (Rohrer and Kobilka, 1998), and their link with hereditary diseases (Stadel *et al.*, 1997).

Activating mutations in $G_{\alpha s}$, $G_{\alpha i2}$ and $G_{\alpha 12}$ have also been correlated with different types of tumours. Almost all known G_α subunits have been inactivated by gene targeting in mice (reviewed in Offermanns and Simon, 1998; Offermans, 2001), and the effects range from the central nervous system, development, the immune system, heart, sensory systems, and platelets. Mice deficient in $G_{\alpha i2}$ display growth retardation and develop a lethal diffuse colitis with clinical and histopathological features closely resembling ulcerative colitis in humans, including development of adenocarcinoma of the colon (Rudolph *et al.*, 1995).

Recent studies indicate that the asynchronous activation of G proteins can lead to the oncogenic transformation of different cell types (reviewed in Dhanasekaran *et al.*, 1998; Radhika and Dhanasekaran, 2001). No mutation of the $G_{\beta\gamma}$ subunit has been associated with disease as yet, but the role played by $G_{\beta\gamma}$ dimers means that deregulation of their activity could potentially contribute to disease states.

1.3. RGS proteins

1.3.1. Overview

The prototype member of the RGS protein family, SST2, was isolated as a negative regulator of the pheromone response in *S. cerevisiae* (Chan and Otte, 1982a; Chan and Otte, 1982b). Members have since been found in fungi, nematodes, and at least 27 mammalian RGS proteins have been discovered (DeVries *et al.*, 2000).

RGS proteins are characterised by a homologous, conserved alpha-helical domain of approximately 120 residues (the RGS domain, or RGS box) that binds G_α subunits and is responsible for their GAP (GTPase-activating protein) activity towards GTP-bound G_α subunits. RGS proteins negatively regulate G protein-coupled signalling pathways by accelerating the intrinsic GTPase activity of the active G_α subunit (Berman *et al.*, 1996; Apanovitch *et al.*, 1998; Snow *et al.*, 1998), driving the reformation of the inactive heterotrimeric G protein.

RGS proteins vary in size, but can be roughly classified into small and large RGS proteins. The former class (including RGS1, RGS2 and RGS4) possess short N- and C-termini flanking their RGS domain, and the latter class (including RGS9 and RGS12) possess longer N- and C-termini that contain additional structural and functional motifs. Sequences outside the RGS domain show great diversity, and contribute to specificity of RGS action, linking them to other signalling networks.

RGS proteins modulate signalling pathways in diverse cell functions, including proliferation, differentiation (Nishizuka *et al.*, 2001), response to neurotransmitters (Jeong and Ikeda, 2000), membrane trafficking (Sullivan *et al.*, 2000) and embryonic development (Wu *et al.*, 2000). RGS proteins are widely expressed, at least one is expressed in every organ, and many tissues express multiple RGS proteins.

1.3.2. SST2 is the prototype RGS protein

The prototype member of the RGS family, SST2, was first identified following genetic screens for negative regulators of the *S. cerevisiae* pheromone response (Chan and Otte, 1982a; Chan and Otte, 1982b). Stimulation of responsive cells with pheromone activates a G protein-linked protein kinase cascade, ultimately leading to G_1 cell cycle arrest and morphological differentiation (Bardwell *et al.*, 1994; Herskowitz, 1995). Cells desensitise to pheromone after approximately 2h of continuous stimulation, resume growth, and return to their pheromone-responsive state. Genetic studies have shown that SST2 is important in the desensitisation process (Dietzel and Kurjan, 1987; Weiner *et al.*, 1993; Dohlman *et al.*, 1996). In the absence of SST2, both *S. cerevisiae* mating types are hypersensitive to pheromone (Dietzel and Kurjan, 1987), and are able to respond to 100-fold lower levels of pheromone compared to wild-type cells. Cells lacking SST2 are also unable to recover from pheromone-induced cell cycle arrest. SST2 is expressed at a low constitutive level, and is transcriptionally induced by pheromone stimulation following a time course that parallels that of desensitisation. SST2 is capable of inhibiting the pheromone response even after reaccumulation of cell surface receptors (Dietzel and Kurjan, 1987). The low constitutive level of SST2 expression is thought to limit sensitivity of naïve yeast to pheromone, and induction of SST2 activity is thought to be responsible for desensitisation to pheromone. SST2 has been shown to be the GAP (GTPase-activating protein) for the *S. cerevisiae* pheromone response G_α subunit, GPA1 (Apanovitch *et al.*, 1998; Dohlman *et al.*, 1996), and has also been shown to undergo proteolytic processing (Hoffman *et al.*, 2000).

1.3.3. Identification of RGS proteins in *A. nidulans* and *C. elegans*

Following the identification of SST2 in *S. cerevisiae*, structural and functional homologues were identified in the fungus *Aspergillus nidulans* and the nematode *Ceanorhabditis elegans*. In *A. nidulans,* the protein FlbA inhibits signalling through the G_α subunit FadA, to regulate cell proliferation and development (Lee and Adams, 1994). FlbA is an early regulator of *A. nidulans* asexual sporulation, and its overexpression leads to activation of BrlA (a regulator of development) and premature initiation of development.

In *C. elegans*, the EGL-10 protein regulates signal transduction pathways that govern egg laying and motility (Koelle and Horvitz, 1996), which are stimulated by G_q signalling and inhibited by G_o signalling (Hajda-Cronin *et al.*, 1999). EGL-10 regulates the *C. elegans* G_α subunit GOA-1 (a homologue of the mammalian $G_{o\alpha}$ proteins).

SST2, FlbA and EGL-10 all contain the conserved RGS domain, although in SST2 and EGL-10 this domain is interrupted by non-conserved insertions, and are quite distantly related to the RGS domains of higher eukaryotes. EGL-10 and human RGS proteins possess a single conserved block of approximately 120 residues.

1.3.4. Discovery of mammalian RGS proteins

Following the isolation of SST2, FlbA and EGL-10, mammalian proteins were quickly discovered that possess the conserved RGS domain.

GAIP (G_α Interacting Protein) was cloned following a two-hybrid screen for $G_{\alpha i}$-interacting proteins (DeVries *et al.*, 1995). RGS1 was identified following screens for B-lymphocyte-specific genes activated in chronic lymphocytic leukaemia cells (Hong *et al.*, 1993), and RGS2, RGS3 and RGS4 were soon discovered (Siderovski *et al.*, 1994; Druey *et al.*, 1996).

The identification and characterisation of human RGS proteins has been aided by their ability to function in heterologous systems, notably the pheromone response in *S. cerevisiae*. This has enabled mammalian proteins to be screened to determine whether they can functionally substitute for SST2 when expressed in *S. cerevisiae* (Druey *et al.*, 1996). Expression of certain mammalian RGS proteins blunts signal transduction through the pheromone response pathway in *S. cerevisiae*, indicating that like SST2, they negatively regulate the pheromone pathway.

All mammalian RGS proteins contain an uninterrupted conserved RGS domain, and some RGS proteins share unique structural features, suggesting the existence of particular RGS subgroups. Zheng (1999) proposed the division of the RGS family into six sub-families, based on the phylogenetic analysis of the RGS domain primary sequences of 62 mammalian and invertebrate RGS proteins. Over 25 mammalian RGS proteins containing the diagnostic RGS domain have been identified to date, which regulate a broad range of G protein-coupled signalling

pathways (reviewed in DeVries, 2000). Mammalian RGS proteins express differential expression patterns, and possess diverse additional functions that link them to other signalling pathways.

1.3.5. Mechanism of RGS GAP activity

Resolution of the crystal structure of rat RGS4 complexed with $G_{i\alpha1}$-Mg^{2+}-GDP-AlF_4^- by Tesmer *et al.* (1997) provided important information concerning the structure of RGS4, and the mechanism of RGS activity. Only the core domain of RGS4 is visible in the crystal, and it forms a four-helix bundle that directly contacts the $G_{i\alpha}$ surface at three 'switch' regions that undergo the greatest conformational change during the GTPase cycle (Sprang, 1997). On binding to $G_{i\alpha1}$, RGS4 reduces the flexibility of all three switch regions. These G_α switch residues contain specific residues critical for GTP hydrolysis, which are intimately associated with the binding and hydrolysis of GTP, and they interact with the most highly conserved regions of RGS4. The interface between $G_{i\alpha1}$ and RGS4 is rich in electrostatic and hydrogen-bonding interactions (Tesmer *et al.*, 1997), and RGS4 appears to accelerate hydrolysis of GTP by stabilising the transition state conformation of switch regions of $G_{i\alpha1}$ through non-covalent interactions.

The possibility of the conserved Asn-128 playing a catalytic role (by interacting with the hydrolytic water molecule or the side chain of $G_{i\alpha1}$ Gln-204) has been proposed, but there is little evidence to suggest that RGS4 offers direct catalytic assistance to GTP hydrolysis (Natochin *et al.*, 1998).

The higher affinity of RGS proteins for the GDP-AlF_4^- complex of G_α than the GTPγS-bound form (Berman *et al.*, 1996) supports the hypothesis that RGS proteins act by stabilising the transition state of G_α in the GTPase cycle.

The NMR solution structure of free RGS4 suggests a significant conformational change upon binding $G_{i\alpha1}$ (Moy *et al.*, 2000), comprised of reorganisation of the packing of the N-terminal and C-terminal helices. There is a significant difference in the secondary structure between the $G_{i\alpha1}$-bound and free forms of RGS4 within the C-terminal helical regions α6 and α7. Moy *et al.* (2000)

suggest a two-stage process composed of a binding and locking step from comparison of the free and bound forms of RGS4.

No RGS proteins have as yet been identified to act as GAPs for $G_{\alpha s}$ subunits, and this has been proposed to be due to structural incompatibility.

1.3.5.1. Mutational analysis of RGS proteins

Mutagenic analyses of RGS4 have shed light on the importance of specific residues within the RGS domain. The mutation of 2 residues (N88 and L159), which, based on the crystal structure of RGS4 complexed with $G_{i\alpha 1}$-GDP-AlF4-, directly contact $G_{i\alpha 1}$ essentially abolished RGS4 binding and GAP activity (Druey and Kerhl, 1997). Two other mutations, one of a contact residue (R167) and the other an adjacent residue (F168) also significantly reduced RGS4 binding to the active G_{α} complex, but in addition directed binding toward the GTPγ-S-bound form. These two mutants had impaired GAP activity, but in contrast to others, behaved as RGS antagonists. These results are consistent with hypothesis that the predominant role of RGS proteins is to stabilise the G_{α} transition state for GTP hydrolysis.

1.3.6. RGS domains in other proteins

A small collection of proteins contain somewhat less conserved RGS domains than RGS proteins. The RGS domain at N-terminal regions of the GPCR kinase, GRK2, specifically interacts with $G_{\alpha q/11}$ and inhibits $G_{\alpha q}$-mediated activation of PLCβ (Carman *et al.*, 1999), suggesting that a subfamily of the GRKs may be bifunctional regulators of GPCR signalling operating directly on both receptors and G proteins. As well as the well-documented role in GPCR desensitisation, GRKs may also regulate signalling at the level of the G_{α} subunit directly.

A newly identified GEF for Rho GTPase, p115RhoGEF (Hart *et al.*, 1996), has an RGS domain at its N-terminus and this domain acts as a specific GAP for $G_{\alpha 12}$ and $G_{\alpha 13}$ (Kozasa *et al.*, 1998; Hart *et al.*, 1998). Thus, p115RhoGEF is a direct link between heterotrimeric G proteins and Rho GTPase, functioning as an effector for $G_{\alpha 12}$ and $G_{\alpha 13}$ in addition to acting as their GAP.

An RGS domain is also present in the protein kinase A (PKA) anchoring protein D-AKAP2 (Huang *et al.*, 1997). AKAPs are adaptor proteins that can assemble signalling complexes at the plasma membrane, including PKA, calcineurin and PKC. D-AKAP2 can bind to two subtypes of regulatory subunits of PKA (PKA anchor), and contains an N-terminal RGS domain for which a G_α target has not yet been identified. G protein signalling pathways and cAMP are linked directly via activation or inhibition of adenylate cyclase isoforms by $G_{\alpha s}$ and $G_{\alpha i}$ respectively, and D-AKAP2 appears to provide an additional link, via PKA anchoring and G_α binding.

1.3.7. Links to other signalling pathways

Many RGS proteins have been found to serve as links to other cellular signalling pathways through non-RGS domains such as DEP, GGL, PDZ and DH/PH domains (DeVries, 2000).

RGS6, RGS7, RGS9, and RGS11 have N-terminal DEP (Dishevelled homology/EGL-10/pleckstrin homology) domains. The DEP domain of the Drosophila protein Dishevelled is responsible for its localisation to membranes. The DEP domain of RGS9 may thus be responsible for its tight membrane association (Cowan *et al.*, 1998; He *et al.*, 1998), although it is not clear whether its DEP domain can regulate membrane association directly or through interaction with other proteins. This family of RGS proteins also have GGL (G_γ-like) domains with homology to the G_γ subunit, which form dimers with the $G_{\beta 5}$ subunit (the least homologous of the G_β subunits) (Sondek and Siderovski, 2001). RGS6, RGS7 and RGS11 complexed with $G_{\beta 5}$ have GAP activity specific for G_o. Analysis of native $G_{\beta 5}$-RGS dimers and their coupled expression argue that *in vivo*, $G_{\beta 5}$ and GGL-containing RGS proteins only exist as heterodimers (Witherow, 2000). Dimers do not bind G_α with very high affinity, but they can still inhibit G protein signalling.

PDZ domains bind to consensus C-terminal motifs in target proteins (Craven and Bredt, 1998), and are involved in the clustering of signalling molecules - an important role in organising protein networks on membranes. RGS12 is the largest RGS protein identified so far, and contains an N-terminal PDZ domain and a C-

terminal PDZ binding domain, although the *in vivo* targets are unknown (Snow *et al.*, 1997; Snow *et al.*, 1998). Results from yeast two-hybrid and *in vitro* interactions have revealed that the chemokine IL-8 receptor B (CXCR-2) can specifically interact with the PDZ domain of RGS12 (Snow *et al.*, 1998), suggesting that RGS12 might be an important scaffold molecule for components of G protein-linked chemokine signalling pathways. RGS12 also contains a C-terminal serine/proline rich coiled-coil structure that could interact with cytoskeleton proteins, and a PID/PTB domain that suggests interaction with a tyrosine-phosphorylated protein (Snow *et al.*, 1998; Mao *et al.*, 1998).

The F-subfamily of RGS proteins is typified by the p115-Rho GEF (Guanine nucleotide Exchange Factor) (Kozasa *et al.*, 1998). Rho GEF bears DH and PH domains C-terminal to a $G_{\alpha 12/13}$-specific RGS domain. Rho GEF not only accelerates $G_{\alpha 12/13}$-GTPase activity, but also acts concomitantly as a G_{α}-effector, since the binding of the RGS domain to G_{α}-GTP stimulates the guanine-nucleotide exchange activity of the DH/PH tandem directed toward the monomeric G-protein Rho (Hart *et al.*, 1998).

RGS12 and RGS14 are presumed to play a role in coordinating cross-talk between heterotrimeric and Ras-superfamily G proteins, as they possess putative Ras-binding and novel G_{α}-binding (GoLoco) domains (Siderovski *et al.*, 1999).

1.3.8. Regulation of RGS activity

The large number of RGS proteins identified in higher eukaryotes requires that their activity must be regulated in some way. Human RGS proteins differ in their expression pattern, subcellular localisation, and interaction with other signalling or regulatory proteins to give them distinct biological functions. Regulation of RGS activity is thought to be achieved via a number of mechanisms, including G_{α} subunit selectivity, intracellular localisation, differential expression, covalent modification and links with other signalling pathways.

1.3.8.1. G_α specificity of RGS proteins

RGS proteins exhibit preferences for the G_α subunits they regulate. For example, RGS2 exhibits a preference for $G_{\alpha q}$ over $G_{\alpha i}$ (Heximer *et al.*, 1997), an effect that is thought to be mediated by unique structural features of its G_α subunit binding interface. Other RGS proteins are more promiscuous towards G_α subunits, for example RGS4 (Diversé-Pierluissi *et al.*, 1999; Cavalli *et al.*, 2000; Rogers *et al.*, 2001).

1.3.8.2. Intracellular localisation of RGS proteins

The intracellular localisation of a number of RGS proteins has been studied by a number of groups. RGS2-GFP localises to the nucleus, plasma membrane and cytoplasm of HEK293 cells (Heximer *et al.*, 2001). Expression of activated G_q increased RGS2 association with the plasma membrane and decreased accumulation in the nucleus, suggesting signal-induced redistribution may regulate RGS2 function. The RGS2 N-terminus is necessary and sufficient for plasma membrane localisation, and contains an amphipathic helix that directs binding to anionic lipid surfaces. This alpha-helical motif is also present in other RGS proteins (RGS3, RGS4, and RGS16). Srinivasa *et al.* (1998a) showed that plasma membrane localisation is required for RGS4 function in *S. cerevisiae*. RGS4 mutants lacking the N-terminal 33 residues were non-functional and unable to localise to the plasma membrane. Localisation to the plasma membrane was restored by addition of a C-terminal membrane targeting sequence to RGS4. At the other extreme, RGS10 is a soluble protein found in the cytosol. RGS3, RGS4, and RGS16 display intermediate behaviour. RGS proteins are thought to localise in the cytoplasm, nucleus or shuttle between the two sites (Chatterjee and Fisher, 2000).

1.3.8.3. Site-specific expression of RGS proteins

Tissue- and cell-specific expression may also play an important role in regulating the function of RGS proteins. RGS1 expression is restricted to B-lymphocytes (Moratz *et al.*, 2000). Region-specific regulation of RGS4 by stress and glucocorticoids has been demonstrated in the brain (Ni *et al.*, 1999.).

1.3.8.4. Alternative splicing of RGS mRNA

The mRNA of a number of the larger RGS proteins is subject to alternative splicing. RGS9 mRNA is alternatively spliced to produce two site-specific RGS proteins (Zhang *et al.*, 1999). The RGS9-1 isoform is found exclusively in the vertebrate visual transduction cascade where it serves as a GAP for the transducin G_{α} subunit ($G_{t\alpha}$). RGS9-2 is a striatal-enriched isoform. Multiple alternatively spliced isoforms of RGS12 have also been identified (DeVries, 2000).

1.3.8.5. Covalent modification of RGS proteins

Covalent modification of a number of RGS proteins has been demonstrated, and fatty acylation is a possible mechanism for the regulation of their activity. One study with GAIP suggests that palmitoylation may be involved in the subcellular localisation and membrane trafficking of GAIP (DeVries *et al.*, 1995, DeVries *et al.*, 1996).

G_{α} subunits are known to be membrane associated, and cytosolic $G_{\alpha s}$ and $G_{\alpha i1}$ translocate to membranes from a cytosolic pool, a process regulated by palmitoylation and myristoylation (Degtyarev, 1994; Wedegaertner, 1996). All G_{α} are myristoylated at an N-terminal glycine or palmitoylated at an internal cysteine, or both. Several RGS proteins are palmitoylated (GAIP, RGS4, RGS16) at their N-terminus, which has been implicated in sub-cellular localisation and membrane association (DeVries *et al.*, 1996; Srinivasa *et al.*; 1998a, Druey *et al.*, 1999). RGS4 is palmitoylated, with Cys-2 and Cys-12 likely sites (Srinivasa *et al.*, 1998a). RGS1, RGS2, and RetRGS also contain cysteine strings that are probable sites for multiple palmitoylation.

Phosphorylation may also serve a regulatory role. RGS9-2 contains several potential phosphorylation sites for protein kinase C, PKA, PKG, tyrosine kinases and casein kinase II. RGS10 activity is regulated through specific and inducible phosphorylation by cAMP-dependent kinase A (PKA). The phosphorylation-mediated attenuation of RGS10 activity correlates with translocation of RGS10 from the plasma membrane and cytosol into the nucleus, as opposed to reducing its GAP activity (Burgon, 2001.).

1.3.8.6. Links with other signalling pathways

The presence of functional motifs within RGS proteins is likely to present a mechanism of regulating RGS activity through links with other signalling pathways (Section 1.3.7). The GAP activity of RGS9 is uniquely potentiated by the G_γ subunit of the effector enzyme cGMP-phosphodiesterase (P_γ) (McEntaffer *et al.*, 1999). In contrast, P_γ attenuates the effects of several other RGS proteins, including RGS16. The mechanism of potentiation of RGS9 GAP activity by P_γ is thought to involve a more rigid stabilisation of the $G_{t\alpha}$ switch regions when $G_{t\alpha}$ is bound to both RGS9 and P_γ.

1.3.9. Implications for disease

Aberrant G protein signalling has been associated with a number of pathological conditions, and the fact that RGS proteins modulate G protein signalling at the G protein level means that altered levels of RGS activity could contribute to pathological states. The majority of information regarding RGS proteins and disease comes from cardiac tissue culture and animal models.

Zhang *et al.* (1998) have shown that RGS3 and RGS4 gene expression is enhanced in two model systems of cardiac hypertrophy, while RGS3 and RGS4 mRNA levels were reduced in failing myocardium. This indicates that RGS proteins may play an important role in regulating G protein signalling in cardiac tissue. Increased $G_{\alpha i}$ protein signalling due to decreased RGS protein activity may contribute to the observed reduction in myocardial contractility in association with heart failure. There is evidence that altered RGS gene expression may contribute to the pathogenesis of cardiac hypertrophy and failure (Rogers et al., 1999), as expression patterns of RGS proteins in cardiac tissue is altered in pathophysiological states and in response to cardiomyocyte dissociation (Zhang *et al.*, 1998; Kardestuncer *et al.*, 1998).

RGS proteins may also be involved in the development of pathophysiological conditions that display alterations in signalling from multiple GPCRs, such as rejection of transplanted organs (Owen *et al.*, 2001) and sepsis (Panetta *et al.*, 1999).

The ability of RGS proteins with altered activity to modulate G protein signalling has been shown by a number of groups. Dominant gain-of-function mutants of SST2 in *S. cerevisiae* block the normal pheromone response, conferring a pheromone-resistant phenotype (Dohlman *et al.*, 1995). The ability to express a hyperactivated RGS protein capable of normalising the level of G_i protein signalling may prove to be beneficial in diseases associated with aberrant G protein signalling. No hyperactivated mammalian RGS protein has yet been isolated.

The ability to modulate protein signalling pathways will undoubtedly be of great clinical significance for any disease in which abnormal levels of signalling contribute to disease pathology. The ubiquitous nature of G protein signalling pathways within intracellular signalling networks means that a diverse array of diseases and conditions could be favourably treated (among them cardiac disease, cancer, and immune and neurological dysfunction) through the expression of RGS proteins with altered activity.

1.4. The *Schizosaccharomyces pombe* pheromone communication pathway

1.4.1. Overview

The fission yeast *Sz. pombe* is used as a simple eukaryotic model to investigate a number of cellular functions, including cytokinesis and signal transduction pathways. The tractability and genetic amenability of *Sz. pombe* enables the consequences of genetic manipulations to be rapidly determined, and enables the consequences of both dominant and recessive mutations to be observed.

The *Sz. pombe* pheromone-responsive signalling pathway is closely related to G protein signalling pathways of more complex eukaryotes, and the simplicity of the *Sz. pombe* pheromone communication pathway means that it can be used to study heterogeneous signalling proteins without the additional complexities present in higher eukaryotic systems. The budding yeast *S. cerevisiae* is also used as a model system to study a large variety of cell functions, including signalling through its pheromone signalling pathway. The *Sz. pombe* system presents another model, which may be more suitable for studying specific signalling proteins, as it is the G_α subunit

that propagates the signal in *Sz. pombe*, in contrast to *S. cerevisiae*, in which the $G_{\beta\gamma}$ subunit propagates the pheromone signal.

1.4.2. Life cycle of *Sz. pombe*

Sz. pombe exists predominately as a haploid organism, replicating by mitotic cell division to produce two identical daughter cells. Sexual activity is repressed during mitotic growth, but when unfavourable conditions are encountered (i.e. nutrient starvation), cells of each mating-type secrete peptides that induce changes in the opposite mating-type to prepare them for mating (reviewed in Davey, 1998). Pheromone signalling results in the expression of proteins responsible for controlling conjugation, meiosis and sporulation, including the pheromone factors and receptors themselves. Expression and release of pheromone factors is initiated following nutrient starvation, and their binding and subsequent intracellular signal transduction induces G_1 arrest in pheromone-responsive cells (Imai and Yamamoto, 1994; Davey and Nielsen, 1994). This cytostatic activity ensures conjugative partners contribute equal amounts of DNA to the zygote. A morphological consequence of pheromone stimulation in responsive cells is that cells continue to grow during the G_1 arrest (Davey, 1991; Davey and Nielsen, 1994), and elongate unidirectionally towards the pheromone source to form an shmoo (Fukui *et al.*, 1986; Leupold, 1987). Two haploid conjugants bind and fuse at the tips of the shmoos, forming the zygote (Egel, 1971; Miyata *et al.*, 1997). Zygotes arrest transiently at the G_1 phase under nutritional starvation, then initiate premeiotic DNA synthesis and proceed through meiosis I and II, resulting in four haploid, encapsulated nuclei that develop to form mature spores. If transferred to rich medium immediately following conjugation, zygotes can grow as diploids.

Two major signal transduction pathways regulate sexual development in *Sz. pombe*, one responding to nutritional conditions and the other to the mating pheromones.

1.4.3. *Sz. pombe* exists in two different mating types

Sz. pombe cells exist as one of two mating types, M (*M*inus) or P (*P*lus). Heterothallic strains are fixed as either M or P cells, while homothallic strains change their mating type approximately once every three generations (Egel, 1973).

The presence of one of two alternative DNA segments at the active mating type locus, *mat1*, specifies the mating-type of the haploid cell (Willer *et al.*, 1995; Yamamoto, 1996). The mating type locus, denoted *mat1-P* for P cells, and *mat1-M* for M cells, consists of two divergently transcribed genes (Egel, 1973; Kelly *et al.*, 1988), termed *mat1-Pc* and *mat1-Pi* (*mat1-Pm*) and *mat1-Mc* and *mat1-Mi* (*mat1-Mm*). Each segment encodes two mating type-specific functions that are required to produce the mating pheromones, pheromone receptors and a product required for meiosis. The Mat-1Pc function produces the P-factor pheromone and the M-factor receptor, while the Mat1-Mc function produces the M-factor pheromone and the P-factor receptor. The expression of these functions provides the means for the cells to initiate communication. The *mat1-Pi* and *mat1-Mi* loci are specifically required for meiosis. The product of *mat1-Mi* is an HMG-family protein, and the product of *mat1-Pi* is a homeobox protein. The P cell and M cell mating type information is also stored at the *mat2* and *mat3* loci respectively, but these are silent and are not expressed (Egel, 1973; Allshire, 1995). Homothallic *Sz. pombe* strains switch mating type as a result of transformation of the material stored at the silent *mat2* and *mat3* loci to the active *mat1* locus.

Secreted pheromones are recognised by their cognate receptors expressed on the cell surface of the opposite mating type. Both pheromone receptors contain seven transmembrane domains, and are coupled to a heterotrimeric G protein. Pheromone binding leads to activation and dissociation of the coupled G protein, and the activated GTP-bound G_α subunit (Gpa1p) activates a downstream protein kinase cascade. Pheromone signalling results in the eventual stimulation of a transcription factor necessary for the expression of genes required to bring about the changes necessary for conjugation.

1.4.4. The *Sz. pombe* pheromones

The *Sz. pombe* pheromones and pheromone receptors are expressed and secreted following nutrient starvation, setting up the pheromone communication system.

P cells respond to M-factor by expressing Map3p (Tanaka *et al.*, 1993), the M-factor receptor. M cells express the P factor receptor, Mam2p (Kitamura and Shimoda, 1991).

1.4.4.1. M-factor

M-factor, produced and secreted by M cells, is encoded by three functionally redundant genes showing strong sequence similarity (*mfm1*, *mfm2*, and *mfm3*). Each of the M-factor genes encodes a precursor containing a single copy of the mature M-factor peptide sequence within it (Davey, 1992, Kjaerulff *et al.*, 1994). Any one of the *mfm* genes is able to produce sufficient M-factor to allow mating to occur (Kjaerulff *et al.*, 1994). The *mfm* genes are only expressed in M cells, and expression is induced under conditions that initiate sexual development in *Sz. pombe*. The *mfm* genes are also induced upon stimulation of M cells with P-factor. M-factor binds to M-factor receptors (encoded by *map3*) on the surface of P cells.

M-factor is a lipophilic nonapeptide, whose C-terminal cysteine residue is carboxymethylated and S-farneysylated (Davey, 1991; Davey *et al.*, 1998). The biosynthesis of mature M-factor involves a number of processing steps. The C-terminus is farnesylated via a thioether linkage, the C-terminal tripeptide is cleaved and the exposed carboxyl group undergoes methyl esterification. The M-factor N-terminal extension is also proteolytically processed. These steps generate the mature pheromone ready for export, and M-factor is transported directly across the plasma membrane by an ATP-dependent transporter belonging to the ABC (ATP binding cassette) superfamily of proteins (Christensen *et al.*, 1997).

1.4.4.2. P-factor

P-factor is a hydrophilic, unmodified peptide of 23 amino acids, encoded by the structural gene *map2* (Imai and Yamamoto, 1994). The *map2* gene product contains four repeats of 23 amino acids flanked by basic residues. Two of these

repeats have identical sequences, but the other two differ from them in 2 and 3 amino acids. The precursor encoded by *map2* contains an N-terminal presequence that targets the protein to the endoplasmic reticulum, and is removed during translocation of the precursor across the ER membrane (Waters *et al.*, 1988; Davey *et al.*, 1994). Further processing occurs in the Golgi body, where the propheromone is split into its individual subunits by an endopeptidase (Krp1p), that cleaves on the C-terminal side of a pair of basic residues (Lys-Arg). Following N- and C-terminal trimming of the subunits, the mature pheromone is released into the medium. Unlike M-factor, P-factor is secreted via the conventional secretory pathway (Davey *et al.*, 1994).

1.4.5. The *Sz. pombe* pheromone communication pathway

Nutrient starvation (more specifically, nitrogen starvation) is a requirement for the initiation of pheromone communication in *Sz. pombe*, and results in a decrease in the intracellular cAMP level. A recently identified gene, *stm1*, has been proposed to function as a sentinel molecule sensing the nutritional state of the cells (Chung *et al.*, 2001). This reduction in the intracellular cAMP concentration acts as a signal for the induction of nitrogen-starvation responsive genes, including those required for mating, meiosis and sporulation (Fukui *et al.*, 1986; Maeda *et al.*, 1990; Mochizuki and Yamamoto, 1992). The G_α homologue, Gpa2p, transmits the nutritional signal to the cAMP cascade. The reduction in cAMP levels that result from nitrogen starvation can be mimicked by disruption of the *cyr1* gene, encoding adenylate cyclase (Davey and Nielsen, 1994). Cells lacking *cyr1* are highly derepressed for sexual development, and can initiate mating and meiosis in nutrient-rich environments. Expression of nitrogen-starvation responsive genes depends upon the function of the *ste11* gene product, whose activity is repressed by high levels of cAMP. The *ste11* gene product is a key transcription factor regulating sexual development in *Sz. pombe*, and is a DNA-binding protein of the high-mobility group (HMG) family. Pheromone-induced genes carry between one and five copies of a *cis* element named the TR box (TTCTTTGTTY) in their 5' non-coding region, which provides a binding site for Ste11p (Sugimoto *et al.*, 1991). The *ste11* gene itself possesses TR-box motifs in its 5' non-coding region that appear to be essential for its expression, and are likely to represent a positive feedback loop. The expression of

mat1-Pc and *mat1-Mc* is dependent upon Stellp activity, and their expression enables cells to communicate via the mating pheromones.

Following binding of pheromone factors to their respective heptahelical receptors on the surface of the cell, the pheromone signal is transmitted to a heterotrimeric G protein coupled to the pheromone receptors (Figure 3). The pheromone receptor-coupled G protein and the downstream signalling pathway are common to both mating types. The G_α subunit is encoded by *gpa1*, and its disruption abolishes the pheromone response (Obara *et al.*, 1991). A G_β subunit is encoded by *gpb1* (Kim *et al.*, 1996), but it is not known whether it associates with Gpa1p (the pheromone pathway G_α subunit) or Gpa2p (the cAMP response pathway G_α subunit). No G_γ subunit has been identified yet.

Once activated by the pheromone receptor, the G_α subunit Gpa1p (Obara *et al.*, 1991), acting in concert with Ras1p, activates a protein kinase cascade that is homologous to MAPK pathways in mammalian cells. Ras1p (a small monomeric G protein related to the mammalian oncoprotein Ras) recruits the MAPKK kinase Byr2p (Wang *et al.*, 1991) to the plasma membrane. Activated Byr2p phosphorylates the downstream MAPK kinase Byr1p (Nadin-Davis and Nasim, 1990), which in turn phosphorylates the MAPK Spk1p (Gotoh *et al.*, 1993). Byr2p is thought to be maintained in an inactive conformation through an inhibitory interaction between its N-terminal regulatory domain and the C-terminal catalytic domain (Tu *et al.*, 1997). The MAPK Spk1p is essential for propagating the pheromone signal, and presumably phosphorylates one or more target proteins. The best candidate for a pheromone-responsive transcription factor is Ste11p, which is a crucial regulator of the mating and meiotic pathway. Activation of Ste11p enables it to induce the expression of pheromone-responsive genes.

1.4.6. Adaptation in the pheromone communication pathway

In common with mammalian cells, desensitisation mechanisms exist within the *Sz. pombe* pheromone signal transduction pathway, that enable responsive cells to desensitise to pheromone even in its continued presence. The G_1 cell cycle arrest caused by pheromone stimulation is transient, and stimulated cells recover and

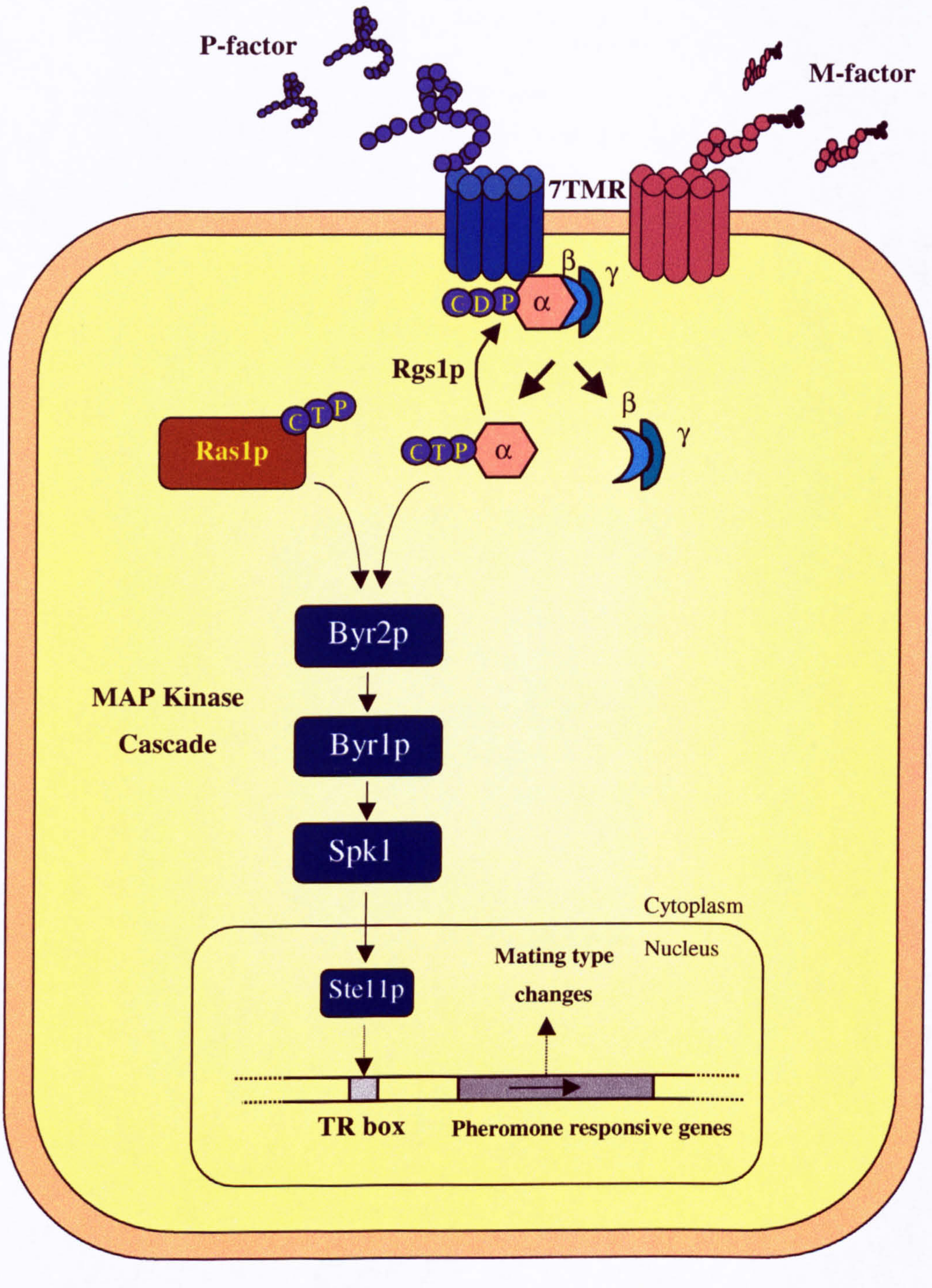


Figure 3. Pheromone communication in *Sz. pombe*

Pheromone receptors are activated by pheromone binding, and in turn activate the G_α subunit, Gpa1p. GTP-bound Gpa1p, in conjunction with Ras1p, activates a protein kinase cascade comprised of Byr2p, Byr1p and Spk1p. Subsequent activation of the transcription factor, Ste11p, results in the expression of genes required for mating.

resume vegetative growth in several hours. Adaptation to pheromone stimulation enables cells that have not mated to inactivate components of the pheromone signalling pathway activated during the initial response, and to resume growth (Davey and Nielsen 1994).

One aspect of the desensitisation process is the degradation of extracellular pheromone. Removal of the pheromone before it binds to cells is the first level of adaptation. M cells express and secrete Sxa2p, a serine carboxypeptidase (Imai and Yamamoto, 1994; Ladds *et al.*, 1996) that degrades extracellular P-factor by removing the C-terminal leucine residue. Sxa2p expression is induced by P-factor stimulation, and thus constitutes a negative feedback mechanism promoting recovery in the absence of a mating partner. Sxa2p is not produced by P cells or unstimulated M cells.

A second adaptation mechanism in the pheromone communication system is likely to be the phosphorylation and internalisation of pheromone receptors. *S. cerevisiae* pheromone receptors are rapidly phosphorylated following pheromone stimulation, and following ubiquitination, the receptors undergo vacuolar degradation (reviewed in Riezman, 1998). *Sz. pombe* pheromone receptors are likewise phosphorylated following stimulation (Chen and Konopka, 1996). The internalisation and recycling mechanisms of *Sz. pombe* pheromone receptors have not yet been elucidated.

At the G protein level, the *Sz. pombe* RGS protein Rgs1p accelerates the GTPase activity of the GTP-bound G_α subunit, Gpa1p. Cells lacking *rgs1* are hypersensitive to pheromone stimulation by both pheromone factors (Watson *et al.*, 1999; Pereira and Jones, 2001) and are sterile, reinforcing the importance of adaptation mechanisms in the pheromone signalling pathway. The *rgs1* gene is a target of Ste11p, possessing three TR-boxes. Like Sxa2p, Rgs1p acts in a negative feedback loop, as it is expressed in response to pheromone signalling and then acts to down-regulate cellular sensitivity towards pheromone.

Covalent modification of G_α subunits has been reported to be a mechanism of regulating their activity in mammalian cells, and it is possible that this also occurs for the *Sz. pombe* G_α subunit. G_α subunits in mammalian cells are myristoylated at an N-terminal glycine residue or palmitoylated at an internal cysteine residue, or

both (Wedegaertner *et al.*, 1995). Fatty acylation of G_α subunits is proposed to be a requirement for their localisation at the plasma membrane, and a mutant of the *S. cerevisiae* G_α subunit that could not be myristoylated was excluded from the plasma membrane (Song and Dohlman, 1996). Regulation of G_α activity via fatty acylation in *Sz. pombe* could be an effective mechanism for controlling transduction of the pheromone signal to the downstream MAPK cascade. Phosphorylation of the *S. cerevisiae* G_β subunit has been reported (Leberer *et al.*, 1992), but it is not known if any of the *Sz. pombe* G protein subunits are regulated by phosphorylation.

As the components of the protein kinase cascade are activated via phosphorylation, each enzyme of the protein kinase cascade could be subject to regulation by phosphatases. While phosphatases have been reported for other protein kinase cascades in *Sz. pombe*, none have been reported that act specifically upon the MAPK cascade responsible for transducing the pheromone signal. The tyrosine-specific Pyp1p and Pyp2p phosphatases dephosphorylate the stress-activated Sty1p MAPK (Millar *et al.*, 1995), and Pmp1p encodes a MAPK phosphatase that acts upon the Pmk1p MAPK involved in chloride homeostasis (Sugiura *et al.*, 1998).

1.4.7. *Sz. pombe* reporter strains have been constructed

Reporter systems are used throughout biological research to investigate the promoter activity of genes. Reporter proteins, whose activity can be easily measured, are driven by the promoter under investigation, and their use has revolutionised the investigation of signal transduction pathways. Such reporter genes are inactive or weakly active with respect to transcription. Upon activation of the upstream promoter (as a result of signalling through the pathway under investigation) the reporter gene is transcribed, and following translation the reporter protein can be quantified by measuring its enzymatic activity.

Reporter gene assays have been developed for both *S. cerevisiae* and *Sz. pombe*, normally using bacterial β-galactosidase or a biosynthetic gene whose product is required for growth on selective media. The *LacZ* gene of *E. coli* encodes β-galactosidase, which catalyses the hydrolysis of various β-galactosides, and its activity can be easily measured using colorimetric, fluorescent and

chemiluminescent detection systems. The *Sz. pombe* auxotrophic gene *ura4* encodes orotidine-5'-monophosphate decarboxylase (Bach, 1987). Uracil is required for growth, and Ura4 activity can be positively and negatively selected using selective growth media. The generation of a temperature-sensitive version of Ura4 is described in Chapter 3.

The reporter assays described in this thesis utilise both the LacZ and Ura4 reporter proteins, driven by the *Sz. pombe sxa2* promoter (created by Dr. Mark Didmon and Dr. Kevin Davis in the Davey laboratory) (Figure 4). Activity of the *sxa2* locus is tightly regulated, and is only induced upon signalling through the pheromone communication pathway. The induction and activity of *sxa2* is used to investigate signalling through the pheromone signal transduction pathway to determine initially whether mammalian RGS proteins are active against the *Sz. pombe* G_α GTPase activity, and to screen for gain-of-function RGS mutants.

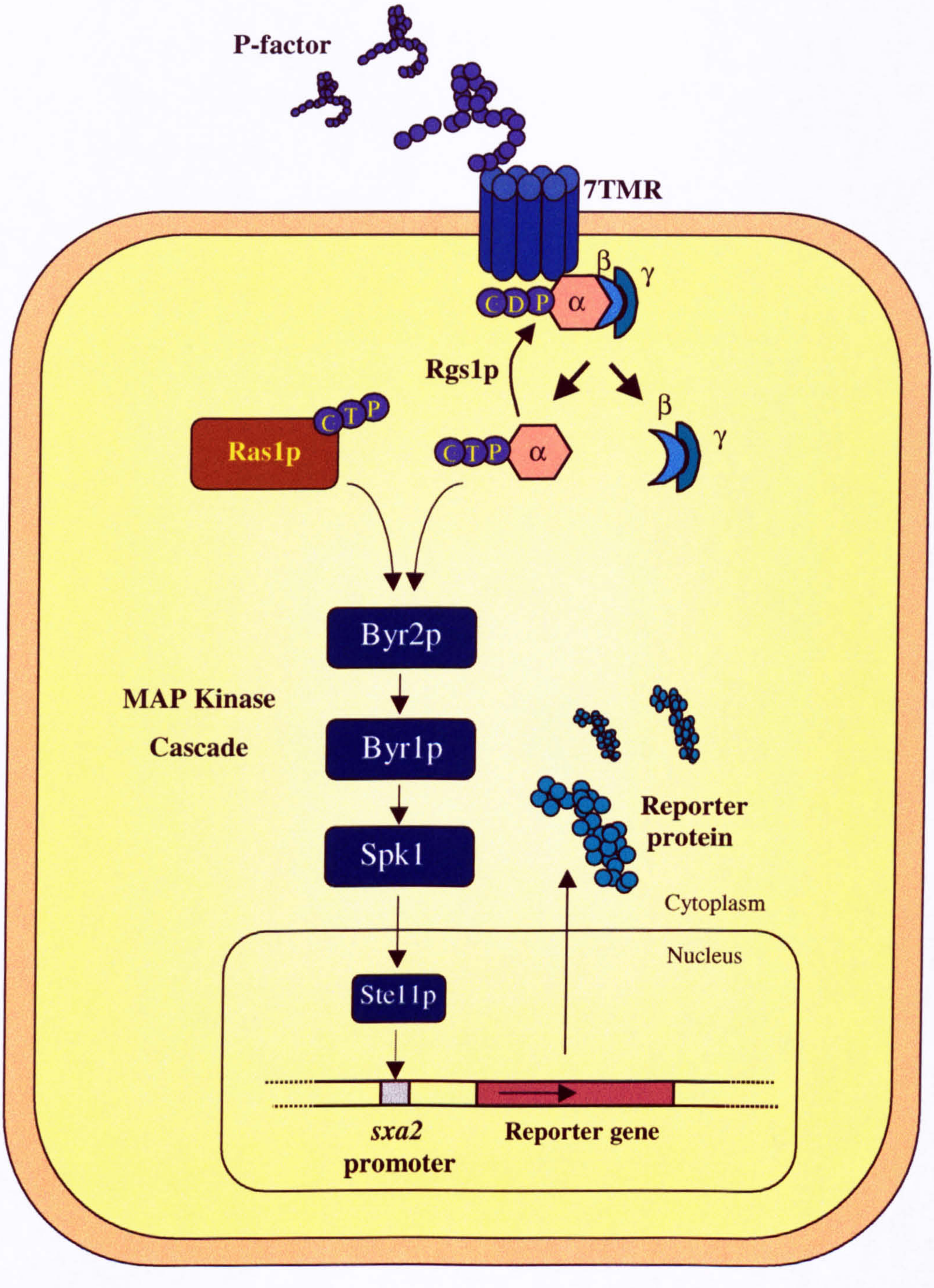


Figure 4. Pheromone-dependent expression of reporter proteins in *Sz. pombe*

In the *Sz. pombe* M cell reporter strains, a marker gene (*LacZ* or *ura4*) is driven by the pheromone-responsive *sxa2* promoter. Expression of reporter genes is dependent upon signalling through the pheromone communication pathway, and thus signalling through the pathway can be monitored by measuring reporter protein activity.

1.5. Aims of the project

To generate a temperature-sensitive *Schizosaccharomyces pombe* Ura4 protein, and to investigate the activity of RGS proteins in the *Sz. pombe* pheromone communication pathway.

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. General laboratory reagents

General laboratory reagents were supplied by the Sigma Chemical Co. (Poole, Dorset) or Merck BDH Laboratory Supplies (Poole, Dorset), and were of analytical grade unless otherwise stated.

2.1.2. Molecular biology reagents

Restriction enzymes, T4 DNA ligase, *Taq* DNA polymerase and bacterial alkaline phosphatase were supplied by Gibco BRL (Paisley, Scotland). T7 DNA polymerase and all nucleotides were supplied by Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire). *Pwo* DNA polymerase and the Random Primed DNA Labelling kit were purchased from Roche Molecular Biochemicals (Lewes, East Sussex). All oligonucleotides were synthesized by AltaBioscience (University of Birmingham, Birmingham).

2.1.3. Electrophoresis reagents

Ultrapure type-I and low melting point agarose were supplied by Gibco BRL (Paisley, Scotland). Acrylamide was supplied by Northumbria Biologicals Limited (Cramlington, Northumbria) as a 30% (w/v) solution of 37.5:1 ratio acrylamide:bisacrylamide for Western blot analysis or a 40% (w/v) solution of 19:1 ratio acrylamide:bisacrylamide for DNA sequencing gels.

2.1.4. Radioisotopes

α^{35}S-dATP (1000Ci/mmol) and α^{32}P-dCTP (6000Ci/mmol) were supplied by Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire)

2.1.5. Photographic Supplies

Kodak Biomax MR autoradiographic film was supplied by Sigma Chemical Co. (Poole, Dorset). Fuji RX autoradiographic film was purchased from Fuji Co.

(Dusseldorf, Germany). Polaroid type 667 Land film was obtained from Polaroid UK Limited (St. Albans, Hertfordshire). DNA gels were visualised using a UVP GDS 8000 gel documentation system with Grab-It software (Ultraviolet Products, Cambridge).

2.1.6. Growth Media

Luria broth, yeast extract and select agar were purchased from Gibco BRL (Paisley, Scotland). All components of Amino Acid selective medium (AA) and Defined Minimal Medium (DMM) were purchased from Sigma Chemical Co. (Poole, Dorset). Plates and liquid media for the selective growth of yeast were made using DMM or AA media. Rich (Yeast Extract) medium was used with appropriate amino acid supplements (250μg/ml) as required. Plates were made with 1.5% Select agar.

The composition of DMM has been described previously (Davey *et al.*, 1995).

Yeast Extract medium.

Per litre;

Yeast extract	5g
Glucose	30g

Selective medium (AA).

Per litre;

Yeast Nitrogen base w/o a.a.	6.7g
Glucose	20g
Amino acid mix	1.5g
Select amino acid mix	0.5g

Amino acid mix;

L-alanine	2g
L-asparagine	2g
L-cysteine	2g

L-glutamine	2g
L-glutamic acid	2g
L-glycine	2g
L-isoleucine	2g
L-lysine	2g
L-phenylalanine	2g
L-proline	2g
L-serine	2g
L-threonine	2g
L-tryptophan	2g
L-tyrosine	2g
L-valine	2g
myo-inositol	2g
para-amino benzoic acid	0.4g

Select amino acid mix (components as required);

Adenine	2g
L-histidine	2g
L-leucine	4g
L-methionine	2g
Uracil	2g

2.1.7. Coulter Channelyser Supplies

Cell numbers and cell volumes were determined using a C256 Coulter Channelyser and Isoton II azide-free electrolyte, both supplied by Coulter Electronics Limited (Luton, Bedfordshire).

2.1.8. P-Factor

P-factor pheromone was synthesized by standard solid-phase methodology using a Biotech instruments BT7300 Peptide synthesiser by AltaBioscience (University of Birmingham, Birmingham).

2.1.9. Antibodies

Rabbit anti-human RGS2 and anti-human RGS4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit horseradish peroxidase antibody was purchased from Sigma Chemical Co. (Poole, Dorset).

2.1.10. Bacterial Strain

General cloning was performed using *Escherichia coli* (*E. coli*) strain DH5 supplied by Stratagene (Cambridge). *E. coli* DH5 genotype: *supE44 hsdR17 endA96 thi-1 relA1 recA1 gyrA96.*

2.1.11. Yeast Strains

The following *Sz. pombe* strains were used or created in this study;

JY383 *mat1-P Δmat2,3::LEU2 leu1-32 ura4-D18*

JY497 *mat1-P Δmat2,3::LEU2 leu1-32 ura4-D18 irp1::ura4*$^{+}$

JY543 *mat1-P Δmat2,3::LEU2 leu1-32 ura4-D18 irp1::ura4*^{ts}

JY544 *mat1-M Δmat2,3::LEU2 leu1*$^{-}$ *ade6-M216 ura4-D18 cyr1::ura4*$^{+}$*::ura4*$^{-}$ *sxa2>lacZ*$_{ORF}$

JY603 *mat1-M Δmat2,3::LEU2 leu1*$^{-}$ *ade6-M216 ura4-D18 cyr1::ura4*$^{+}$*::ura4*$^{-}$ *sxa2>ura4*$_{ORF}$

JY606 *mat1-P Δmat2,3::LEU2 leu1-32 ura4-D18, irp1::ura4*^{ts} *prk1::ura4*$^{+}$

JY629 *mat1-M Δmat2,3::LEU2 leu1*$^{-}$ *ade6-M216 ura4-D18 cyr1::ura4*$^{+}$*::ura4*$^{-}$ *sxa2>lacZ*$_{ORF}$ *Δrgs1::ura4*$^{+}$

JY727 *mat1-M Δmat2,3::LEU*$^{-}$ *leu1*$^{-}$ *ade6-M216 ura4-D18 cyr1::ura4*$^{+}$*::ura4*$^{-}$ *sxa2>ura4*$_{ORF}$ *Δrgs1::ura4*$^{+}$

JY1153 *mat1-M Δmat2,3::LEU2 leu1*$^{-}$ *ade6-M216 ura4-D18 cyr1::ura4*$^{+}$*::ura4*$^{-}$ *sxa2>lacZ*$_{ORF}$ *rgs1::huRgs4*

JY1189 *mat1-M Δmat2,3::LEU2 leu1*$^{-}$ *ade6-M216 ura4-D18 cyr1::ura4*$^{+}$*::ura4*$^{-}$ *sxa2>lacZ*$_{ORF}$ *rgs1::rgs1*$^{Val305\rightarrow Ile}$

JY1190 *mat1-M Δmat2,3::LEU2 leu1⁻ ade6-M216 ura4-D18 cyr1::ura4⁺::ura4⁻ sxa2>lacZ$_{ORF}$ rgs1::rgs1*$^{His171→Arg}$

JY1191 *mat1-M Δmat2,3::LEU2 leu1⁻ ade6-M216 ura4-D18 cyr1::ura4⁺::ura4⁻ sxa2>ura4$_{ORF}$ rgs1::rgs1*$^{Val305→Ile}$

JY1192 *mat1-M Δmat2,3::LEU2 leu1⁻ ade6-M216 ura4-D18 cyr1::ura4⁺::ura4⁻ sxa2>ura4$_{ORF}$ rgs1::rgs1*$^{His171→Arg}$

JY1193 *mat1-M Δmat2,3::LEU2 leu1⁻ ade6-M216 ura4-D18 cyr1::ura4⁺::ura4⁻ sxa2>lacZ$_{ORF}$ rgs1::huRgs1*

JY1194 *mat1-M Δmat2,3::LEU2 leu1⁻ ade6-M216 ura4-D18 cyr1::ura4⁺::ura4⁻ sxa2>ura4$_{ORF}$ rgs1::huRgs1*

2.2. Methods

2.2.1. General molecular biology techniques

DNA manipulations were carried out using standard methods (Sambrook *et al.*, 1989) and DNA modifying enzymes were used according to manufacturers recommendations. DNA fragments were analysed by electrophoresis on 1% agarose gels stained with 0.5μg/ml ethidium bromide. DNA fragments were recovered from 0.8-1.0% low melting point (LMP) agarose gels using standard methods (Sambrook *et al.*, 1989) or using the Geneclean II kit supplied by Anachem (Luton, Bedfordshire).

2.2.2. Polymerase Chain Reaction (PCR)

2.2.2.1. Purified DNA

1μg each of sense and antisense oligonucleotide primer were added, and between 1 and 5ng template DNA used. Total reaction volume was typically 50μl. In general, a denaturation step of 94°C held for 30s was followed by a 55°C annealing step, held for 1 min. The extension step at 72°C was typically held for 1 min per 1kb of product. In general, 30 cycles of amplification were used per reaction. Reactions were concluded with a 7 min incubation at 72°C. *Taq* DNA polymerase was used for screening bacterial and yeast strains and for PCR mutagenesis. *Pwo* DNA polymerase was used for amplifying fragments for cloning.

2.2.2.2. Screening plasmid DNA from bacterial cells

A single bacterial colony was suspended in 100μl water and stored at 4°C. 1μl of this suspension was used as the template in a 10μl PCR reaction.

2.2.2.3. Screening crude preparations of yeast genomic DNA

A 10ml yeast culture was grown to mid-log phase (1-2x10^7cells/ml), and 1ml harvested via centrifugation (13,000g for 10s). Cells were washed once in 500μl 5xTE (50mM Tris pH7.5, 5mM EDTA) and the cells pelleted via centrifugation (13,000g for 10s). 450μl supernatant was removed and 0.5ml acid-washed glass

beads (425-600μm, supplied by Sigma Chemical Co., Poole, Dorset) added. The mixture was vortexed for 1 min, after which the yeast homogenate and glass beads were transferred to a 0.5ml Eppendorf tube. The bottom of the 0.5ml tube was pierced, placed inside a 1.5ml Eppendorf tube and centrifuged at 13,000g for 10s. 450μl water was added to the lysate collected in the 1.5ml Eppendorf tube. Typically, 1μl of the resulting solution was used in a 50μl PCR reaction.

2.2.2.4. Random mutagenesis using *Taq* DNA polymerase

In order to generate random mutations in DNA products using *Taq* polymerase, a modified *Taq* polymerase buffer was used: 660mM Tris-HCl, pH8.8, 66mM $MgCl_2$, 166mM $(NH_4)_2SO_4$, 100mM β-mercaptoethanol, 0.2mM dATP, 0.2.mM dCTP, 0.2mM dGTP, 2mM dTTP. Products obtained following 30 cycles of amplification (94°C for 30s, 55°C for 30s, 72°C for 1 min) contain an average of 1 error per 1Kb.

2.2.3. Double stranded DNA sequencing

2.2.3.1. Dideoxynucleotide chain-termination method

Double-stranded DNA constructs were sequenced by the dideoxynucleotide chain-termination (Sanger *et al.*, 1977).

18μl template DNA (approximately 1-2μg of 5Kb DNA) was denatured for 3 min at room temperature with 2μl 10M NaOH. 8μl 5M ammonium acetate and 100μl ethanol were then added, and DNA precipitated at –70°C for 10 min. DNA was pelleted by centrifugation at 4°C (13,000g for 10 min), washed with 80% ethanol, and resuspended in 7μl water. 1μl oligonucleotide primer (6.6ng/μl) and 2μl Sequencing buffer were added, and the solution incubated at 37°C for 20 min. Reactions were radioactively labelled by the addition of 5.5μl Labelling mix, and incubated at room temperature for 5 min. 3μl of the radioactively labelled mix were added to each of four micro-titre plate wells (purchased from Becton Dickinson, France) containing 2μl of either ddA, ddC, ddG or ddT Termination mixes, and reactions incubated for 5 min at 37°C. Reactions were terminated by the addition of 4μl Stop solution. Samples were denatured at 80°C for 2 min prior to loading on 6% polyacrylamide sequencing gels.

Sequencing buffer;	200mM Tris pH7.5, 100mM $MgCl_2$, 250mM NaCl
Labelling mix;	0.55μM each dCTP, dGTP, dTTP, 18mM DTT, 0.5U T7 DNA polymerase/μl, 1μCi [γ^{35}S-dATP (1000Ci/mmol)]μl.
Termination mix;	80μM each dATP, dGTP, dCTP, dTTP, 8μM ddNTP (required dideoxynucleotide), 50mM NaCl.
Stop solution;	95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol.

2.2.3.2. Automated cycle sequencing

Double stranded DNA was also sequenced using the ABI-PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). PCR reactions were carried out in 10μl reactions in 0.2ml thin-walled tubes. 4μl Terminator Ready Reaction Mix was added to 100ng plasmid DNA and 3.2pmole oligonucleotide primer. 30 cycles of amplification were carried out (96°C for 30s, 50°C for 15s, 60°C for 4 min), following which DNA was ethanol-precipitated and incubated at –20°C for 1h. DNA was pelleted by centrifugation at 13,000g for 30 min, before the supernatant was removed, and the pellet dried. Reactions were electrophoresed by the departmental sequencing service using ABI Prism 373 & 377 automated sequencers. Sequence chromatograms were viewed using Chromas software (v1.61; Technelysium Pty Ltd., Helensvale, Australia)

2.2.4. Bacterial transformation

2.2.4.1. Chemically competent cells

Chemically competent *E. coli* DH5 (Stratagene, Cambridge) were produced and transformed with plasmid DNA as described by Hanahan (1985).

2.2.4.2. Electrocompetent cells

Electrotransformation of electrocompetent *E. coli* was carried out using a MicroPulser electroporation system purchased from Bio-Rad (Hemel Hempstead, Hertfordshire). Electrocompetent *E. coli* were prepared and electroporated in accordance with manufacturers instructions.

2.2.5. Yeast Transformation

Circular plasmid DNA and linear DNA fragments were transformed into *Sz. pombe* using the lithium acetate method described by Okazaki *et al.* (1990).

2.2.6. Preparation of Yeast Genomic DNA

Yeast genomic DNA was prepared as described by Guthrie and Fink (1991).

2.2.7. Southern Blot Analysis

Southern blot analysis was carried out using standard methodology (Sambrook *et al.*, 1989). Digested DNA was blotted to Hybond ECL nitrocellulose purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire), and detected using α^{32}P-dCTP-labelled DNA probes prepared with the Random Primed DNA Labelling kit.

2.2.8. Western Blot Analysis

2.2.8.1. Preparation of crude yeast extracts

10ml yeast cultures were cultured at 29°C to mid-log phase (typically 1x 10^7 cells/ml) in DMM. Cells were harvested by centrifugation (1,500g for 5 min),

washed once with TEN buffer (100mM Tris pH6.8, 10mM EDTA) and transferred to a 2ml Eppendorf tube. Cells were pelleted by centrifugation at 1,500g for 5 min. All but 50µl of the supernatant was removed and 25µl 10X inhibitor stock (20mM PMSF, 20mM TLCK [Sigma Chemical Co., Poole, Dorset], 1 protease inhibitor cocktail tablet [Boerhinger Mannheim, Germany], in 1ml distilled sterile water) added. 0.5ml glass beads were added to the yeast homogenate and the tubes vortexed for 1 min, before being transferred to a 0.5ml tube. The bottom of the 0.5ml tube was pierced, and placed within a 1.5ml Eppendorf before a centrifugation step at 13,000g for 10s. 33µl sample buffer (5% SDS, 125mM Tris pH 6.8, 8M urea, ~0.02% bromophenol blue) was added to the crude yeast homogenate, and the crude extracts heated at 100°C for 5 min prior to SDS-PAGE electrophoresis. Typically, 10µl crude yeast extract was subjected to SDS-PAGE electrophoresis.

2.2.8.2. SDS-PAGE electrophoresis

SDS polyacrylamide gels were cast and run using the Bio-Rad Mini Protean II gel electrophoresis system (Hemel Hempstead, Hertfordshire) according to the manufacturers instructions.

2.2.8.3. Western Blot Analysis

Proteins separated by SDS-PAGE electrophoresis were transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire) using a Semi-Phor Transfer Dry Blotting Unit (Hoefer Scientific Instruments, San Francisco, CA). Once transfer was complete the membrane was placed into TBS (100mM Tris, 150mM NaCl) containing 4% Marvel (non-fat dried milk) for 30 min with gentle shaking. The membrane was washed twice in TTBS (TBS containing 0.3 % Tween20 (v/v)). The primary antibody was added to the membrane at the appropriate dilution (antibody specific) in blocking solution (TBS containing 2% marvel, 0.3 % Tween20), and left for 1h with gentle shaking. The membrane was washed for 3 x 5 min in TTBS. The secondary antibody was added to the membrane at a 1:10000 dilution in TTBS and left for 1h with gentle shaking. The membrane was washed for 3 x 15 min in TTBS. Proteins were detected using the ECL™ Western Blotting Detection Reagents (Amersham Pharmacia Biotech,

Little Chalfont, Buckinghamshire), which were used in accordance with the manufacturers instructions.

2.2.8.4. Silver staining of SDS-PAGE gels

Silver staining of SDS-PAGE gels was performed using the Silver Stain Plus Kit purchased from Bio-Rad (Hemel Hempstead, Hertfordshire) according to the manufacturers instructions.

2.2.9. Detection of lacZ (β-galactosidase) activity

Liquid β-galactosidase assays of *Sz. pombe* cultures were performed using a method modified from Dohlman *et al.* (1995).

Sz. pombe M cells were cultured to a density of ~$5x10^5$ cells/ml in DMM and 500µl aliquots transferred to 2ml Safe-Lock Eppendorf tubes containing 5µl P-factor pheromone (diluted in HPLC-grade methanol). Tubes were incubated at 29°C for 16h on a rotating wheel, following which 50µl were transferred to 750µl Z^+-buffer containing 2.25mM *o*-nitrophenyl-D-galactoside (ONPG; purchased from Sigma Chemical Co., Poole, Dorset) and incubated on a rotating wheel at 29°C for a further 90 min. Reactions were halted by addition of 250µl 2M Na_2CO_3, and β-galactosidase activity calculated as the ratio of ONPG product formed (Optical Density at 420nm) to assayed cells (determined using a Coulter Channelyser) using the formula $OD_{420}/10^6$ assayed cells.

Z^+-buffer; 0.1M $NaPO_4$ (pH7.0)
10mM KCl
1mM $MgSO_4$
50mM β-mercaptoethanol
0.5% (v/v) chloroform
0.005% (w/v) SDS
2.25mM ONPG

2.2.10. Detection of Ura4 (orotidine 5'-monophosphate decarboxylase) activity

Orotidine 5'-monophosphate decarboxylase (Ura4) activity was detected using plate-based viability assays (Grimm *et al.*, 1988). Cells possessing Ura4 activity were selected on AA selective agar plates lacking uracil. Cells without Ura4 activity were selected using AA selective agar plates containing uracil and 5-fluoroorotic acid (5-FOA; purchased from Toronto Research Chemicals Inc., Ontario, Canada). 5-FOA is converted into a toxic compound by Ura4 (Grimm *et al.*, 1988).

2.2.11. Hydroxylamine Mutagenesis of Double Stranded DNA

Plasmid DNA was treated with hydroxylamine using a method modified from Busby *et al.* (1982).

20μg plasmid DNA was incubated in 500μl hydroxylamine solution (1M NH_2OH, 50mM $Na_2P_2O_7$, pH7.0, 100mM NaCl, 2mM EDTA) at 75°C for 2h. DNA was precipitated with potassium acetate and propan-2-ol on ice for 30 min, and recovered by centrifugation (13,000g at room temperature for 30 min). Hydroxylamine-treated DNA was purified by LMP gel electrophoresis and the GeneClean II kit supplied by Anachem (Luton, Bedfordshire), followed by standard phenol/ethanol DNA precipitation (Sambrook *et al.*, 1989).

2.3. Generation of DNA Constructs and Yeast strains

2.3.1. Generation of *Sz. pombe ura4* constructs

Figure 5 shows the construction of the *irp1*::*ura4*^{ts} integration construct. The *ura4*^{ts} cassette was inserted between the 5' and 3' *irp1* non-coding regions as a *Bam*HI fragment. Digestion of this construct with *Pst*I and *Xba*I liberates a linear integration fragment. Figure 6 shows the construction of the *prk1*::*ura4*$^{+}$ integration cassette. The *ura4*$^{+}$ cassette was cloned between the *prk1* 5' and 3' non-coding regions as a *Bam*HI cassette. Liberation of the integration fragment was achieved by digestion of the construct with *Pvu*II. The construction of the *ura4* expression constructs is illustrated in Figure 7. The *ura4*$^{+}$ and *ura4*^{ts} open reading frames (ORFs) were amplified and cloned into the *Bam*HI restriction site of the *Sz. pombe* pREP3X and pREP81X expression vectors (Maundrell, 1990). These expression vectors contain the *nmt1* promoter upstream of the multi-cloning site. Lower expression levels are achieved with pREP81X as a result of a mutated *nmt1* promoter. Expression constructs were digested with appropriate restriction enzymes to ensure the correct orientation of the *ura4* ORFs for translation (ATG immediately downstream of the *nmt1* promoter).

2.3.2. Generation of RGS expression constructs

Figures 8-14 show the construction of RGS protein expression constructs. Typically, RGS open reading frames were amplified and cloned into the *Bam*HI site of pKS. The ORFs were then sequenced and sub-cloned into the *Bam*HI site of pREP3X. The human *Rgs3* ORF and the *Sz. pombe rgs1* C-terminus were directly cloned into the *Eco*RV site of a pREP3X derivative. Restriction digests of the expression constructs confirmed correct orientation of the ORFs for translation.

2.3.3. Generation of human RGS1 and human RGS4 integration constructs

Figure 15 and Figure 16 show the construction of human *Rgs1* and human *Rgs4* integration constructs. The human *Rgs1* and *Rgs4* ORFs were amplified using oligonucleotide primers containing *Sz. pombe rgs1* 5' and 3' non-coding sequences.

Amplified fragments were cloned into a construct containing additional *rgs1* 5' and 3' non-coding sequences to generate integration constructs in which the human *Rgs1* and *Rgs4* ORFs exactly replace the *Sz. pombe rgs1* ORF within the *rgs1* 5' and 3' non-coding regions. Restriction of the integration constructs with *Spe*I and *Nde*I liberate linear fragments for homologous integration.

2.3.4. Generation of RGS site-directed mutant constructs

Figure 17 and Figure 18 show the creation of two site-directed *rgs1* mutations. Figure 19 shows the creation of a human *Rgs4* site-directed mutation.

Site-directed RGS mutants were amplified using divergent oligonucleotide primers that introduced a specific nucleotide at the desired position in the RGS ORF. A construct containing the *rgs1* ORF flanked by its 5' and 3' non-coding regions was used as the template for creation of the *Sz. pombe rgs1* site-directed mutants. The human *Rgs4* integration construct described in Figure 16 was used as the template for creating the human *Rgs4* site-directed mutant (Figure 19). The resulting fragments were circularised to create constructs in which the mutant RGS ORF was flanked with *rgs1* 5' and 3' non-coding regions. Restriction with *Spe*I and *Nde*I liberated linear fragments for homologous integration.

All constructs were sequenced to ensure no mutations had been introduced during the amplification and cloning steps.

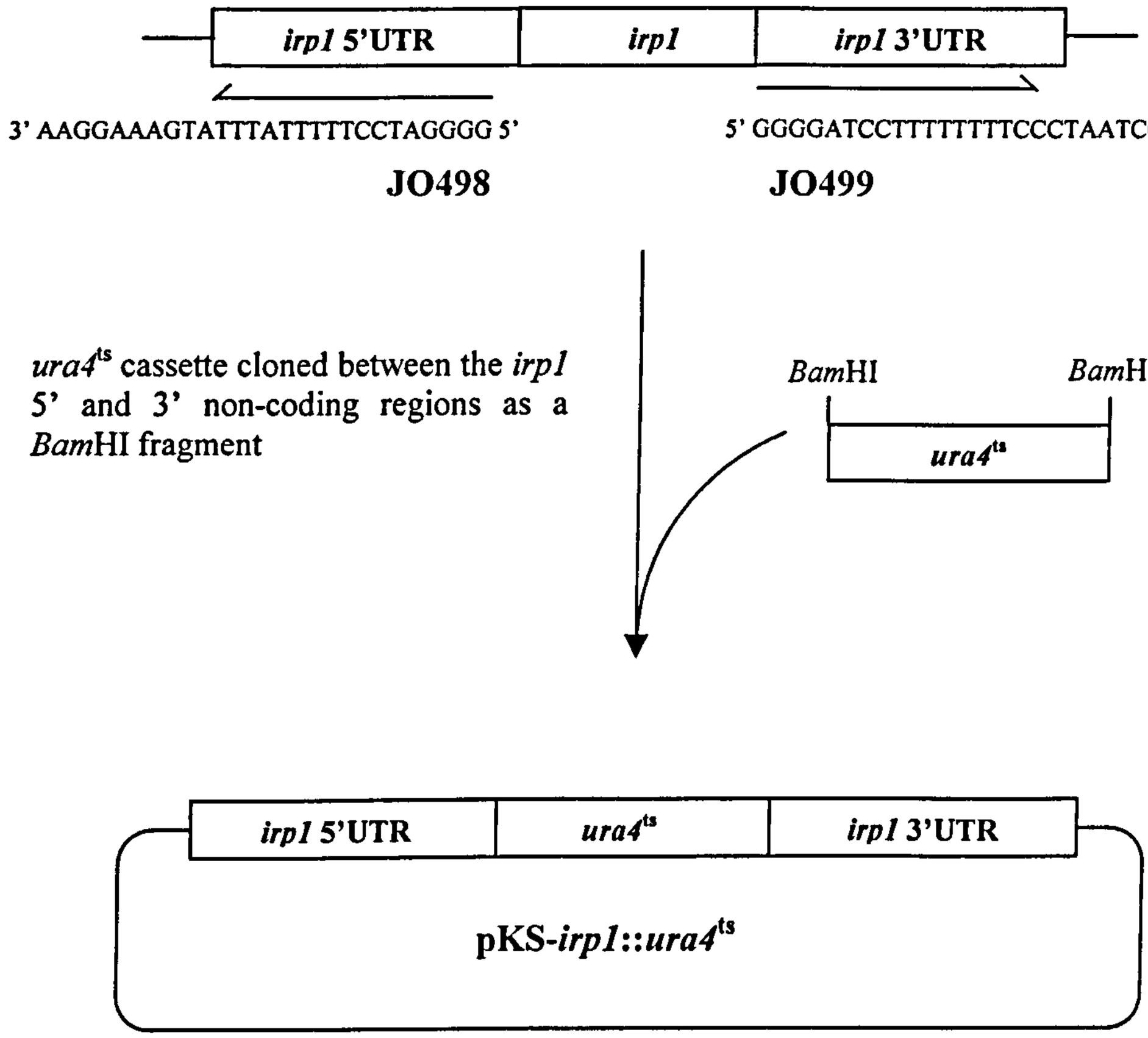


Figure 5. Generation of an *irp1*::*ura4*^{ts} disruption construct

The *irp1* 5' and 3' non-coding regions were amplified using JO498 and JO499, which contain terminal *Bam*HI sites. The *ura4*^{ts} cassette amplified from JY395 genomic DNA was cloned between the *irp1* 5' and 3' non-coding regions in pKS as a *Bam*HI fragment.

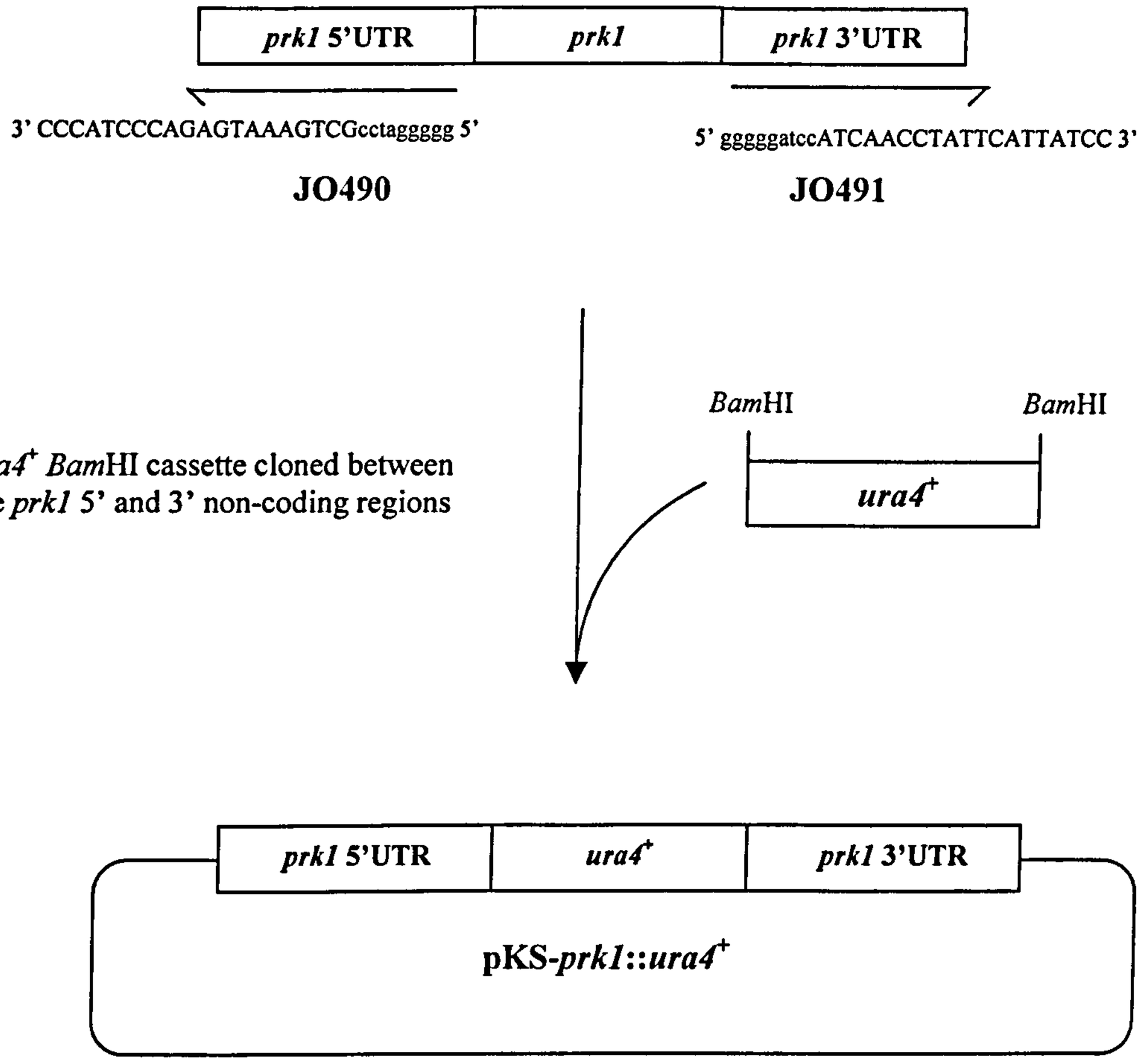


Figure 6. Generation of a *prk1*::*ura4*$^+$ disruption construct

JO490 and JO491 were used to amplify the *prk1* 5' and 3' non-coding regions in pKS. Both primers contain terminal *Bam*HI sites. The *ura4*$^+$ cassette was cloned between the *prk1* 5' and 3' non-coding regions in pKS as a *Bam*HI fragment.

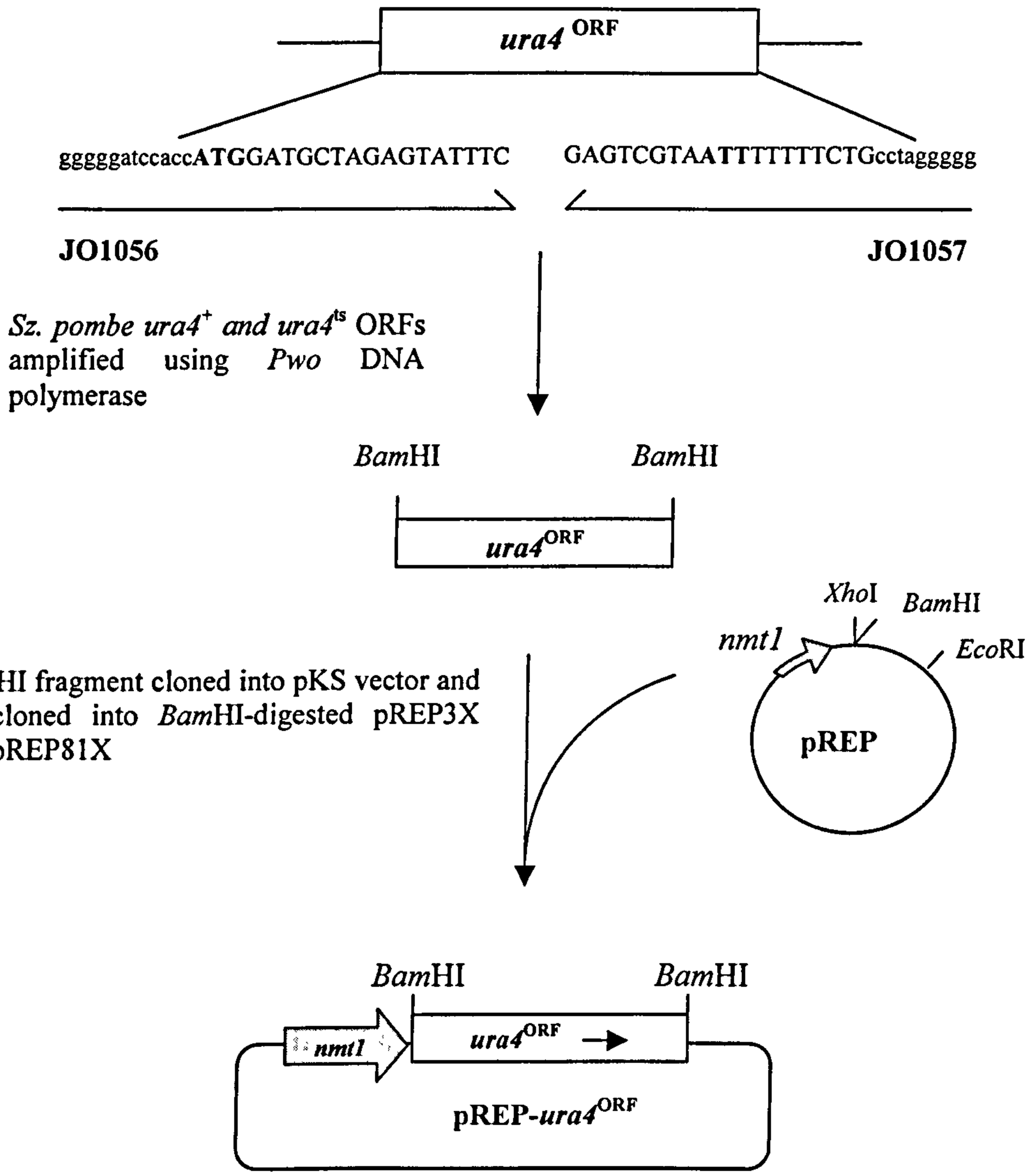


Figure 7. Creation of the *ura4*$^{+}$ and *ura4*^{ts} expression constructs

JO1056 and JO1057 were used to amplify the *Sz. pombe ura4* open reading frame from using *Pwo* DNA polymerase. JO1056 and JO1057 contain terminal *Bam*HI sites to facilitate cloning. JO1056 contains the sequence CCACC to potentially increase the efficiency of translation (Kozak, 1984; Kozak, 1986). Lower case letters indicate primer sequence not found in the *ura4* ORF.

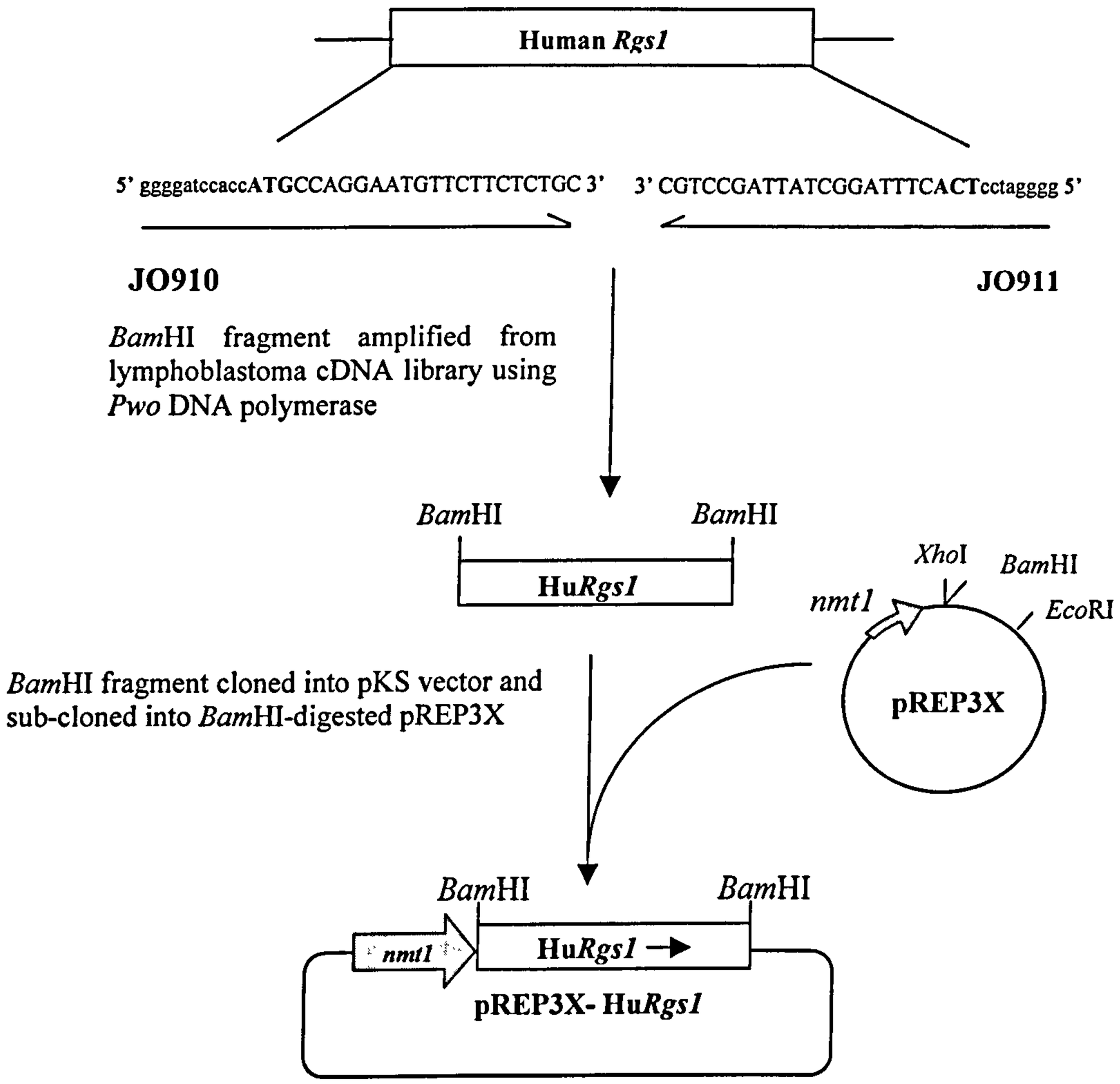


Figure 8. Creation of the human RGS1 expression construct

JO910 and JO911 were used to amplify the human *Rgs1* open reading frame from a lymphoblastoma cDNA library using *Pwo* DNA polymerase. JO910 and JO911 contain terminal *Bam*HI sites to facilitate cloning. The amplified fragment was digested with *Bam*HI and cloned into the *Bam*HI site of pKS. JO910 contains the sequence CCACC to potentially increase the efficiency of translation (Kozak, 1984; Kozak, 1986). Lower case letters indicate primer sequence not found in the human *Rgs1* open reading frame. The human *Rgs1* ORF was cloned into the *Bam*HI restriction site of pKS, sequenced, and sub-cloned into the *Bam*HI restriction site of pREP3X.

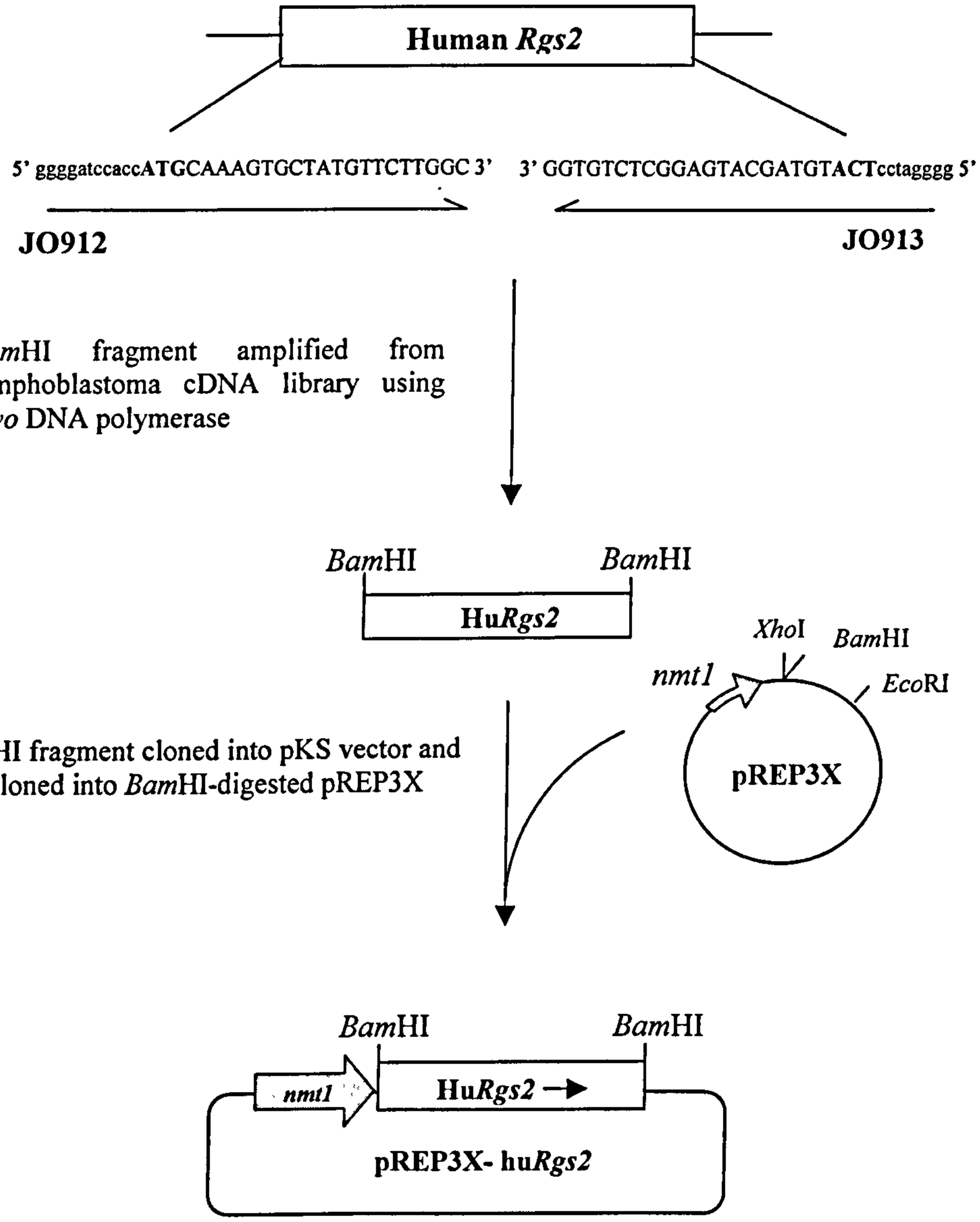


Figure 9. Creation of the human RGS2 expression construct

JO912 and JO913 were used to amplify the human *Rgs2* open reading frame from a lymphoblastoma cDNA library using *Pwo* DNA polymerase. JO912 and JO913 contain terminal *Bam*HI sites to facilitate cloning. JO912 contains the sequence CCACC to potentially increase the efficiency of translation (Kozak, 1984; Kozak, 1986). Lower case letters indicate primer sequence not found in the human *Rgs2* open reading frame. The human *Rgs2* ORF was cloned into the *Bam*HI restriction site of pKS, sequenced, and sub-cloned into the *Bam*HI restriction site of pREP3X.

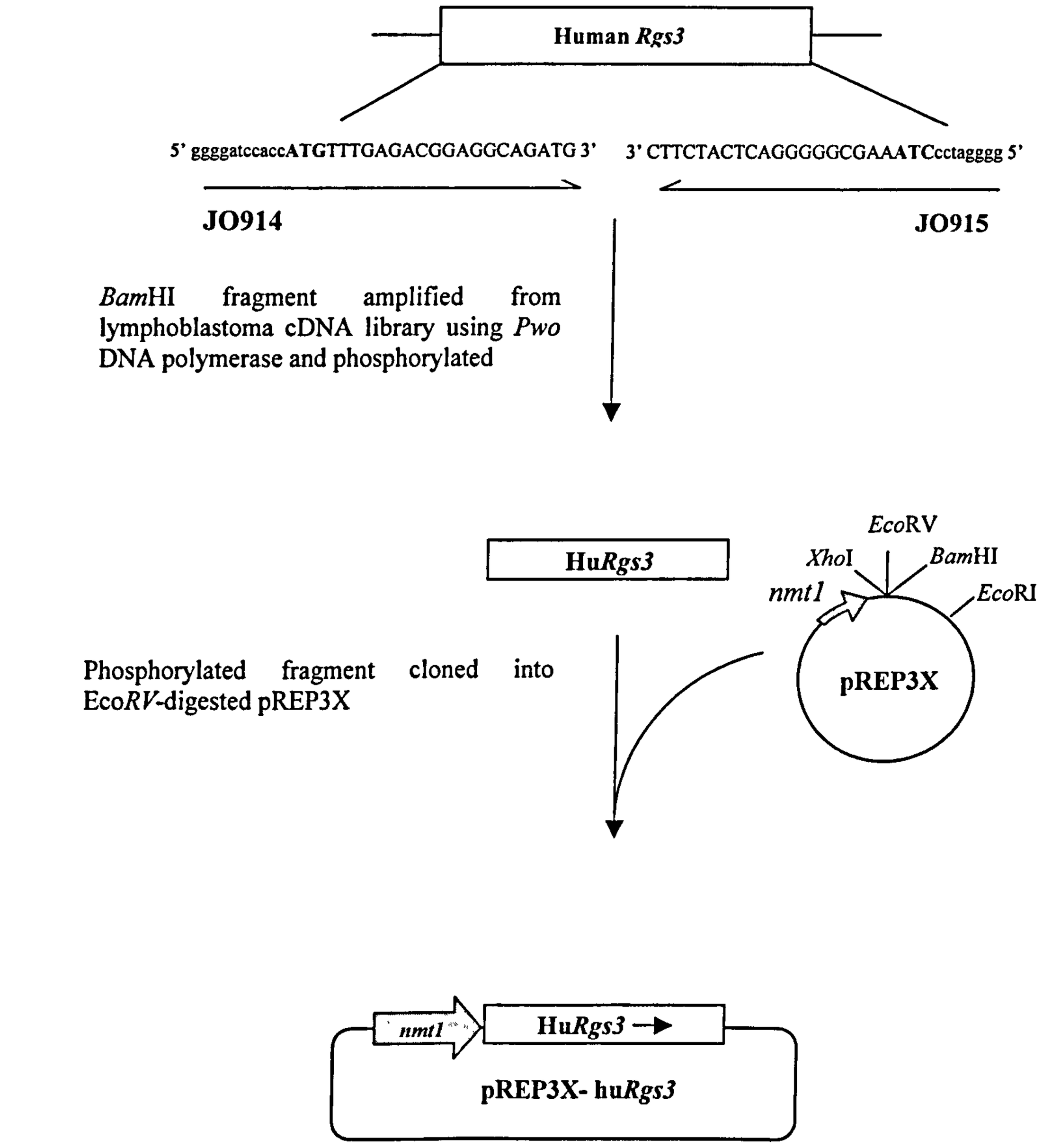


Figure 10. Creation of the human RGS3 expression construct

JO914 and JO915 were used to amplify the human *Rgs3* open reading frame from a lymphoblastoma cDNA library using *Pwo* DNA polymerase. JO914 contains the sequence CCACC to potentially increase the efficiency of translation (Kozak, 1984; Kozak, 1986). Lower case letters indicate primer sequence not found in the human *Rgs3* open reading frame. The amplified fragment was phosphorylated and cloned into the *Eco*RV site of the expression vector pREP3X.

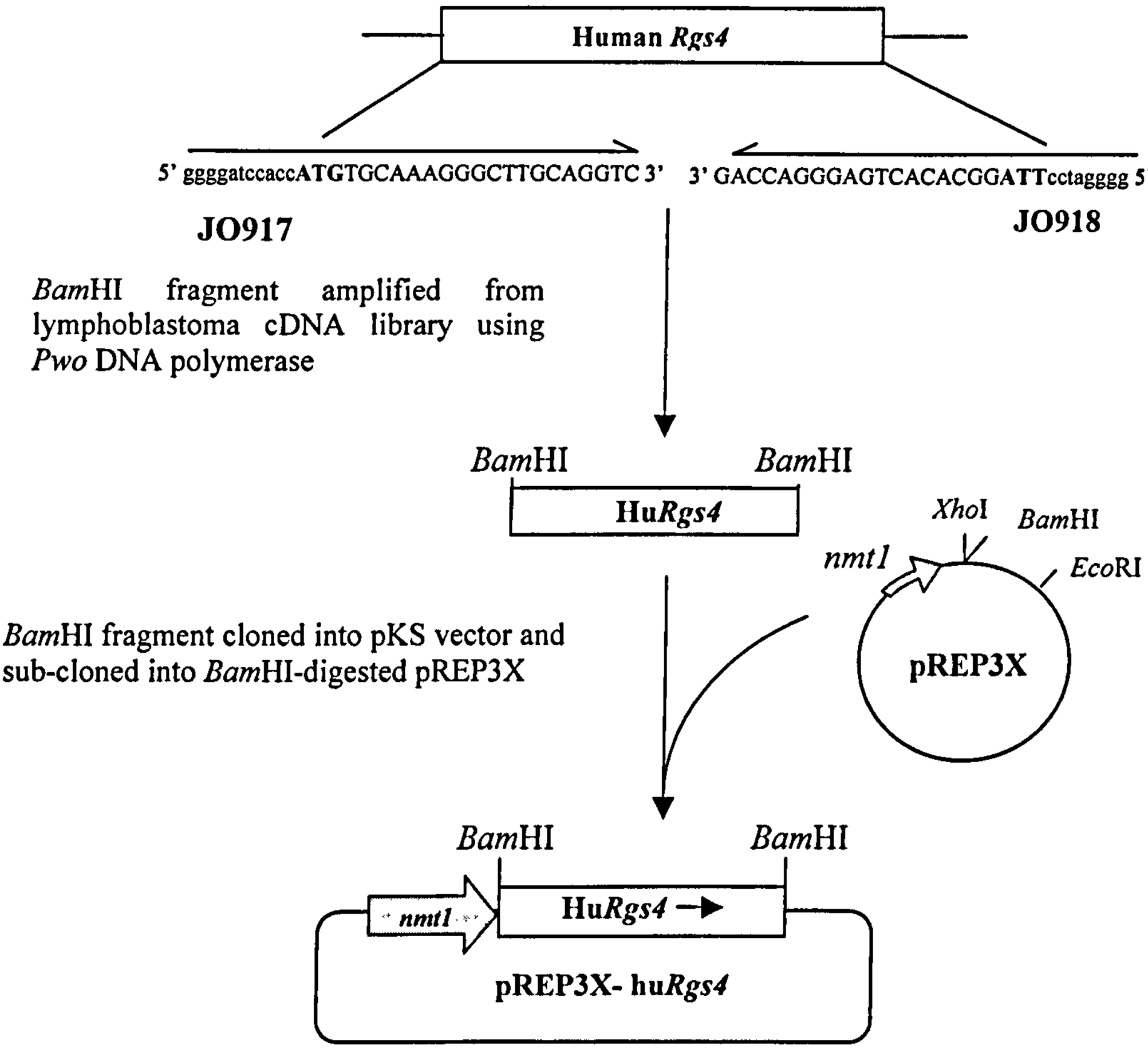


Figure 11. Creation of the human RGS4 expression construct

JO917 and JO918 were used to amplify the human *Rgs4* open reading frame from a lymphoblastoma cDNA library using *Pwo* DNA polymerase. JO917 and JO918 contain terminal *Bam*HI sites to facilitate cloning. JO917 contains the sequence CCACC to potentially increase the efficiency of translation (Kozak, 1984; Kozak, 1986). Lower case letters indicate primer sequence not found in the human *Rgs4* open reading frame. The human *Rgs4* ORF was cloned into the *Bam*HI restriction site of pKS, sequenced, and sub-cloned into the *Bam*HI restriction site of pREP3X.

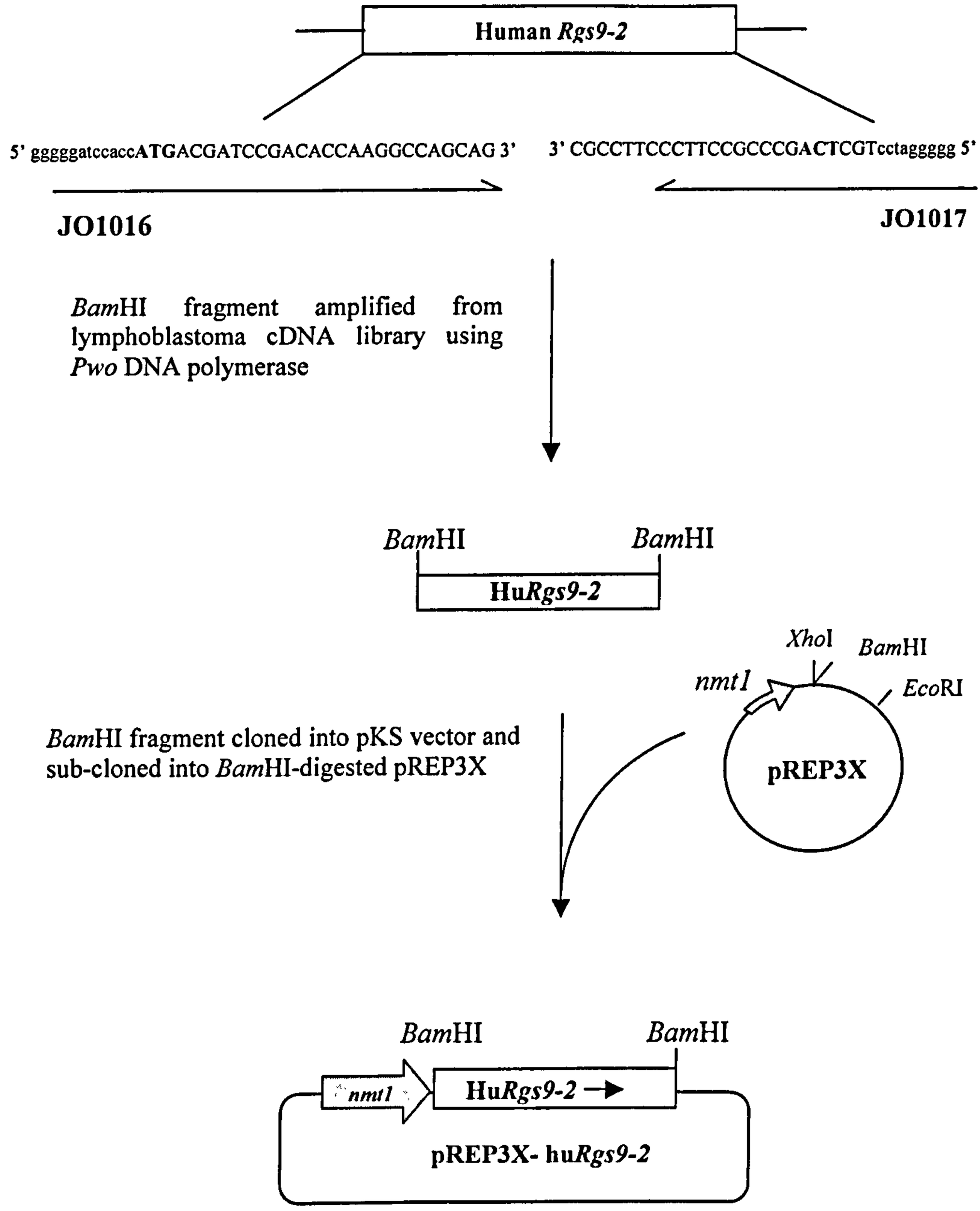


Figure 12. Creation of the human RGS9-2 expression construct

JO1016 and JO1017 were used to amplify the human *Rgs9-2* open reading frame from a pKS-*Rgs9-2* construct (Rahman *et al.*, 1999). JO1016 and JO1017 contain terminal *Bam*HI sites to facilitate cloning. JO1016 contains the sequence CCACC to potentially increase the efficiency of translation (Kozak, 1984; Kozak, 1986). Lower case letters indicate primer sequence not found in the human *Rgs9-2* open reading frame. The human *Rgs9-2* ORF was cloned into the *Bam*HI restriction site of pKS, sequenced, and sub-cloned into the *Bam*HI restriction site of pREP3X.

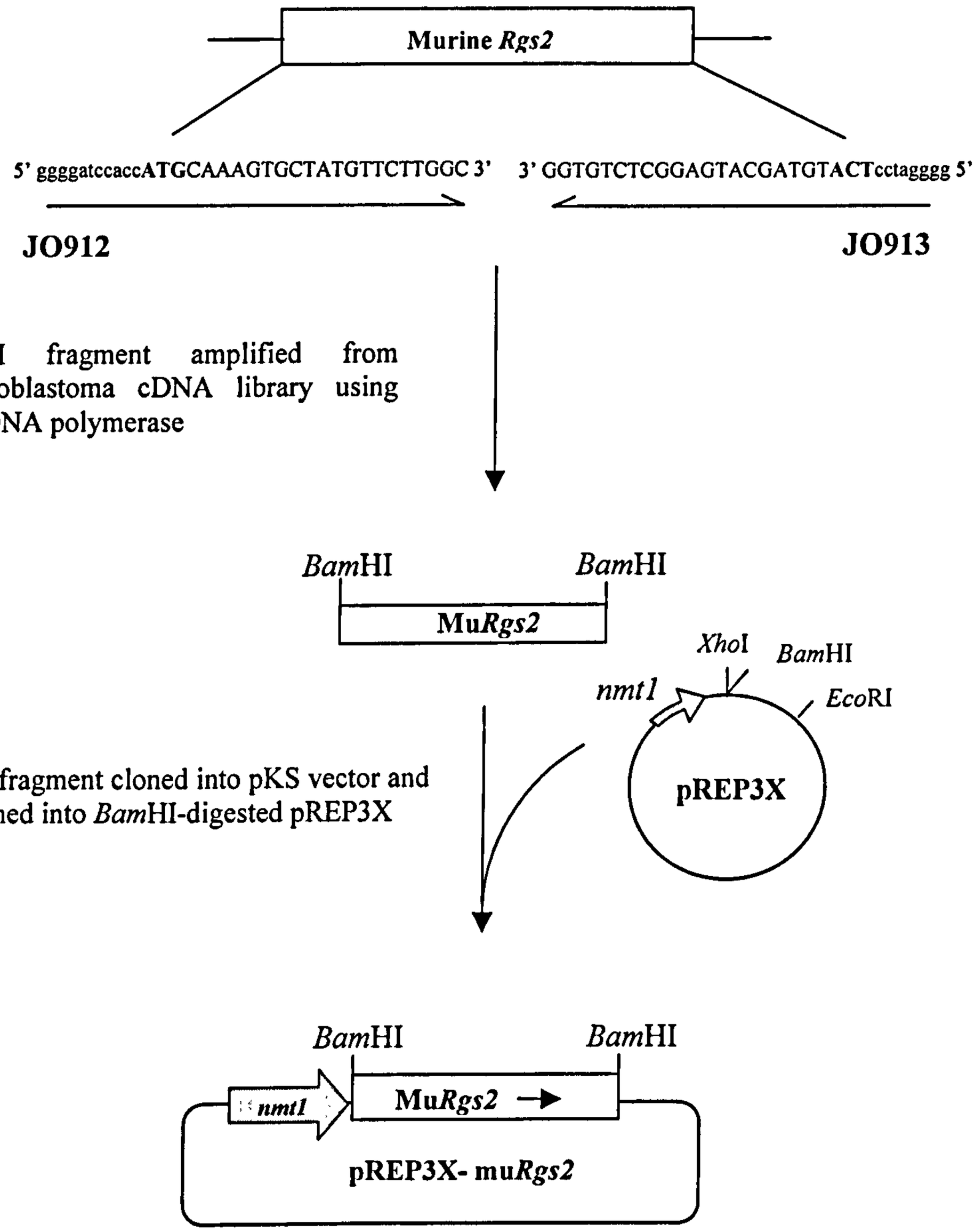


Figure 13. Creation of the murine RGS2 expression construct

JO912 and JO913 were used to amplify the murine *Rgs2* open reading frame from a pKS-mu*Rgs2* construct (Chen *et al.*, 1997). JO912 and JO913 contain terminal *Bam*HI sites to facilitate cloning. JO912 contains the sequence CCACC to potentially increase the efficiency of translation (Kozak, 1984; Kozak, 1986). Lower case letters indicate primer sequence not found in the murine *Rgs2* open reading frame. The murine *Rgs2* ORF was cloned into the *Bam*HI restriction site of pKS, sequenced, and sub-cloned into the *Bam*HI restriction site of pREP3X.

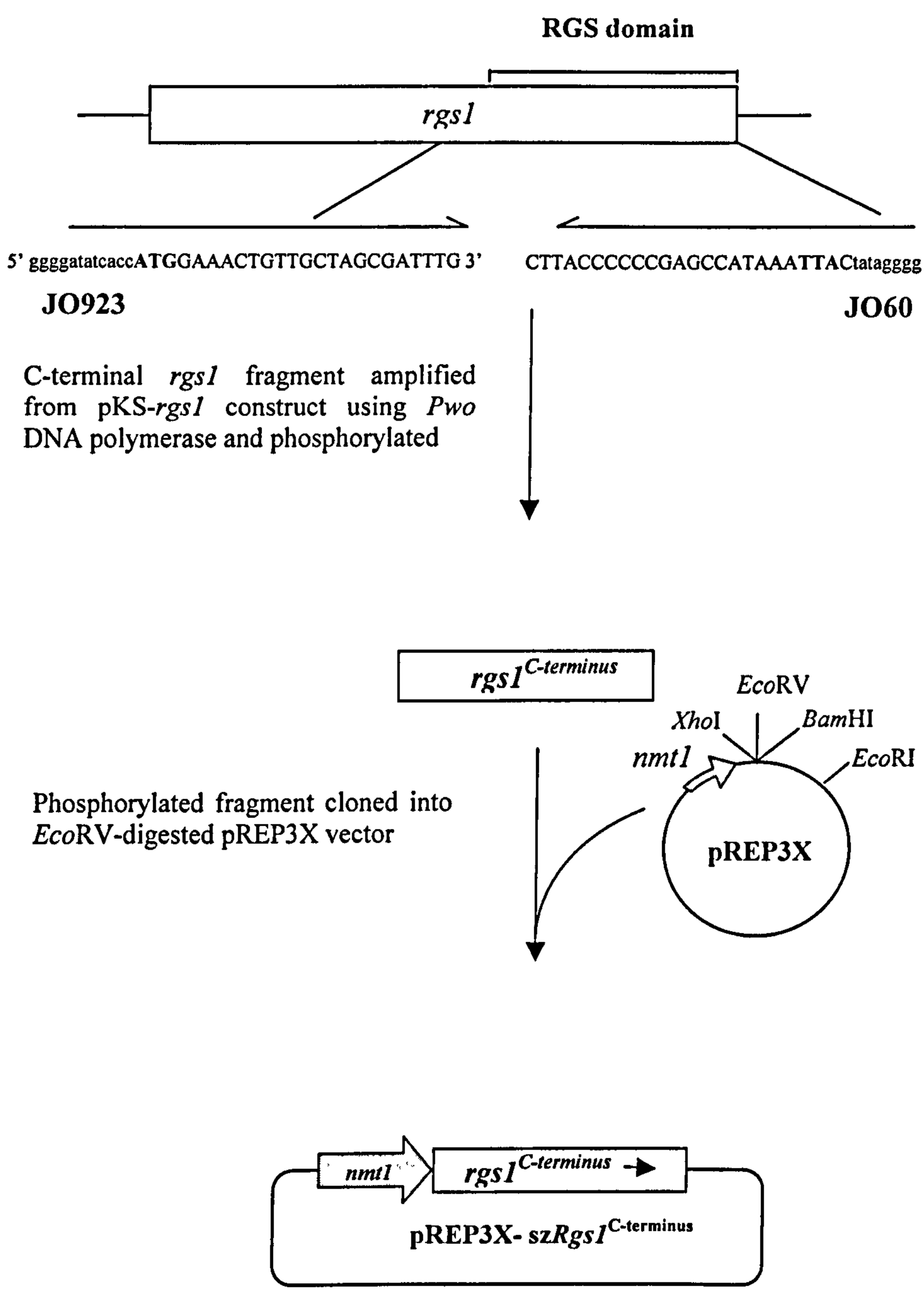
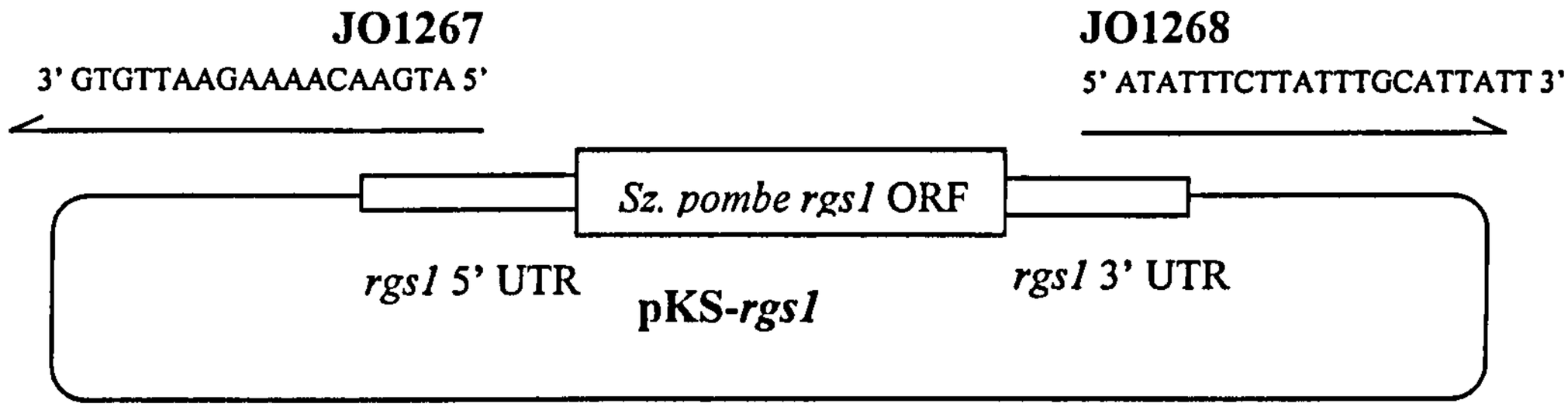


Figure 14. Creation of the *Sz. pombe* Rgs1 C-terminus expression construct

JO923 and JO600 were used to amplify the C-terminus of the *Sz. pombe rgs1* open reading frame from a pKS-*rgs1* construct using *Pwo* DNA polymerase. JO923 initiates at an initiating at nucleotide position +820 relative to the *rgs1* initiator codon (this corresponds to an internal Met residue at residue 274 in the szRgs1p primary sequence) and contains the sequence CCACC to potentially increase the efficiency of translation (Kozak, 1984; Kozak, 1986). Lower case letters indicate primer sequence not found in the *rgs1* open reading frame. The amplified fragment was phosphorylated and cloned into the *Eco*RV site of the expression vector pREP3X.



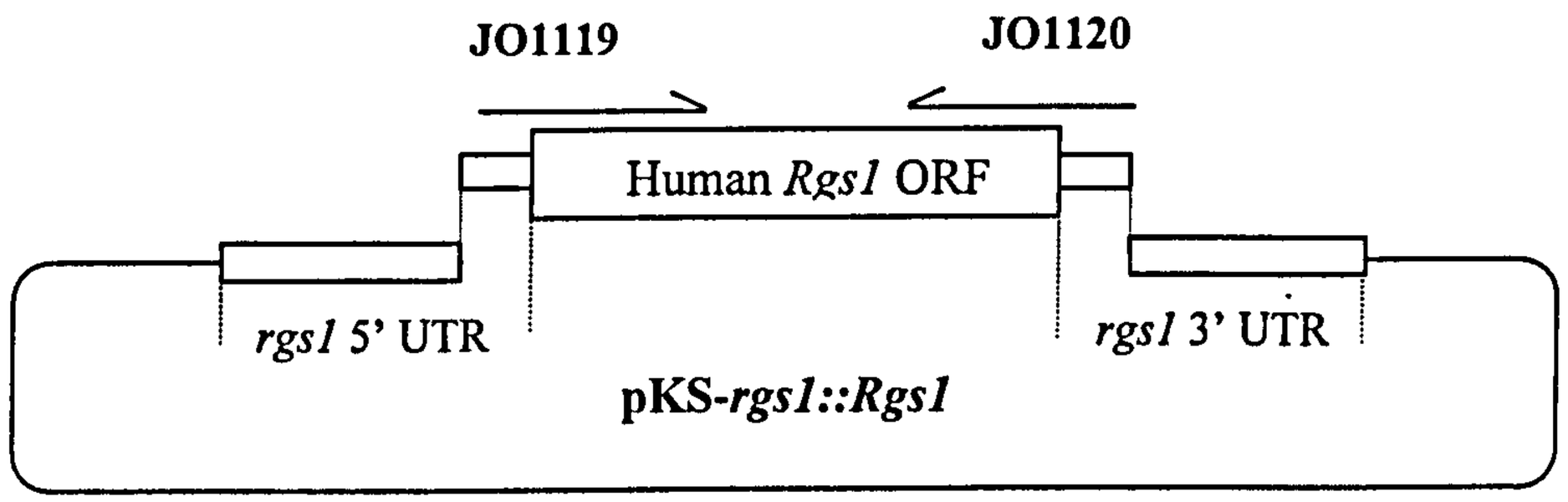
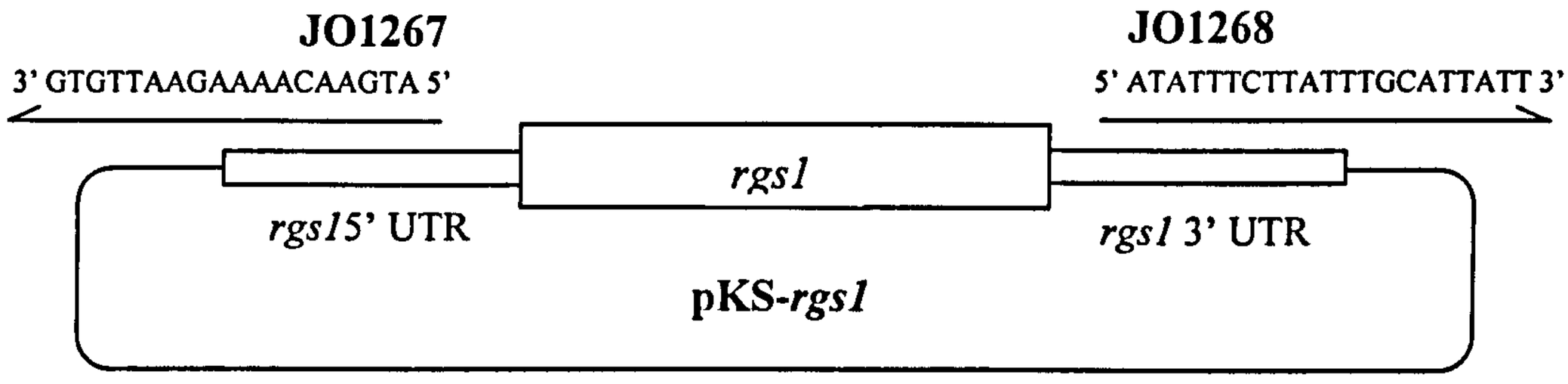


Figure 15. Creation of the human RGS1 integration construct

JO1119 and JO1120 were used to amplify the human *Rgs1* ORF flanked with *Sz. pombe rgs1* 5' and 3' non-coding regions. This fragment was cloned into a fragment amplified with JO1267 and JO1268, which provided additional *Sz. pombe rgs1* 5' and 3' non-coding regions.



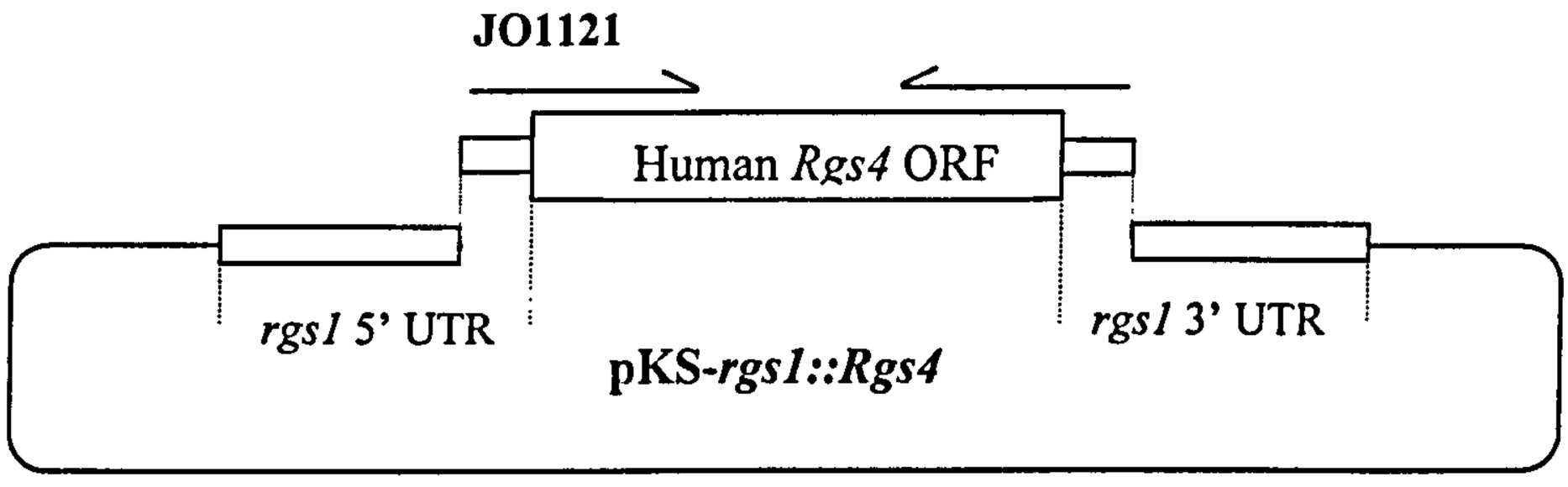


Figure 16. Creation of the human RGS4 integration construct

JO1121 and JO1122 were used to amplify the human *Rgs4* ORF flanked with *Sz. pombe rgs1* 5' and 3' non-coding regions. This fragment was cloned into a fragment amplified with JO1267 and JO1268, which provided additional *Sz. pombe rgs1* 5' and 3' non-coding regions.

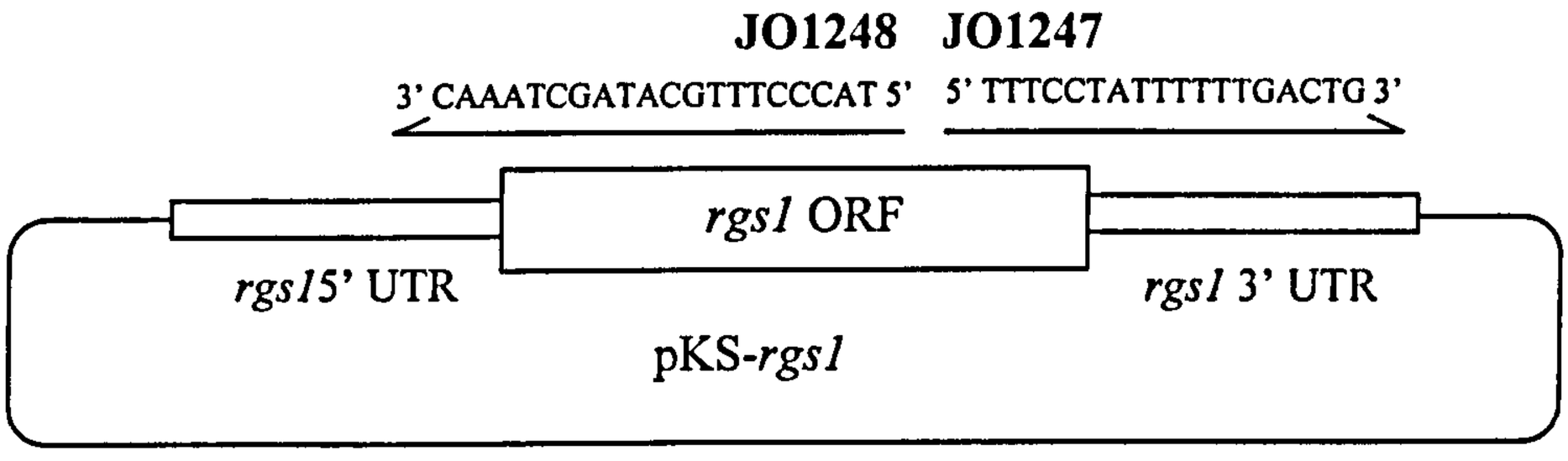


Figure 17. Creation of the *Sz. pombe* Rgs1$^{Val305\rightarrow Ile}$ integration construct

The oligonucleotide primers JO1247 and JO1248 were used in conjunction with a DNA template containing the *rgs1* ORF flanked by its 5' and 3' non-coding regions to generate the G→A mutation at nucleotide +913 in the *Sz. pombe rgs1* ORF, relative to the initiator codon. JO1248 introduces an adenine nucleotide at position 913 in the resulting *rgs1* ORF (replacing guanine in the wild-type *rgs1* ORF). The fragment was amplified with *Pwo* DNA polymerase and circularised.

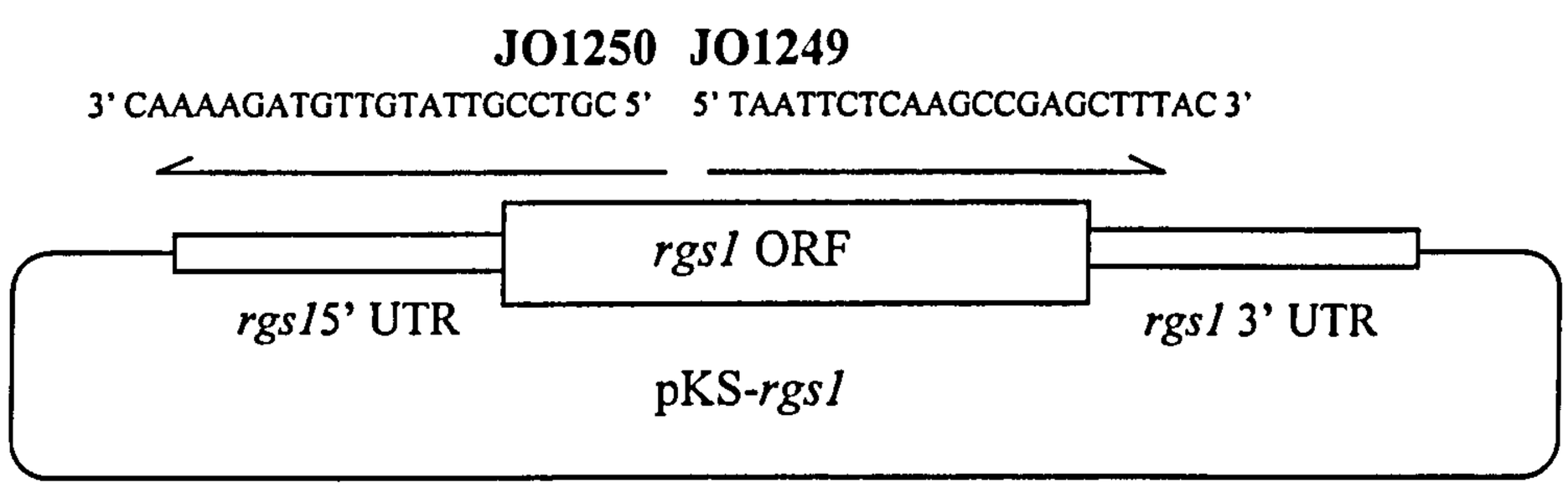


Figure 18. Creation of the *Sz. pombe* Rgs1$^{His171 \rightarrow Arg}$ integration construct

The oligonucleotide primers JO1249 and JO1250 were used in conjunction with a DNA template containing the *rgs1* ORF flanked by its 5' and 3' non-coding regions to generate the A→G mutation at nucleotide position +512 in the *Sz. pombe rgs1* ORF relative to the initiator codon. JO1250 introduces a guanine nucleotide at position 512 in the resulting *rgs1* ORF (replacing adenine in the wild-type *rgs1* ORF). The fragment was amplified with *Pwo* DNA polymerase and circularised.

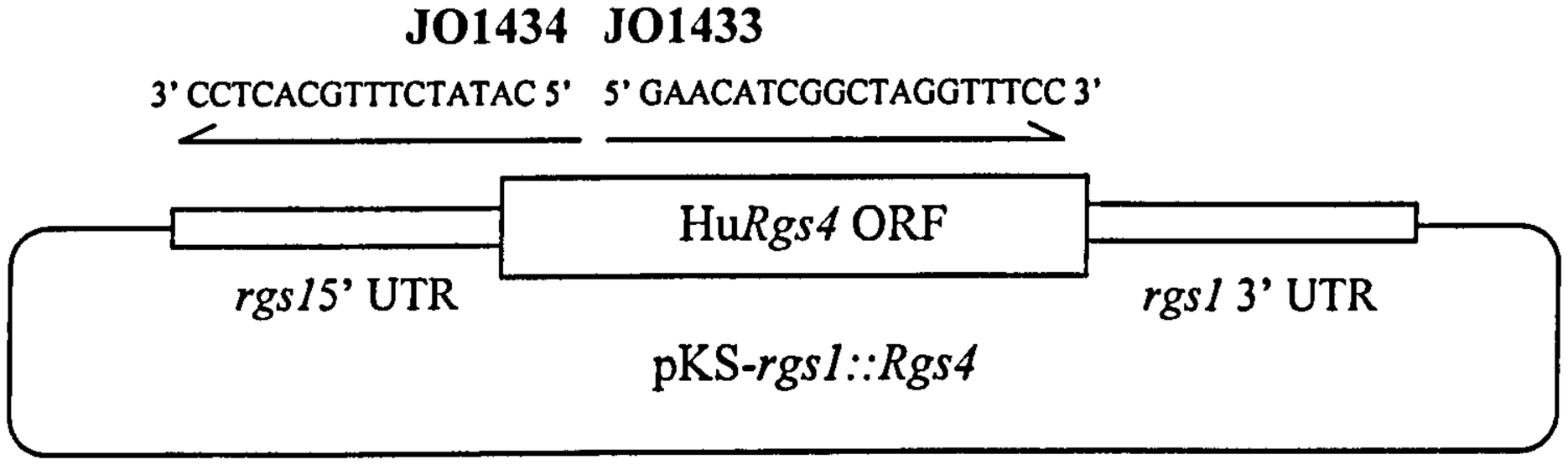


Figure 19. Creation of the Human RGS4$^{Lys20\rightarrow Arg}$ integration construct

The oligonucleotides JO1433 and JO1434 were used to incorporate a guanine nucleotide at position 58 in the human *Rgs4* integration construct. JO1433 introduces a guanine nucleotide at nucleotide position 58 in the resulting *Rgs4* ORF (replacing adenine in the wild-type *Rgs4* ORF). The fragment was amplified with *Pwo* DNA polymerase and circularised.

Chapter 3. Isolation of a temperature-sensitive Ura4 marker

3.1. Introduction

The study of gene function in the fission yeast *Sz. pombe* is commonly undertaken by the disruption of genes, in which the target gene is replaced by a selectable marker via homologous recombination. This is achieved by flanking the marker gene to be integrated with appropriate *Sz. pombe* genomic sequences. The marker gene is then introduced into the recipient genome as a result of homologous integration. While non-homologous recombination is rarely observed when constructs bear appropriate flanking regions (Grimm *et al.*, 1988; Keeney and Boeke, 1994), the transformed DNA can sometimes be maintained extra-chromosomally.

One commonly used auxotrophic marker in *Sz. pombe* is the *ura4* gene that encodes orotidine-5'-monophosphate decarboxylase (Bach, 1987), an enzyme involved in the pyrimidine biosynthetic pathway. Ura4 has a number of advantages over other *Sz. pombe* markers, including the availability of compounds for negative selection (i.e. loss of Ura4p function). One such compound is 5-fluoroorotic acid (5-FOA, [Boeke *et al.*, 1984]), a fluoropyrimidine analogue that is lethal to cells expressing a functional *ura4* gene. Following gene disruption with *ura4*, the marker may be subsequently replaced with another gene of choice. 5-FOA can thus be used to select for the loss of the *ura4* marker.

3.1.1. *Sz. pombe ura4-D18* deletion mutants enable directed integration of the *ura4* cassette

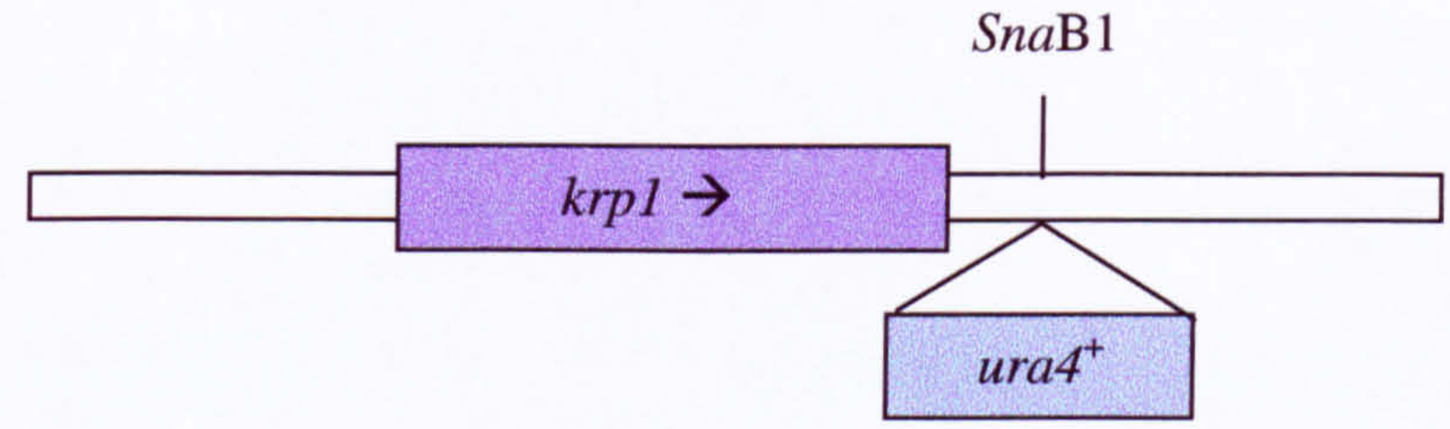
One drawback to certain *Sz. pombe* auxotrophic markers is the residue of homologous sequences within the recipient isolates. In this sense, *ura4* has an immense advantage in that the *Sz. pombe* deletion mutant *ura4-D18* (Grimm *et al.*, 1988) possesses no homologous *ura4* cassette sequences within its genome. Thus, in strains possessing the *ura4-D18* deletion (which have had a 1.8kb *Hind*III fragment encompassing the *ura4* coding region and 5' and 3' non-coding regions deleted), integration is targeted by virtue of the sequences flanking the *ura4* cassette. The combination of *Sz. pombe* strains with the *ura4-D18* deletion and integration constructs carrying the *ura4* cassette makes this a frequently used marker.

The limiting number of selective markers available for *Sz. pombe* places restrictions upon experimental designs, and so it was envisaged that a temperature-sensitive version of the Ura4p marker might aid and simplify multiple, sequential integration events within a single strain. For example, if an isolate has gene A disrupted with the temperature-sensitive *ura4* cassette, gene B could be then be disrupted by a wild-type *ura4* cassette, and integrants identified by their ability to grow at the restrictive temperature in the absence of uracil. The development of this alternative *Sz. pombe* marker would enable sequential gene disruptions and/or replacements to be carried out with the minimum of intermediate procedures.

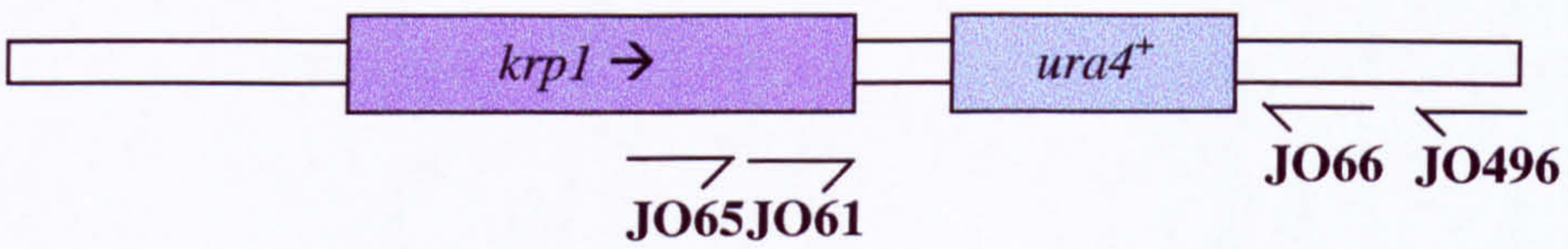
3.2. Generation of an *ura4*^{ts} cassette

Prior to the commencement of this work, a construct was made in the Davey laboratory for the disruption of the *krp1* gene. The *krp1* gene encodes a dibasic endopeptidase required for cell viability in *Sz. pombe* (Davey *et al.*, 1994). From this construct, successful attempts were made to isolate a temperature-sensitive version of Krp1p (Ladds *et al.*, 2000). In the *krp1* disruption construct an *ura4* cassette is cloned into a *Sna*BI restriction site in the 3' non-coding region of *krp1* (Figure 20), creating the *krp1>ura4* disruption construct.

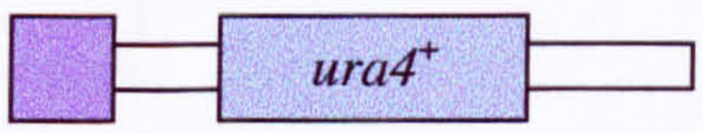
The *krp1>ura4* disruption construct was used as a template to isolate a temperature-sensitive *ura4* allele. A 3kb fragment containing the *ura4* cassette flanked either side by approximately 600bp from the *krp1* gene was amplified from the *krp1>ura4* construct using the oligonucleotide primers JO65 and JO66 and *Taq* DNA polymerase. The sense primer JO65 contains 17 bases complementary to the *krp1* open reading frame initiating at nucleotide position +1035 relative to the *krp1* initiator codon. The antisense primer JO66 contains 17 bases complementary to the *krp1* 3' non-coding region initiating at nucleotide position +2695 relative to the *krp1* initiator codon. The conditions used for the PCR amplification of the *krp1>ura4* construct introduces errors at a rate of approximately 0.1% (Section 2.2.2.4).



ura4$^+$ cassette cloned into *Sna*B1 site downstream of *krp1* open reading frame



PCR amplification of *krp1>ura4* construct using JO65 and JO66 with *Taq* DNA polymerase. Conditions used introduce errors at rate of ~ 0.1%. PolyA tails were removed by performing a round of PCR using *Pwo* DNA polymerase after the *Taq* PCR steps.



JY383 was transformed with the *Taq* polymerase-amplified *krp1>ura4* construct generating the yeast strain JY395

Figure 20. Generation of a *krp1>ura4* disruption construct

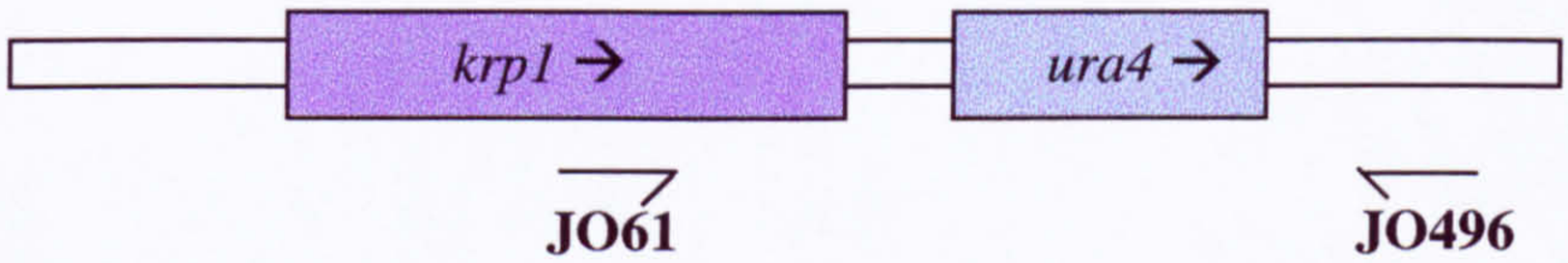
The *ura4* cassette was cloned into a *Sna*BI site in the 3' non-coding region of *krp1*. JY383 (*mat1-P*, *leu1-32*, *ura4-D18*) was transformed with a DNA fragment containing the *ura4* cassette flanked either side by ~600bp of the *krp1* allele (amplified with *Taq* DNA polymerase under conditions that introduce errors at a rate of approximately 0.1%).

The amplification mixture was used to transform directly JY383 (*mat1-P*, *leu1-32*, *ura4-D18*). Transformants were plated onto AA plates lacking uracil and incubated at 23°C. Transformants were then replicated onto AA plates containing or lacking uracil and incubated at 37°C to identify which transformants exhibited a temperature-sensitive phenotype.

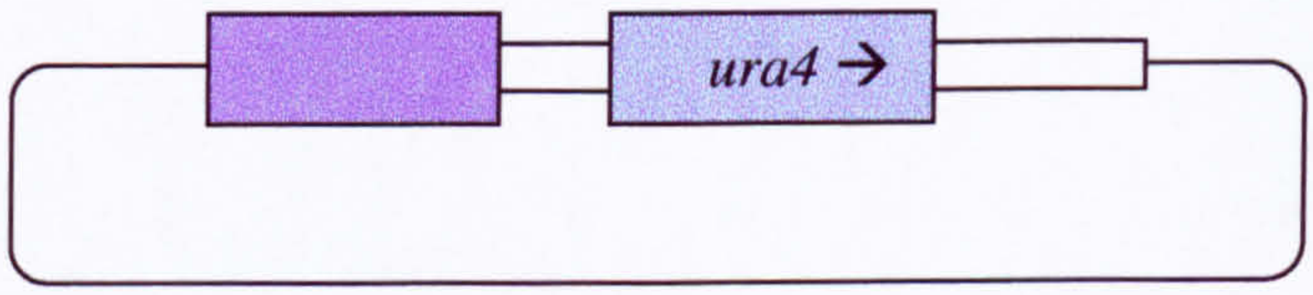
The uracil-dependency of one strain (JY395) appeared to be temperature-sensitive. At 37°C, this strain was able to grow on AA plates containing uracil but unable to grow on AA plates lacking uracil. A fragment containing the *ura4* cassette was amplified from JY395 genomic DNA, using the oligonucleotide primers JO61 and JO496 and *Pwo* DNA polymerase (Figure 21). The sense primer JO61 contains 17 bases complementary to the *krp1* open reading frame initiating at nucleotide position +1484 relative to the *krp1* initiator codon. The antisense primer JO496 contains 28 bases complementary to the *krp1* 3' non-coding region initiating at nucleotide position +2966 relative to the *krp1* initiator codon.

3.2.1. A single point mutation generates a temperature-sensitive Ura4p

The *krp1>ura4* construct amplified with the oligonucleotide primers JO61 and JO496 from JY395 genomic DNA was cloned into the *Pvu*II site of pKS and sequenced by the dideoxynucleotide method. The region corresponding to the *ura4* 795bp open reading frame (ORF) had a single point mutation at nucleotide position +782 relative to the *ura4* initiator codon. This T-to-C substitution results in a leucine to proline substitution at amino acid residue 261 in the primary sequence of Ura4^{ts}p. In order to verify that the temperature-sensitive phenotype was due to the Leu261→Pro mutation, and not due to fortuitous mutations elsewhere in the host chromosome, JY383 was re-transformed with the *krp1>ura4*^{ts} construct. Transformants exhibiting a temperature-sensitive requirement for uracil had undergone successful homologous integration at the *krp1* locus, as confirmed by PCR analysis and Southern blot analysis (carried out in collaboration with Dr. Kevin Davis) (Figure 22).



PCR amplification of the *krp1>ura4* construct from JY395 genomic DNA using JO61 and JO496 in conjunction with *Pwo* DNA polymerase. Amplified products were cloned into the cloning vector pKS



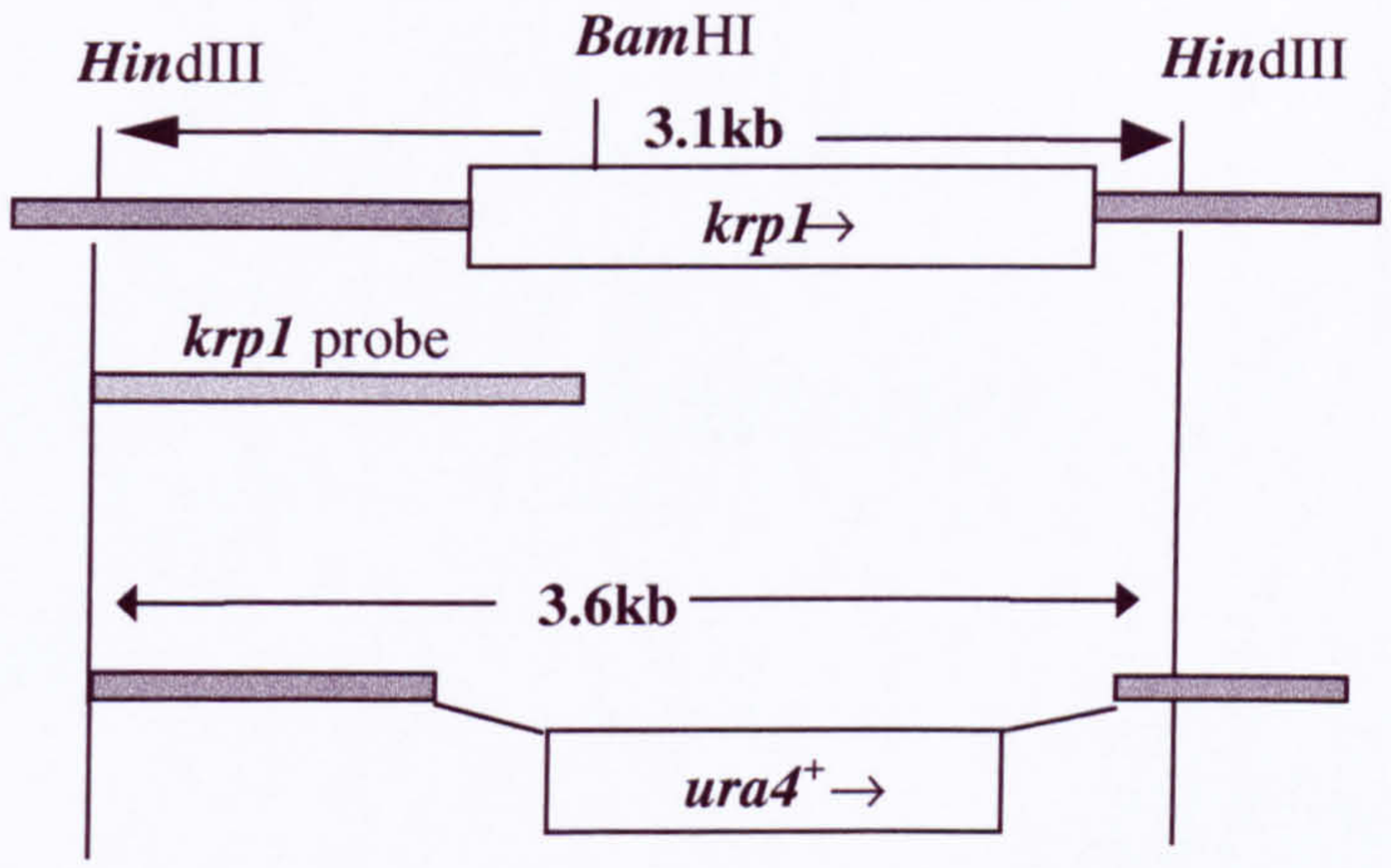
The *ura4* cassette was sequenced by the dideoxynucleotide method

		CAG	CAA	AGA	CTT	TCT	CAG	CAT	
		GTC	GTT	TCT	GAA	AGA	GTC	GTA	
Position within Ura4p	**258**	Gln	Gln	Arg	**Leu**	Ser	Gln	His	**264**
		CAG	CAA	AGA	CCT	TCT	CAG	CAT	
		GTC	GTT	TCT	GGA	AGA	GTC	GTA	
Position within Ura4p	**258**	Gln	Gln	Arg	**Pro**	Ser	Gln	His	**264**

A T-to-C mutation at nucleotide +782 in the *ura4* ORF changes Leu261 to Pro in the *ura4*^{ts} cassette.

Figure 21. The *ura4*^{ts} ORF possesses a mutation at nucleotide 782 that results in a leucine-to-proline substitution at amino acid residue 261 in Ura4p

The *ura4* cassette amplified from JY395 genomic DNA (a strain exhibiting temperature-sensitive uracil-dependency) was cloned into pBluescript and sequenced. A mutation at nucleotide +782 in the *ura4* open reading frame results in a leucine-to-proline substitution at residue 261 in the primary sequence of Ura4^{ts}p. The *ura4*^{ts} cassette is identical to the wild-type *ura4* cassette except for the T-to-C mutation at nucleotide +782 in the *ura4* open reading frame (relative to the initiator codon).



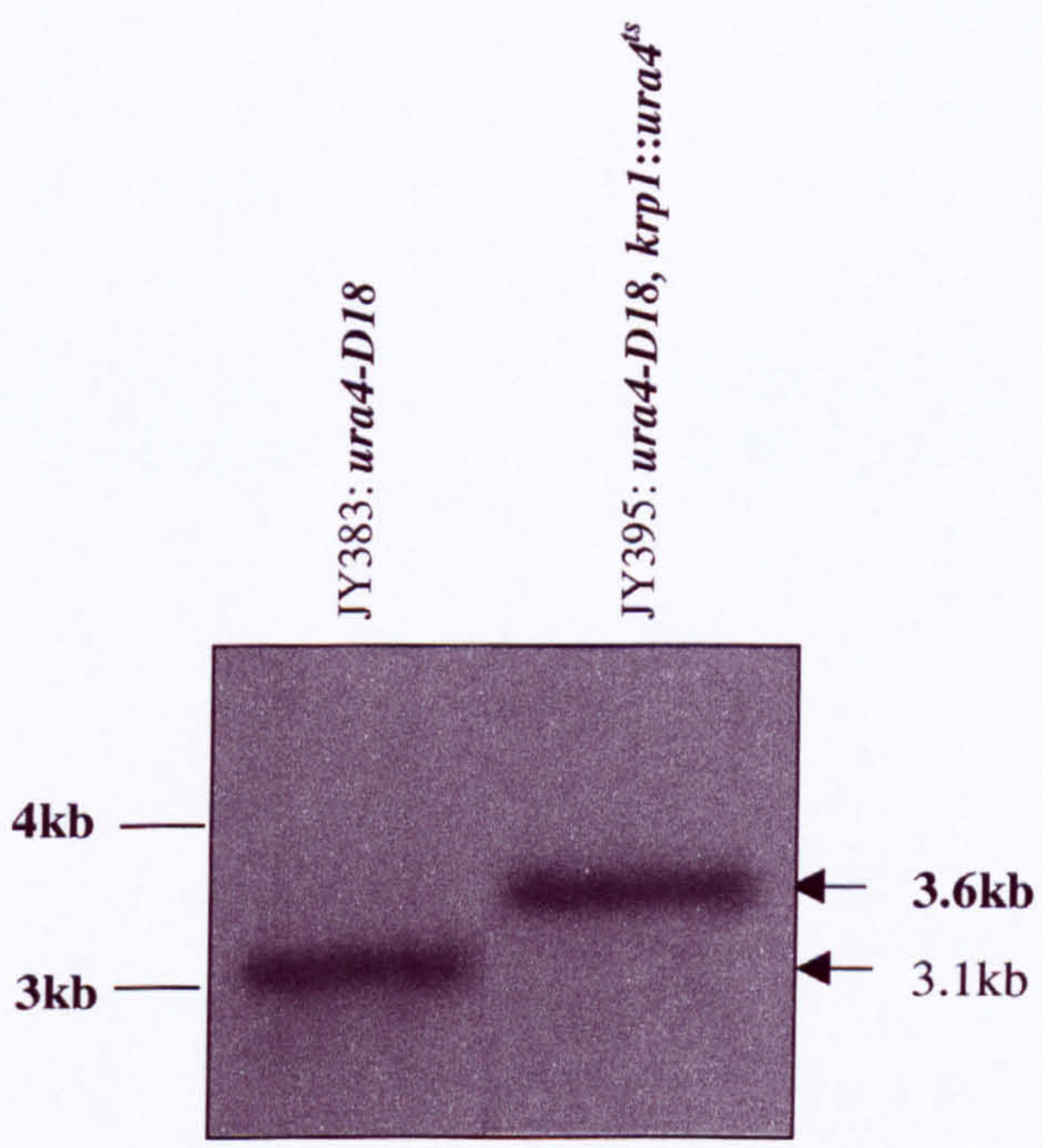


Figure 22. Disruption of the *krp1* locus with the *ura4* cassette

Genomic DNA was prepared from a *krp1*$^{+}$ strain (JY383) and a *krp1*::*ura4* strain (JY395) and digested with *Hind*III. Restricted DNA was separated on a 1% agarose gel, blotted onto a nitrocellulose filter and probed with a 1kb *Hin*dIII/*Bam*HI DNA fragment from the *krp1* locus (indicated above). The wild-type *krp1*$^{+}$ allele gives a band of 3.1kb, while the *krp1>ura4* allele gives a band of 3.6kb.

The substitution of leucine for proline in the primary amino acid sequence of Ura4p can be expected to have profound effects upon the secondary and tertiary structures of Ura4p. Proline is unique in that it is covalently bound to the nitrogen atom of the polypeptide backbone, and as a result, has no amide hydrogen for use as a donor in hydrogen bonding or in resonance stabilization of the peptide bond of which it is part. The peptide bond preceding a proline residue is more likely to adopt a *cis*-configuration due to constraints on rotation about the N-C^{α} bond of the polypeptide backbone.

3.3. Disruption of *irp1* with the *ura4*^{ts} cassette in a *ura4-D18* strain

3.3.1. Generation of the *irp1*::*ura4*^{ts} disruption construct

In order to demonstrate the value of *ura4*^{ts} for gene disruption, a construct was prepared to disrupt the *irp1* gene in JY383 (*mat1-P*, *leu1-32*, *ura4-D18*) with the *ura4*^{ts} cassette described in Section 3.2 (Figure 23). Irp1p (insulinase-related protease) is a member of the insulin-degrading family of proteases and loss of *irp1* has no apparent effect on normal cell behaviour (Hughes and Davey, 1997).

The 5' non-coding region of *irp1* was amplified from JY383 genomic DNA using the oligonucleotide primers JO497 and JO498 (Figure 23). The sense primer JO497 contains 18 bases complementary to the *irp1* 5' non-coding region, initiating at nucleotide position -1228 relative to the *irp1* initiator codon. The anti-sense primer JO498 contains 27 bases complementary to the region immediately upstream of the *irp1* initiator codon and includes a terminal *Bam*HI site. The *irp1* 5' non-coding region was cloned between the *Pst*I and *Bam*HI restriction sites of pKS.

The 3' non-coding region of *irp1* was amplified using the oligonucleotide primers JO499 and JO500. The sense primer JO499 contains 26 bases complementary to the *irp1* 3' non-coding region immediately downstream of the *irp1* stop codon, initiating at position +2911 relative to the *irp1* initiator codon. JO499 also includes a terminal *Bam*HI site. The antisense primer JO500 contains 18 bases complementary to the *irp1* 3' non-coding region initiating at nucleotide position +4281 relative to the *irp1* initiator codon.

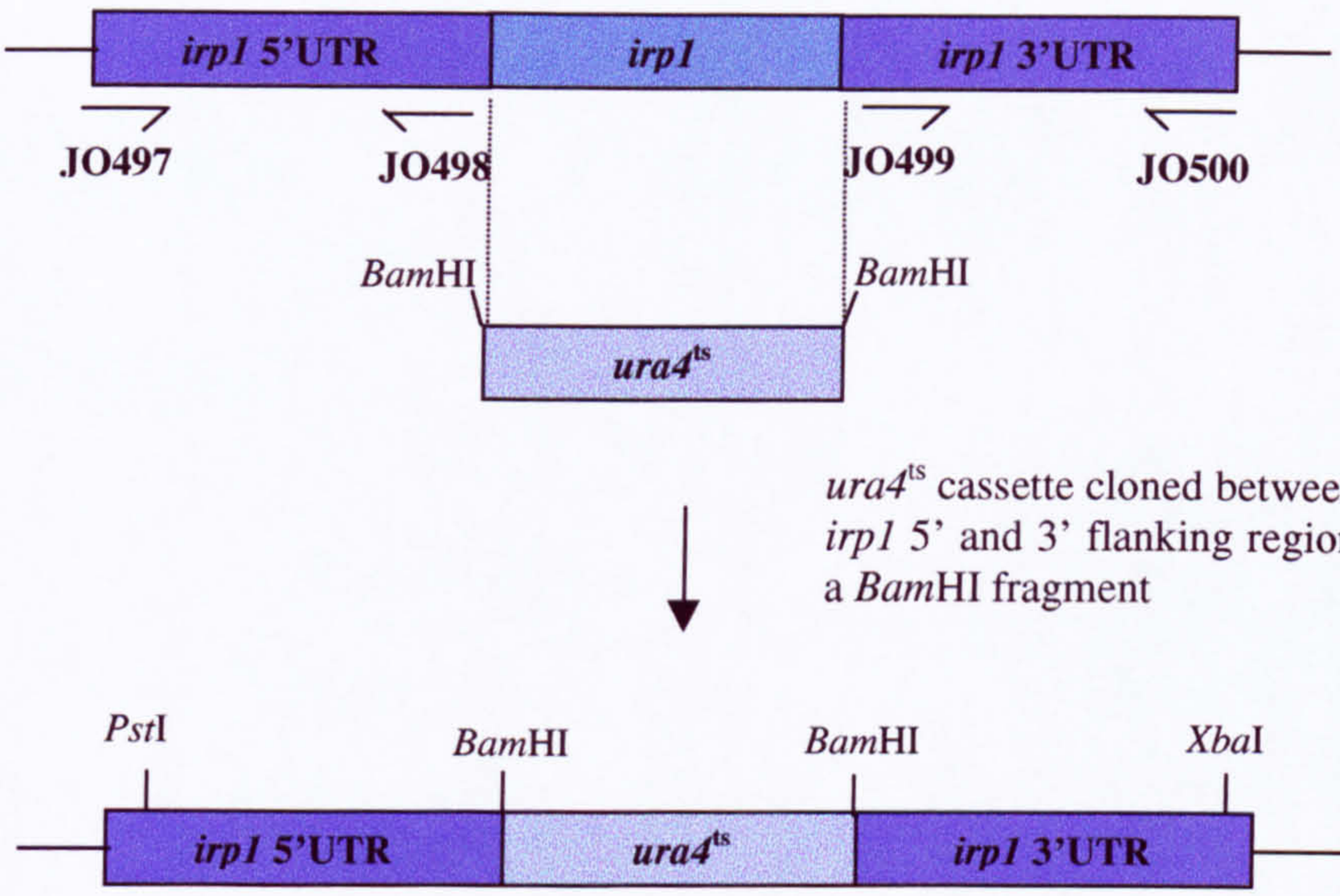


Figure 23. Generation of an *irp1*::*ura4*^{ts} disruption construct

The *ura*4^{ts} cassette amplified from JY395 genomic DNA was cloned between the *irp1* 5' and 3' non-coding regions in pBluescript as a *Bam*HI fragment. JY383 was transformed with the *irp1*::*ura4*^{ts} disruption construct liberated by digestion with *Pst*I and *Xba*I.

The *irp1* 3' non-coding region was cloned between the *Bam*HI and *Xba*I sites of the pKS construct containing the *irp1* 5' non-coding region to create a construct in which the *irp1* 5' and 3' non-coding regions are separated by a *Bam*HI restriction site. The *ura4*^{ts} cassette was liberated from the *krp>ura4*^{ts} construct by digestion with *Bam*HI, and the resulting fragment cloned into the *Bam*HI site separating the *irp1* 5' and 3' non-coding regions to create the *irp1*::*ura4*^{ts} disruption construct.

3.3.2. Disruption of *irp1* with the *ura4*^{ts} cassette

The *irp1*::*ura4*^{ts} disruption fragment was liberated by digestion with *Pst*I and *Xba*I. JY383 (*mat1-P*, *leu1-32*, *ura4-D18*) was transformed with the *irp1*::*ura4*^{ts} disruption fragment and plated onto AA plates lacking uracil at 29°C. Transformants were replicated to AA plates containing or lacking uracil at 37°C. Approximately 10% (five of 52 tested) of the transformants capable of growth on uracil-deficient plates had a temperature-sensitive requirement for uracil, and Southern blot analysis confirmed that each of these resulted from a single homologous recombination event at the *irp1* locus. 90% of the transformants capable of growth on AA plates lacking uracil did not exhibit a temperature-sensitive requirement for uracil, and stability assays suggested that in many of these isolates the *ura4*^{ts} gene was maintained as a plasmid.

3.3.3. Southern blot analysis confirming *irp1* disruption with *ura4*^{ts}

Southern blot analysis confirmed that the *ura4*^{ts} cassette had integrated correctly and exclusively at the *irp1* locus in JY383, generating the strain JY543 (*ura4-D18, irp1*::*ura4*^{ts}) (Figure 24).

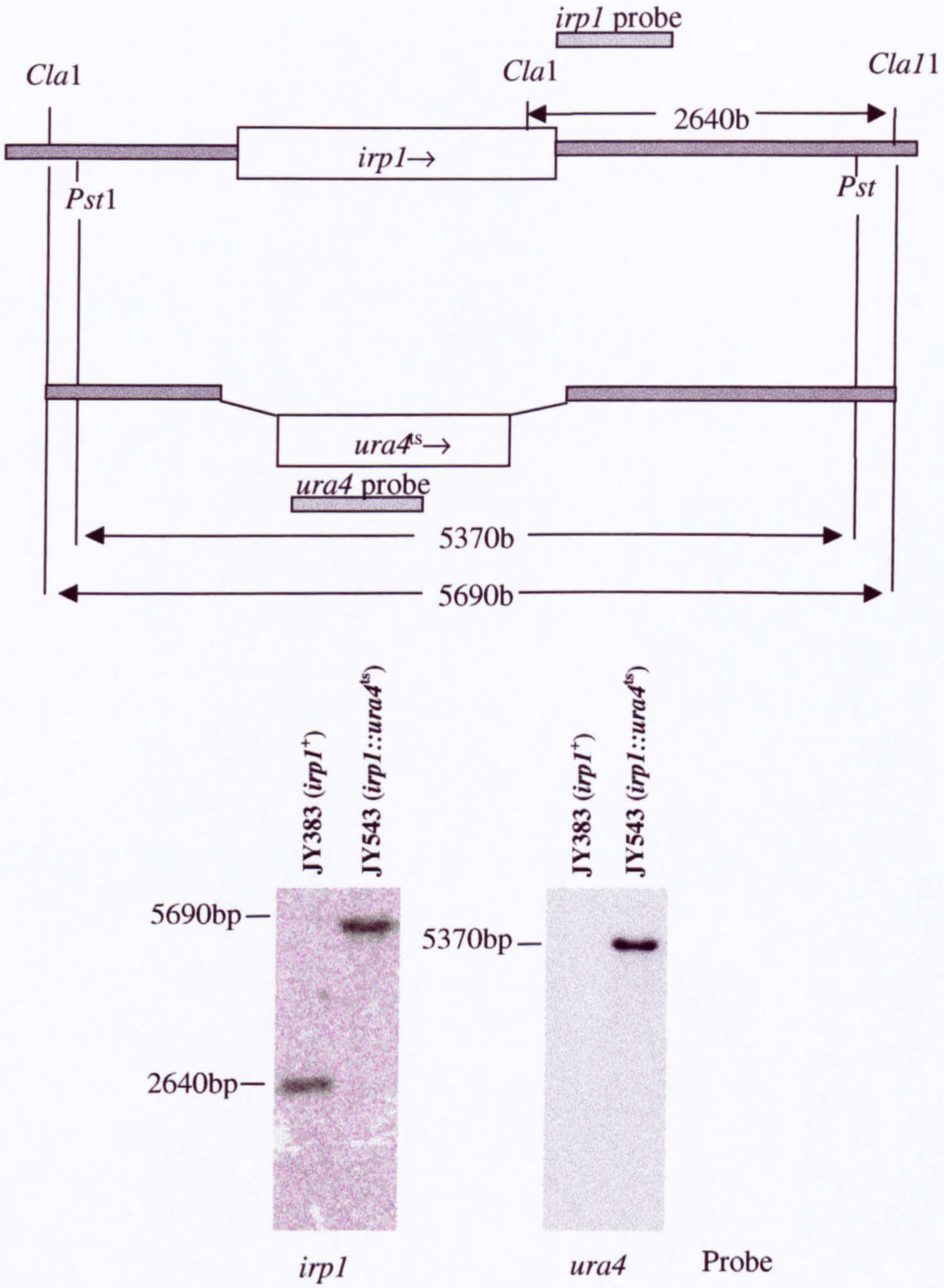


Figure 24. Southern blot analysis confirming *irp1* disruption with *ura4*^{ts}

Genomic DNA was prepared from JY383 (*irp1*$^{+}$) and JY543 (*irp1*::*ura4*^{ts}), and the DNA digested with *Cla*I. Digested DNA was separated on a 1% agarose gel, blotted onto a nitrocellulose filter, and probed with an *irp1* 3' fragment. A band of 5690bp indicated homologous recombination had occurred at the *irp1* locus. A band of 2640bp indicated an undisrupted *irp1* locus. JY383 and JY543 genomic DNA was also digested with *Pst*I and probed with an *ura4* ORF fragment. A band of 5370bp indicated homologous integration of the *ura4*^{ts} cassette at the *irp1* locus.

3.3.4. Growth characteristics for *irp1*::*ura4*$^{+}$ and *irp1*::*ura4*^{ts} strains

Growth characteristics were determined for an *irp1*::*ura4*$^{+}$ strain, JY497 (*ura4-D18, irp1*::*ura4*$^{+}$), previously created in the laboratory, and the *irp1*::*ura4*^{ts} strain JY543 (*ura4-D18, irp1*::*ura4*^{ts}) when shifted from 29°C to 37°C.

Growth characteristics of JY497 (*irp1*::*ura4*$^{+}$) and JY543 (*irp1*::*ura4*^{ts}) were determined in minimal media containing alanine and leucine in the presence or absence of uracil when shifted from 29°C to 37°C. Briefly, 50ml medium were inoculated at a cell density of $2x10^5$ cells/ml, and cells were incubated in a shaking incubator at 29°C. Once strains reached a density of $1x10^6$ cells/ml, cultures were shifted quickly to a 37°C shaking water bath to minimize the time taken to shift temperatures (control experiments indicated that media took less than 10 min to reach 37°C). Cell counts were determined at various time-points post-shift using a Coulter Channelyser.

Neither strain exhibited a lag in cell division upon the shift to 37°C (Figure 25), and both JY497 (*irp1*::*ura4*$^{+}$) and JY543 (*irp1*::*ura4*^{ts}) cells retained the typical wild-type *Sz. pombe* barrel-like morphology throughout the experiment (determined by microscopic observation, not shown). JY543 cells were slightly larger than JY497 cells at 37°C (JY543 had a median cell volume of 108μm^3 compared to 96μm^3 for JY497). The median cell volume is the volume that divides the distribution of cells into two equal groups, such that 50% of the cells are larger than the median volume, and is a useful measure of cell size in a non-synchronous culture.

From Figure 25, it can be seen that when shifted to 37°C, JY497 (*irp1*::*ura4*$^{+}$) had comparable doubling times (2h) in the absence and presence of uracil. The doubling time of 4h seen for JY543 (*irp1*::*ura4*^{ts}) was slower than that of JY497 (*irp1*::*ura4*$^{+}$) at 37°C. This is likely due to the reduced activity of Ura4^{ts}p in JY543 once intracellular stores of uracil had been depleted. The absence of uracil in the medium did not adversely affect the doubling time of JY543 in the first 8h following the shift to 37°C.

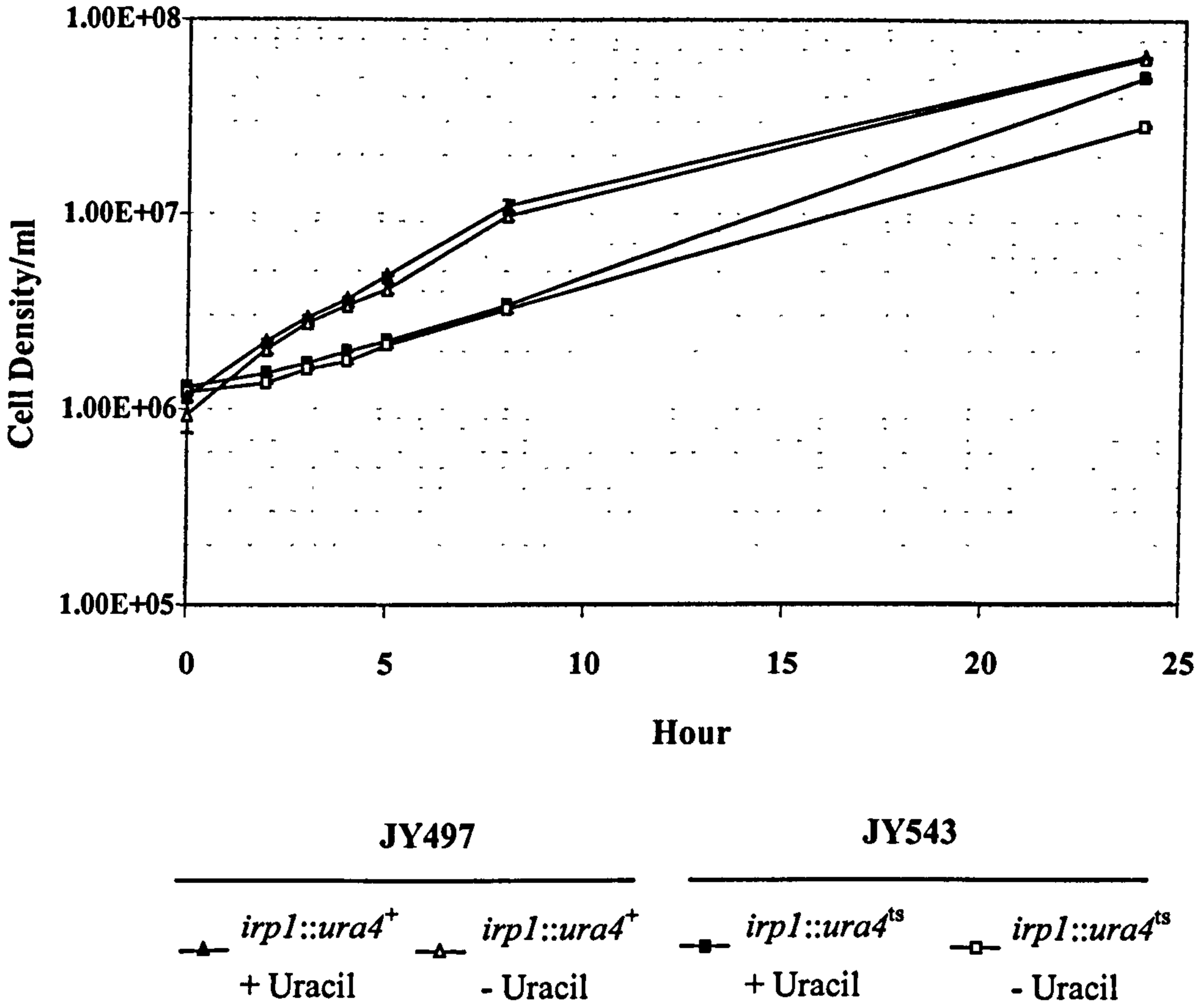


Figure 25. Growth characteristics of *irp1*::*ura4*$^{+}$ and *irp1*::*ura4*^{ts} strains at 37°C

Growth characteristics of JY497 (*irp1*::*ura4*$^{+}$) and JY543 (*irp1*::*ura4*^{ts}) when shifted from 29°C to 37°C were determined in minimal media containing alanine and leucine in the presence or absence of uracil. JY497 (*irp1*::*ura4*$^{+}$) has a generation time of 2h in the absence and presence of uracil. JY543 (*irp1*::*ura4*^{ts}) has a generation time of 4h in the absence and presence of uracil for the first 8h following the shift to 37°C. JY543 growth in the absence of uracil slows between 8h and 24h compared to JY543 growth in the presence of uracil.

JY543 (*ura4-D18, irp1*::*ura4*^{ts}) was isolated as a consequence of its temperature-sensitive requirement for uracil when plated on AA plates lacking uracil at 37°C. However, in liquid culture, the temperature-sensitive uracil requirement of JY543 was not immediately apparent. In the first 8h following the cultures being shifted to 37°C, JY543 exhibited the same doubling time regardless of the presence or absence of uracil in the medium. This is not altogether unexpected however, as previous to the temperature shift, JY543 cells may have expressed sufficient levels of Ura4^{ts}p to generate an intracellular pool of uracil sufficient to sustain growth in the first 8h of the growth assay. Thus it would be expected that the restrictive temperature would only be seen to adversely affect growth at later time-points. This can be seen in Figure 25, as by 24h the cell density of JY543 grown in the presence of uracil was double that of JY543 grown in the absence of uracil. It is possible that inoculating JY543 cells grown in uracil-deficient medium at 37°C into fresh uracil-deficient medium at 37°C would demonstrate the long-term detrimental effect of this restrictive temperature upon the growth of JY543 in liquid medium.

To determine the viability of JY497 (*irp1*::*ura4*$^{+}$) and JY543 (*irp1*::*ura4*^{ts}) on solid media, 1x10^{5} cells were plated on AA plates lacking uracil and incubated at either 23°C or 37°C. JY497 was capable of forming colonies at both temperatures, while JY543 was only capable of growth at 23°C (Figure 26). No growth was observed for JY543 at 37°C.

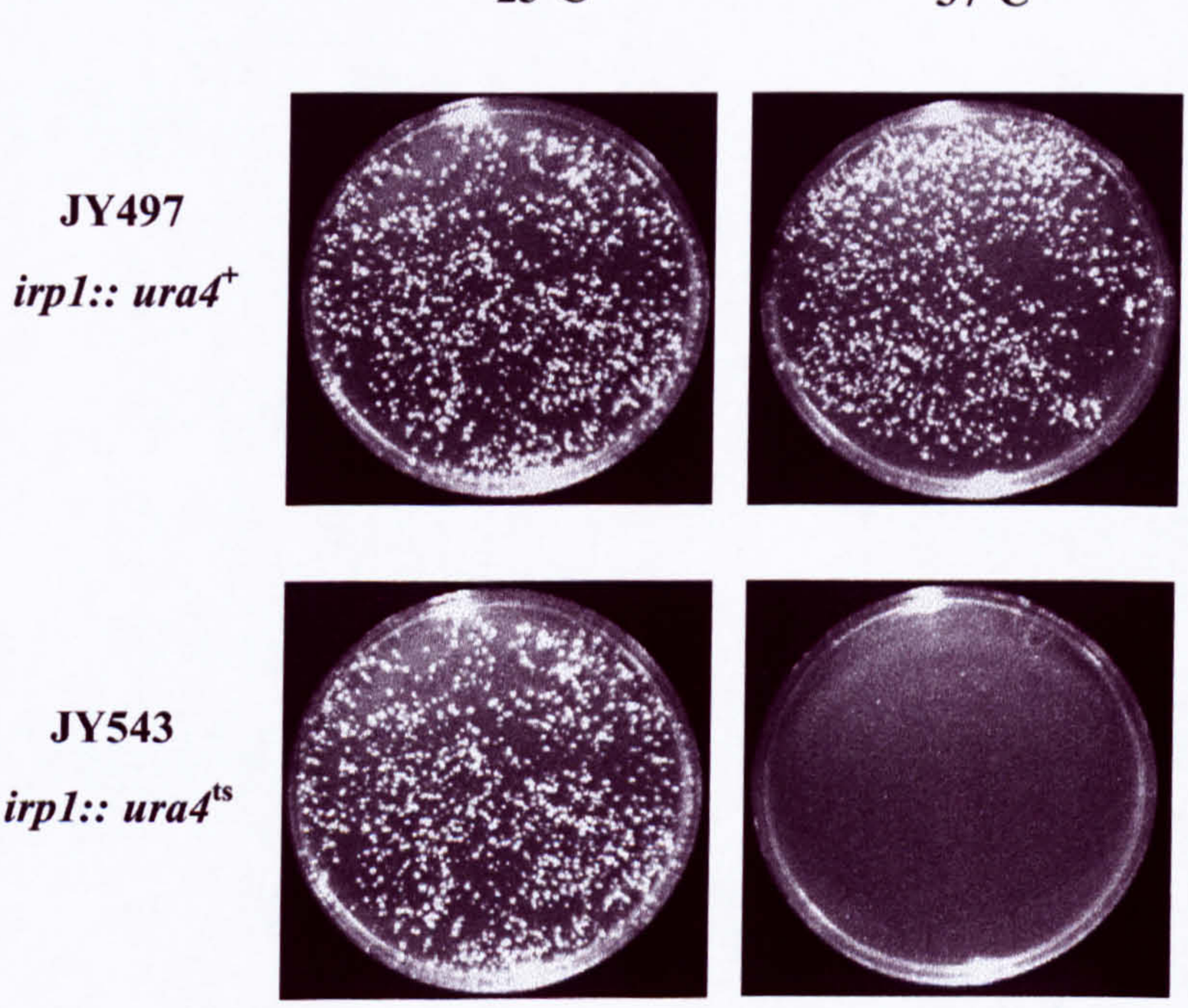


Figure 26. JY543 is incapable of growth on AA plates lacking uracil at 37°C

JY497 (*irp1*::*ura4*$^{+}$) and JY543 (*irp1*::*ura4*^{ts}) were cultured overnight in AA medium lacking uracil, and x10^{6} cells plated on AA medium lacking uracil. Plates were incubated at 23°C or 37°C for 3d. JY497 was capable of forming colonies at both 23°C and 37°C, while JY543 formed colonies at 23°C but not 37°C.

3.4. Disruption of *prk1* with the *ura4*$^{+}$ cassette in the *irp1*::*ura4*^{ts} strain

One aim of creating a temperature-sensitive *ura4* allele was to enable sequential gene disruptions to be carried out. Disruption of the *irp1* gene with the *ura4*^{ts} cassette in JY543 (*ura4-D18, irp1*::*ura4*^{ts}) caused the strain to exhibit a temperature-sensitive requirement for uracil when plated on uracil-deficient medium at 37°C. The next stage of the investigation was to use the wild-type *ura4* cassette to disrupt another gene, *prk1*, in a strain already possessing a single genomic copy of the *ura4*^{ts} cassette (JY543).

Prk1p (protein kinase) is the *Sz. pombe* homologue of the *Saccharomyces cerevisiae* UME5p protein kinase (Watson and Davey, 1998) that is involved in regulating gene transcription in response to a variety of stimuli. Disruption of *prk1* has been shown to have little effect on cell behaviour other than increasing the tendency of cells to flocculate (Watson and Davey, 1998).

3.4.1. Generation of the *prk1*::*ura4*$^{+}$ disruption construct

A construct containing the *prk1* 5' and 3' non-coding regions had previously been constructed in the Davey laboratory by Dr. Peter Watson. A construct containing the *prk1* ORF flanked by its 5' and 3' non-coding region was used as a template to generate a construct in which the *prk1* 5' and 3' non-coding regions are separated by a *Bam*HI site. The antisense primer JO491 includes 28 bases complementary to the 5' non-coding region immediately upstream of the *prk1* initiator codon and a terminal *Bam*HI site. The sense primer JO490 includes 29 bases complementary to the 3' non-coding immediately downstream of the *prk1* stop codon and a terminal *Bam*HI site. The amplified fragment was circularised to generate a construct containing the *prk1* 5' and 3' non-coding regions separated by a *Bam*HI site. The *ura4*$^{+}$ cassette was introduced between the *prk1* non-coding regions as a *Bam*HI fragment (Figure 27).

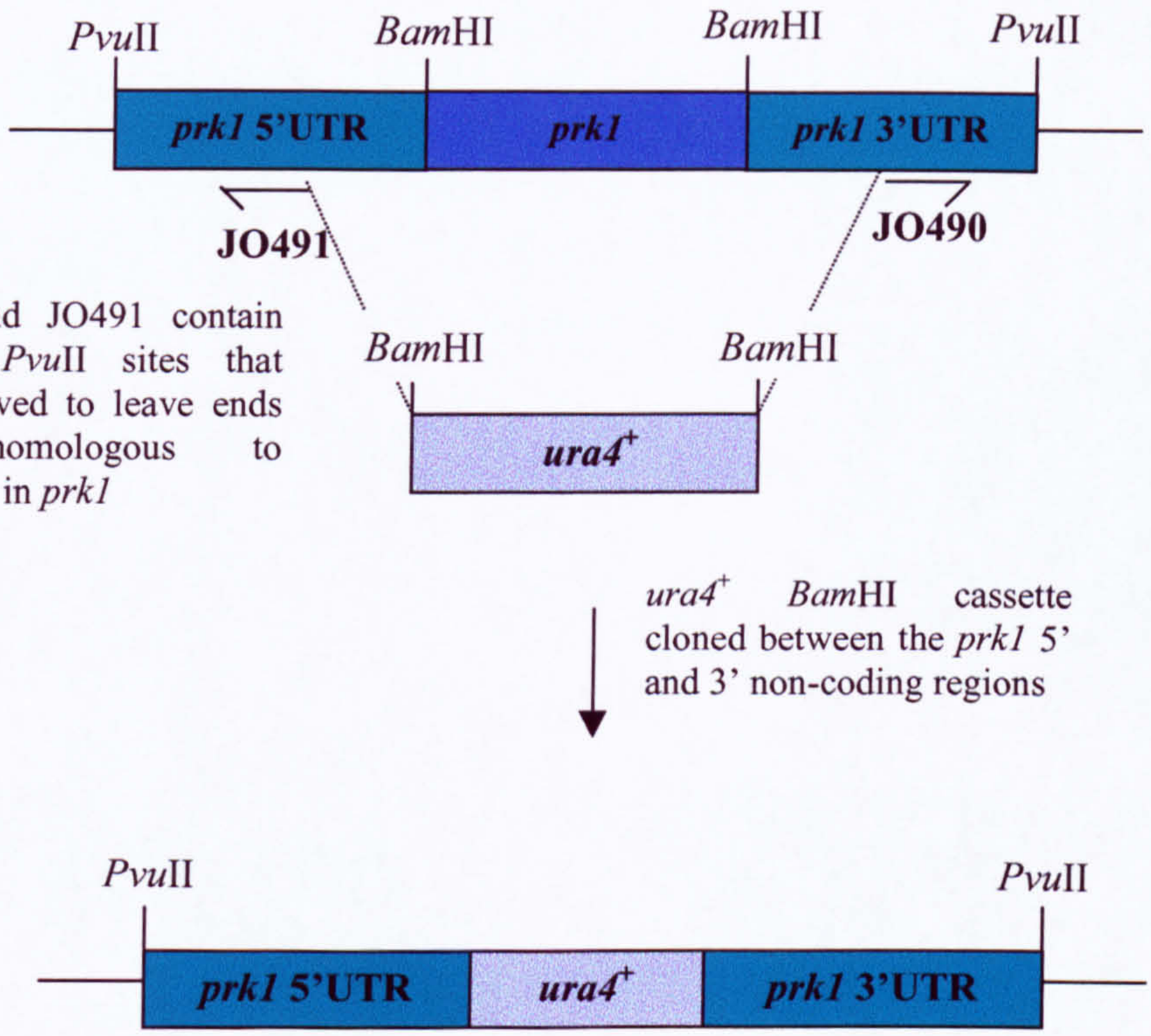


Figure 27. Generation of a *prk1*::*ura4*$^+$ disruption construct

The *ura4*$^+$ cassette was cloned between the *prk1* 5' and 3' non-coding regions in pBluescript as a *Bam*HI fragment. JY383 was transformed with the *prk1*::*ura4*$^+$ disruption construct liberated by digestion with *Pvu*II.

3.4.2.Disruption of *prk1* with the wild-type *ura4*$^+$ cassette in JY543 (*irp1*::*ura4*^{ts})

JY543 (*ura4-D18, irp1*::*ura4*^{ts}) was transformed with a linear *Pvu*II fragment containing the *prk1*::*ura4*$^+$ disruption construct. Transformants were plated on uracil-deficient AA plates and incubated at 37°C for 3d. Plating transformants at 37°C was carried out to prevent the growth of any JY543 cells in which the *prk1*::*ura4*$^+$ construct had not successfully integrated at the *prk1* locus. Transformants were screened for integration of the *ura4*$^+$ cassette at the *prk1* locus using oligonucleotide primers specific for the *prk1* 5' non-coding region and the *ura4*$^+$ cassette. Southern blot analysis was carried out for isolates in which integration of the *ura4*$^+$ cassette at the *prk1* locus had been confirmed by PCR.

Southern blot analysis confirmed that the wild-type *ura4*$^+$ cassette had integrated correctly and exclusively at the *prk1* locus in JY543 (Figure 28), generating JY606 (*ura4-D18, irp1*::*ura4*^{ts}, *prk1*::*ura4*$^+$).

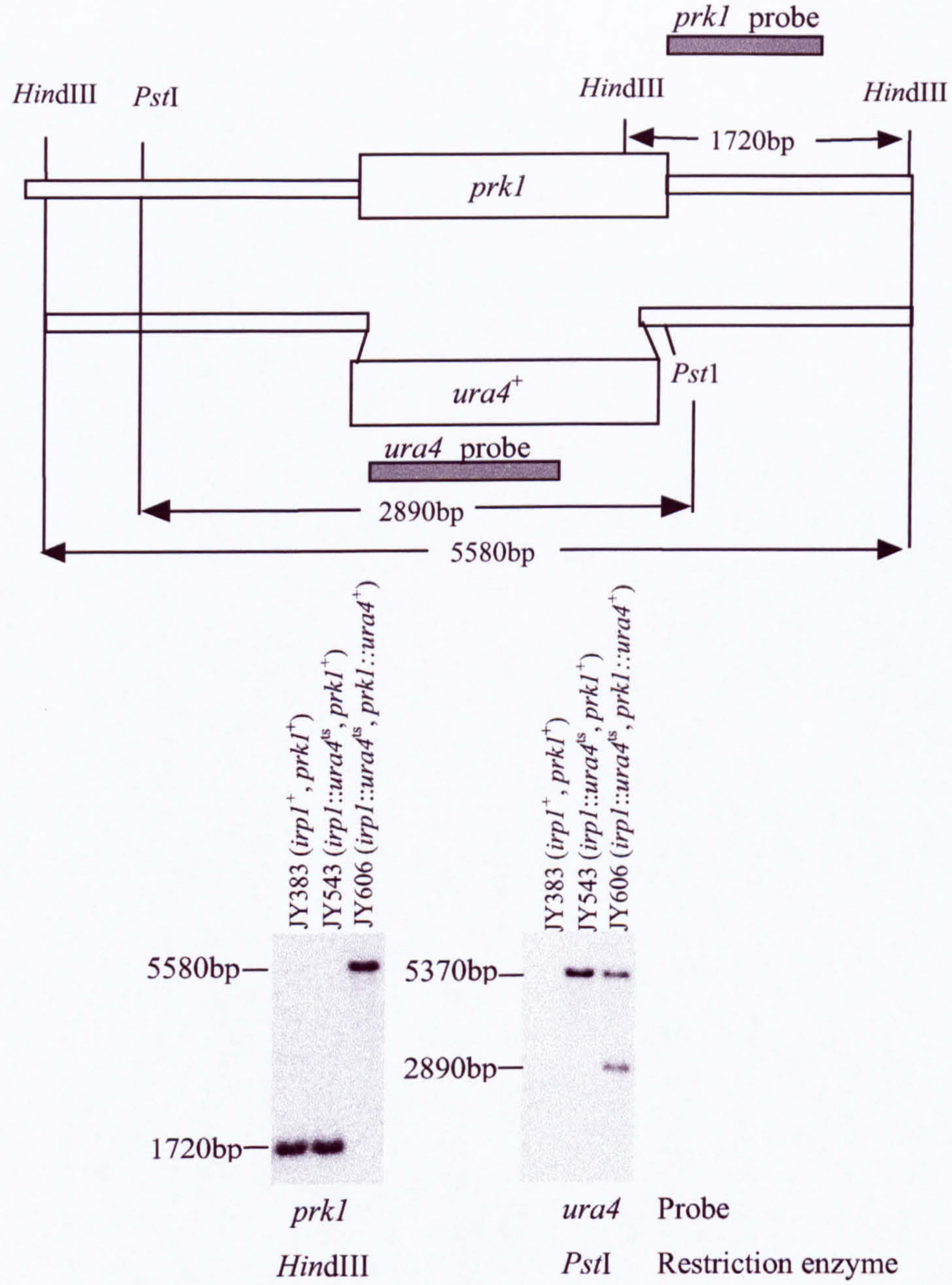


Figure 28. Disruption of *prk1* with *ura4*$^+$ in the *irp1*::*ura4*^{ts} strain

Genomic DNA from JY383 (*irp1*$^+$, *prk1*$^+$), JY543 (*irp1*::*ura4*^{ts}, *prk1*$^+$) and JY606 (*irp1*::*ura4*^{ts}, *prk1*::*ura4*$^+$) was digested with *Hin*dIII. Digested DNA was separated on a 1% agarose gel, blotted onto a nitrocellulose filter, and probed with a *prk1* 3' fragment. The presence of the *ura4*$^+$ cassette at the *prk1* locus was indicated by a band of 5580bp, demonstrating homologous recombination had occurred at the *prk1* locus. In the absence of homologous integration occurring a band of 1720bp indicated an undisrupted *prk1* locus. Genomic DNA from JY383, JY543 and JY606 was also digested with *Pst*I and probed with an *ura4* open reading frame fragment. Homologous integration of the *ura4*$^+$ cassette was indicated by a band of 2890bp (a band of 5370bp results from the integration of *ura4*^{ts}) while the undisrupted *prk1* locus gave no band of 2890bp (a band of 5370bp results from the integration of the *ura4*^{ts} cassette).

3.5. Uracil requirement of *ura4*$^{+}$ and *ura4*^{ts} strains

The temperature-dependent uracil requirement of the following strains were determined:

JY383 (*ura4-D18, irp1*$^{+}$, *prk1*$^{+}$),

JY497 (*ura4-D18, irp1*::*ura4*$^{+}$, *prk1*$^{+}$),

JY543 (*ura4-D18, irp1*::*ura4*^{ts}, *prk1*$^{+}$),

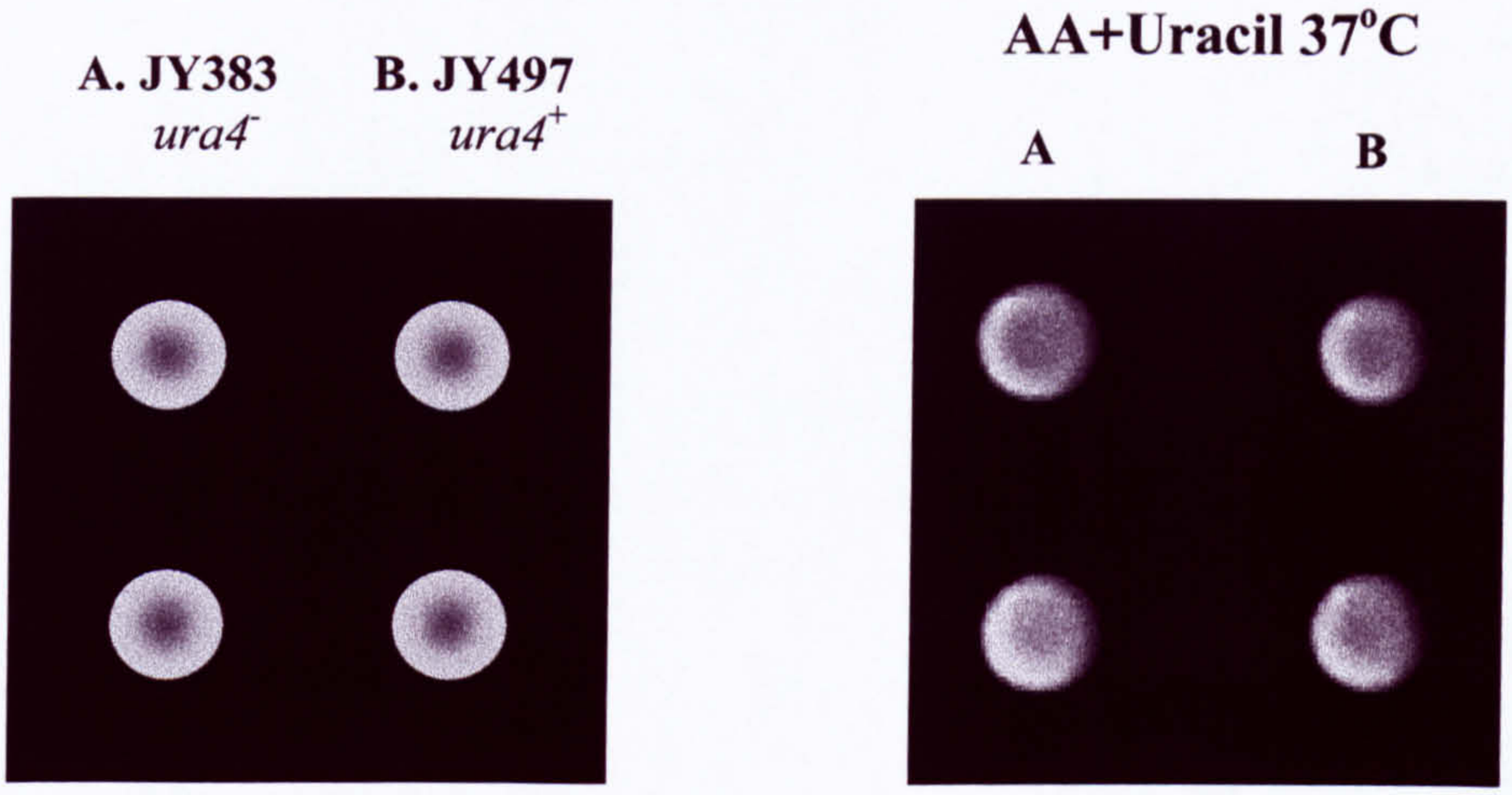
JY606 (*ura4-D18, irp1*::*ura4*^{ts}, *prk1*::*ura4*$^{+}$).

Patches of cells were plated onto selective plates and incubated at 23°C or 37°C. Briefly, a small colony was suspended in 20µl water, and 2µl spotted onto an AA plate containing or lacking uracil. Plates were incubated at 23°C or 37°C (Figure 29).

JY497 and JY606 contain a genomic copy of the wild-type *ura4* cassette and grew on AA plates in the absence of uracil at both 23°C and 37°C. JY383 lacks a genomic copy of *ura4* and grew on AA plates containing uracil at 37°C, but did not grow on AA plates lacking uracil at either 23°C or 37°C. This confirmed that the absence of an Ura4p function did not enable sustained growth on AA plates lacking uracil at either temperature.

JY543 was capable of growth on AA plates lacking uracil at 23°C and AA plates containing uracil at 37°C. JY543 was however incapable of sustained growth on AA plates lacking uracil at 37°C. Thus, at 37°C the activity of the temperature-sensitive *ura4*^{ts} expressed from a single genomic copy appeared to be too low to enable sustained growth on solid AA media lacking uracil.

JY383 and JY543 appeared to undergo mitotic growth for a limited number of divisions under conditions expected to inhibit growth (seen as a faint discs of growth in Figure 29). This can be explained by the build-up of an intracellular pool of uracil prior to cells being plated onto selective plates.



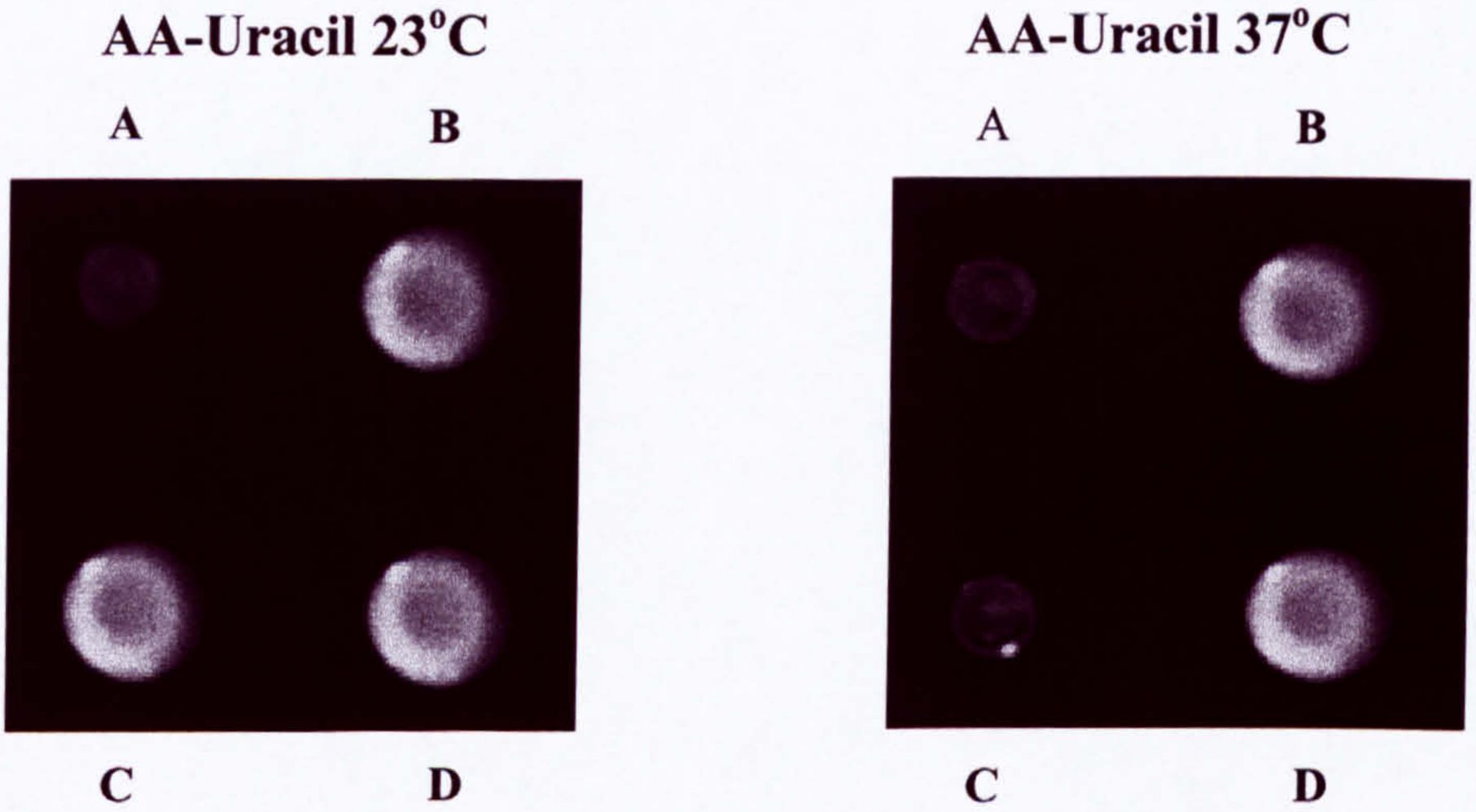


Figure 29. Viability of strains expressing Ura4^{+}p and Ura4^{ts}p at 23°C and 37°C

The viability of JY383 (*irp1*$^{+}$, *prk1*$^{+}$), JY497 (*irp1*::*ura4*$^{+}$, *prk1*$^{+}$), JY543 (*irp1*::*ura4*^{ts}, *prk1*$^{+}$) and JY606 (*irp1*::*ura4*^{ts}, *prk1*::*ura4*$^{+}$) at 23°C and 37°C was determined on AA plates containing or lacking uracil. The absence of Ura4 activity in JY383 prevented growth on AA plates lacking uracil at both 23°C and 37°C. Disruption of the *irp1* gene with the *ura4*$^{+}$ cassette in JY497 enabled growth in the absence of uracil at 23°C and 37°C. The *irp1* gene in JY543 is disrupted with the *ura4*^{ts} cassette, and this prevented JY543 growth on AA plates lacking uracil at 37°C while enabling growth on AA plates lacking uracil at 23°C. Expression of the wild-type Ura4p in JY606 enabled growth on uracil-deficient AA plates at both 23°C and 37°C.

JY543 (*irp1*::*ura4*^{ts}) showed a temperature-sensitive requirement for uracil when grown on solid media. This probably indicates that Ura4^{ts}p exhibits decreased activity at 37°C compared to that at 23°C, as a consequence of a leucine-to-proline substitution at amino acid residue 261. On solid media lacking uracil, the reduced Ura4^{ts}p activity at 37°C was insufficient to enable sustained growth of JY543.

3.6. Overexpression of Ura4^{+}p and Ura4^{ts}p

It appeared that in single copy there was insufficient Ura4^{ts}p activity to enable growth on solid media lacking uracil at 37°C. In liquid media lacking uracil, JY543 (*irp1*::*ura4*^{ts}) was initially capable of growth following a shift to the restrictive temperature of 37°C. To further characterise Ura4^{ts}p activity, the *ura4*$^{+}$ and *ura4*^{ts} ORFs were cloned into repressible *Sz. pombe* expression vectors to determine the growth characteristics of JY383 (*ura4-D18*) expressing the wild-type Ura4p and the temperature-sensitive Ura4^{ts}p.

3.6.1. The *Sz. pombe* pREP expression vectors

The pREP expression vector series (Maundrell, 1993) includes thiamine-repressible *nmt1* promoter TATA-box mutants that enable the expression level of a protein to be determined by the choice of construct and presence or absence of thiamine in the growth media. The pREP3X vector possesses the full-strength *nmt1* promoter, whilst the pREP81X vector (possessing a mutated TATA-box within the *nmt1* promoter) results in lower levels of expression (Basi *et al.*, 1993). Expression from both vectors can be repressed by the addition of thiamine in growth media.

3.6.2. Growth characteristics of JY383 transformed with pREP-*ura4* constructs

The *ura4*$^{+}$ and *ura4*^{ts} open reading frames were cloned into pREP3X and pREP81X downstream of the *nmt1* promoter (Figure 30). The sense primer JO1056 contains 31 bases complementary to the *ura4* ORF initiating at nucleotide position +1 relative to the *ura4* initiator codon and contains a terminal *Bam*HI site. The antisense primer JO1057 contains 28 bases complementary to the *ura4* ORF initiating at nucleotide position +803 relative to the *ura4* initiator codon, and contains a terminal *Bam*HI site. The amplified fragments were restricted with *Bam*HI and cloned into the *Bam*HI site of pREP3X and pREP81X.

JY383 (*ura4-D18*) was transformed with the *ura4* expression constructs, and transformants were plated on AA plates lacking leucine (to select for cells transformed with the expression constructs) and incubated at 23°C.

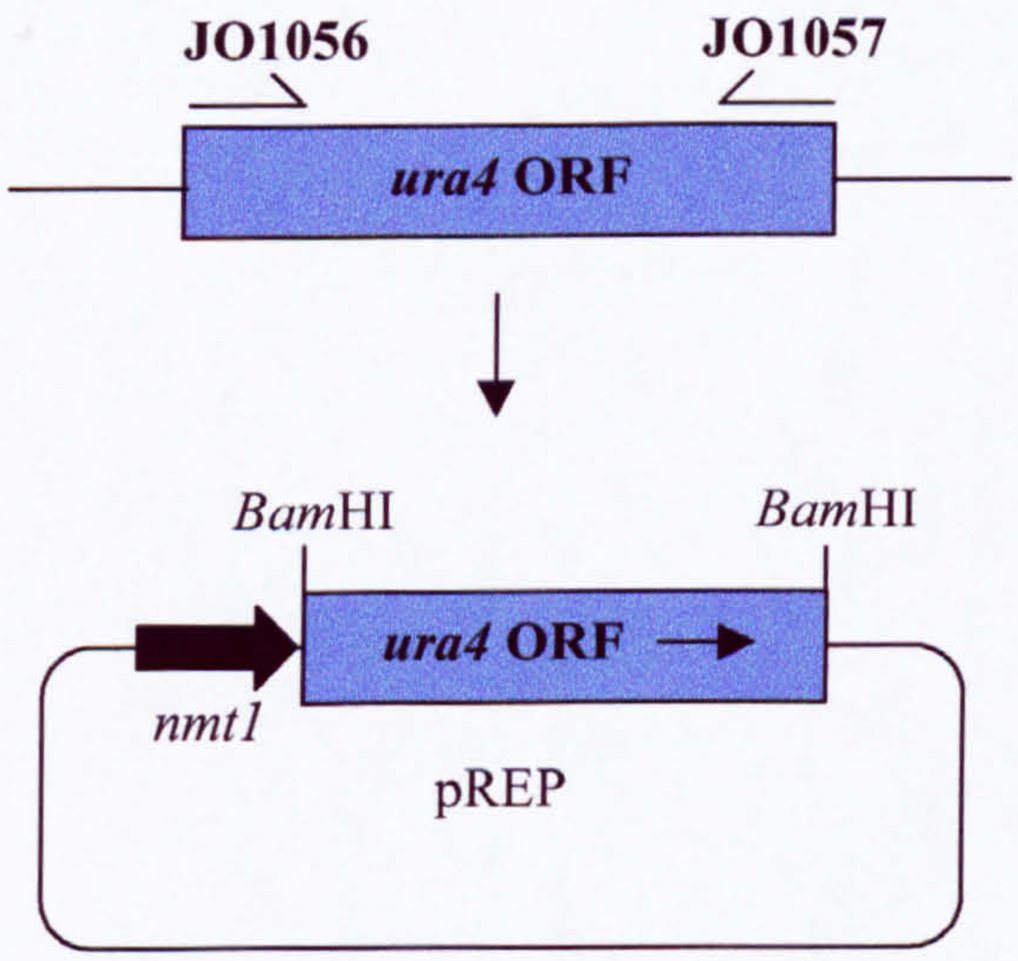


Figure 30 Cloning the *ura4* ORFs into the *Sz. pombe* pREP vectors

The wild-type and temperature-sensitive *ura4* ORFs were amplified using the primers JO1056 and JO1057, which contain terminal *Bam*HI sites. The *ura4*$^{+}$ and *ura4*^{ts} ORF *Bam*HI fragments were cloned into the *Bam*HI site of pREP3X and pREP81X.

Growth characteristics of JY383 transformed with pREP3x-*ura4*$^{+}$, pREP3x-*ura4*^{ts}, pREP81x-*ura4*$^{+}$ and pREP81X-*ura4*^{ts} were determined in minimal media containing adenine (lacking uracil and leucine) in the presence or absence of thiamine at 29°C or 37°C. 50ml medium was inoculated at a cell density of $2x10^5$ cells/ml, and cells were cultured in a shaking incubator at 29°C. Once strains reached a cell density of approximately $1x10^6$ cells/ml, cultures were either left at 29°C or transferred to a 37°C shaking water bath (control experiments indicated media took less than 10 min to reach 37°C). Cell density was monitored using a Coulter Channelyser.

Figure 31 shows the effects of temperature and thiamine upon the mitotic growth of JY383 (*ura4-D18*) cells transformed with pREP3X-*ura4*$^{+}$ (JY1108). At 29°C the generation time of JY1108 increased from 2½h in the absence of thiamine to 6h in the presence of thiamine, indicating repression of Ura4p expression from the *nmt1* promoter. In the absence of thiamine, the shift from 29°C to 37°C resulted in a lag in mitotic growth, possibly as a consequence of the metabolic burden of expressing heat-shock proteins. Following the temperature shift, growth resumed after approximately 6h, after which time JY1108 exhibited a generation time of 3h. The presence of thiamine had a greater detrimental effect upon growth at 37°C than at 29°C, halting mitotic growth. The slower generation time of JY1108 cultured at 29°C in the presence of thiamine indicates that reduced intracellular Ura4p activity retarded growth at 29°C, as a consequence of lower Ura4^{+}p expression levels (resulting from repression of *nmt1* promoter activity). At 37°C the reduced level of Ura4^{+}p expression as a result of thiamine repression was insufficient to sustain growth.

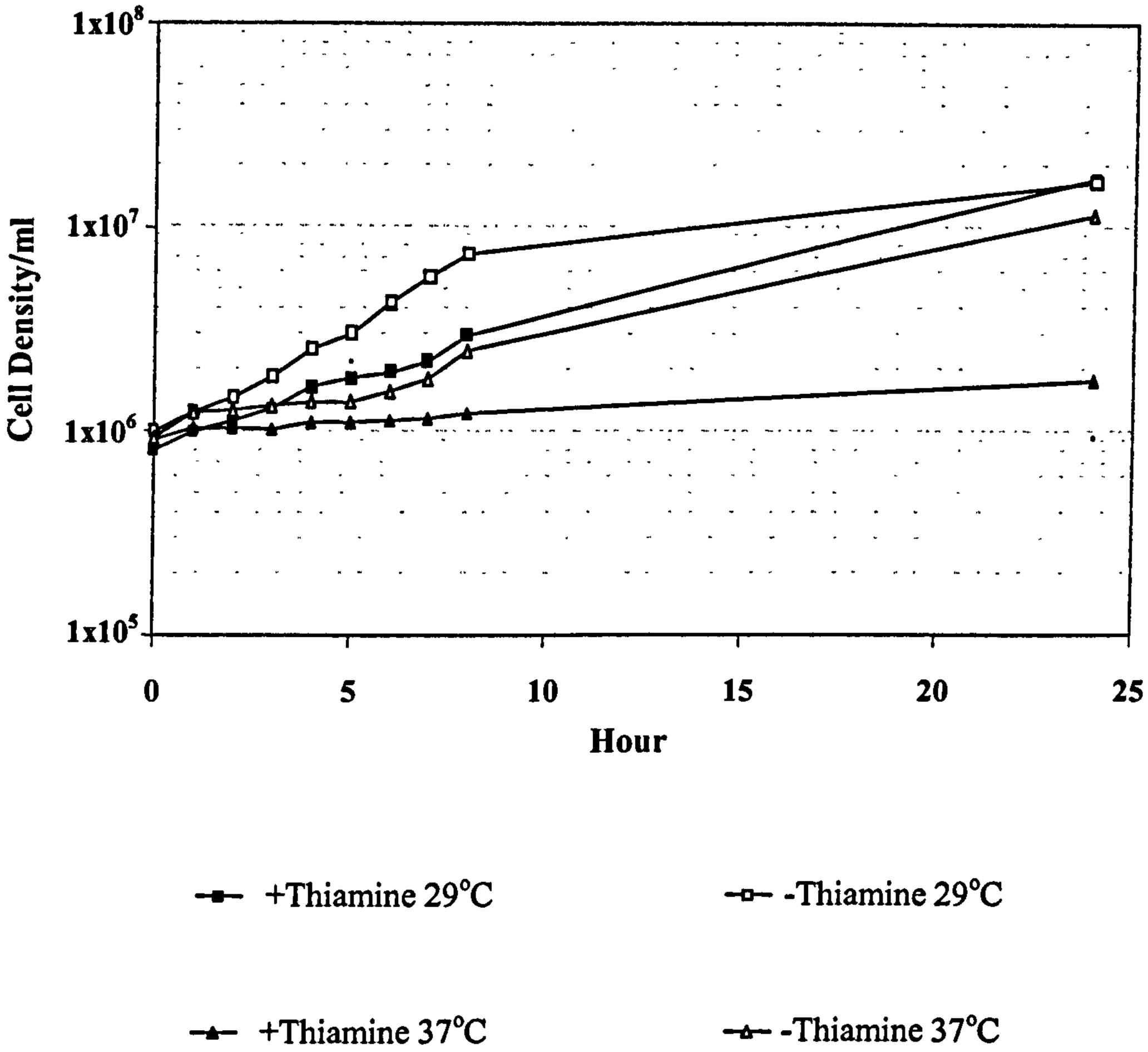


Figure 31. Growth characteristics of JY383 transformed with pREP3X-*ura4*$^+$

Growth characteristics of JY383 transformed with pREP3x-*ura4*$^+$ (JY1108) were determined in minimal medium containing adenine (lacking uracil and leucine) in the presence or absence of thiamine at both 29°C and 37°C. The addition of thiamine slowed growth at both 29°C and 37°C. JY1108 exhibited a lag in mitotic growth when shifted to 37°C in both the presence and absence of thiamine. Growth was not resumed when thiamine was present in the medium at 37°C. At 29°C JY1108 had a generation time of 2.5h in the absence of thiamine and 6h in the presence of thiamine. At 37°C a generation time of 4h was observed in the absence of thiamine and 24h in the presence of thiamine.

JY1109 (pREP81X-*ura4*$^+$) exhibited very slow generation times under all conditions, ranging from 7h in the absence of thiamine at 29°C to 16h in the presence of thiamine at 37°C (Figure 32). The presence of thiamine slowed growth at both 29°C and 37°C. The low level of Ura4$^+$p expression from pREP81X is illustrated in this experiment, as even in the absence of thiamine the generation time of JY1109 was much slower than that seen for JY1108 (pREP3X-*ura4*$^+$) at 29°C (7h compared to 2.5h). The repressive effect of thiamine upon Ura4p expression from pREP81X is indicated by the reduced generation times of JY1109 at both 29°C and 37°C in the presence of thiamine. The use of pREP81X to direct Ura4p expression indicates that a reduced level of intracellular Ura4 activity can have a detrimental effect upon the generation time of JY383.

Figure 33 shows the effects of temperature and thiamine upon the growth of JY383 cells transformed with pREP3X-*ura4*^{ts} (JY1110). The shortest generation time (3½h) was achieved at 29°C in the absence of thiamine. In the presence of thiamine at 29°C the generation time of JY1110 increased to approximately 6h. Upon the shift to 37°C, JY1110 exhibited a lag in mitotic growth. At 37°C in the absence of thiamine JY1110 growth resumed after approximately 8h (with a generation time of approximately 7h). In the presence of thiamine at 37°C JY1110 did not resume growth within 24h, and the cells remained at a density of approximately $1x10^6$ cells/ml.

When Ura4^{ts} was expressed from pREP3X in JY1110, cells exhibited slower generation times at 29°C in the absence of thiamine compared to JY1108, in which the wild-type Ura4$^+$p is expressed from pREP3X. This indicates that when expressed from pREP3X the activity of Ura4^{ts}p is reduced compared to that of the wild-type Ura4 protein, and as a result JY1110 (pREP3X-*ura4*^{ts}) cells exhibited slower generation times than JY1108 (pREP3X-*ura4*$^+$).

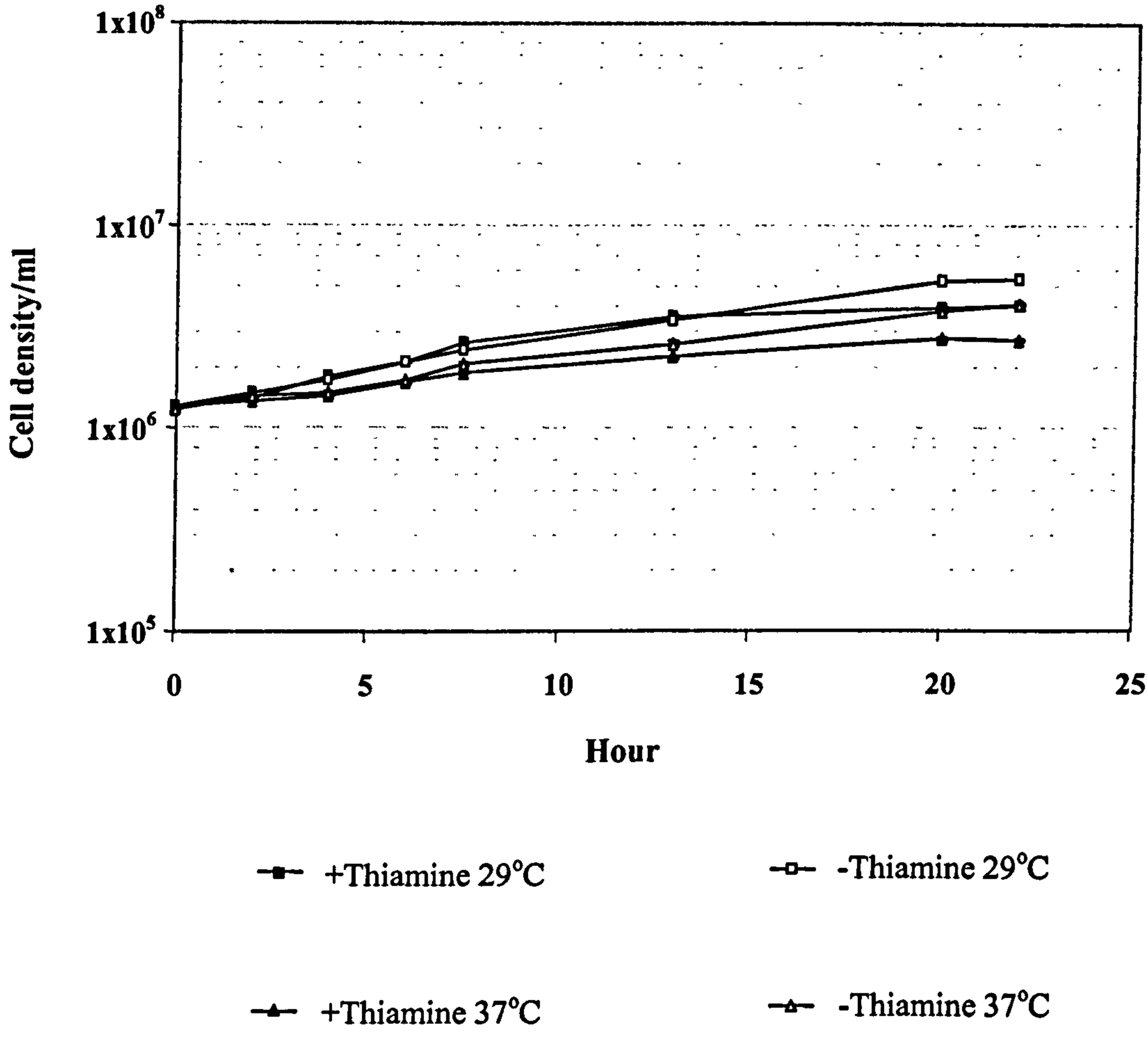


Figure 32. Growth characteristics of JY383 transformed with pREP81X-*ura4*$^+$

Growth characteristics of JY383 transformed with pREP81X-*ura4*$^+$ (JY1109) were determined in minimal medium containing adenine (lacking uracil and leucine) in the presence or absence of thiamine at both 29°C and 37°C. JY1109 exhibited slow generation times under all conditions. The presence of thiamine in media further slowed JY1109 growth. JY1109 had a generation time of 7h in the presence and absence of thiamine. At 37°C JY1109 had a generation time of 12h in the absence of thiamine and 16h in the presence of thiamine.

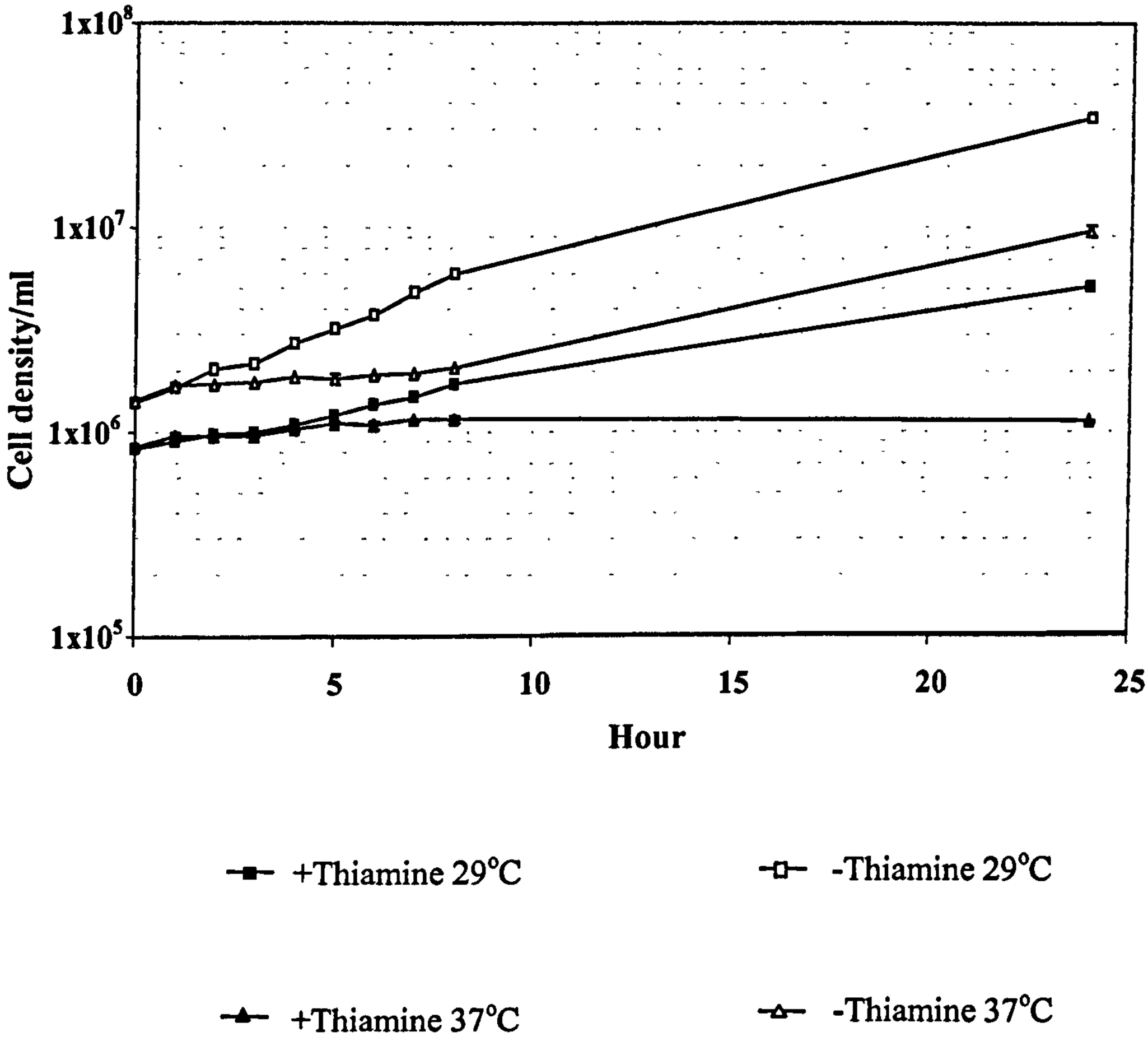


Figure 33. Growth characteristics of JY383 transformed with pREP3X-*ura4^{ts}*

Growth characteristics of JY383 transformed with pREP3X-*ura4^{ts}* (JY1110) were observed in minimal medium containing adenine (lacking uracil and leucine) in the presence or absence of thiamine at both 29°C and 37°C. At 29°C JY1110 had a doubling time of 3½h in the absence of thiamine and 6h in the presence of thiamine. At 37°C, JY1110 exhibited a lag in mitotic growth, which was not resumed in the presence of thiamine. In the absence of thiamine at 37°C JY1110 exhibited a doubling time of 7h.

JY383 transformed with the pREP81X-*ura4*^{ts} expression construct (JY1111) was incapable of growth at either 23°C or 37°C, regardless of the presence or absence of thiamine. This indicates that the low Ura4^{ts}p expression level directed by pREP81X reduced Ura4 activity to a level that was insufficient to support growth. The fact that JY1109 (pREP81X-*ura4*$^{+}$) was capable of growth at 29°C (albeit with a slow generation time of 7h) supports the hypothesis that Ura4^{ts}p exhibits a lower level of Ura4 activity compared to wild-type Ura4p.

In the presence of uracil, all strains were capable of growth (data not shown) and exhibited similar generation times at 29°C (3.5h) and 37°C (2.5h).

3.6.3. Colony formation of JY383 transformed with pREP-*ura4* constructs

To further investigate the activity of Ura4^{ts}p, the ability of JY383 transformed with *ura4*$^{+}$ and *ura4*^{ts} expression constructs to form colonies on selective plates lacking uracil was determined. Briefly, cells were inoculated in minimal medium lacking uracil and leucine. The absence of leucine selects for transformed cells, and the absence of uracil ensures that growth is only achieved by cells with a sufficient level of intracellular Ura4 activity. Strains were cultured at 23°C overnight, and 1 x 10^5 cells were plated onto selective plates (containing or lacking thiamine). Plates were incubated at 23°C or 29°C to determine whether increased temperature affects the activity of Ura4^{ts}p.

High expression levels of Ura4^{+}p (expressed from the pREP3X expression vector) enabled JY1108 growth at both 23°C and 29°C in the presence and absence of thiamine (Figure 34). At low Ura4^{+}p expression levels (when Ura4^{+}p expression levels were directed by the pREP81X *nmt1* promoter), some JY1109 growth was observed at 29°C, but this was visibly less than that seen for pREP3X-driven Ura4^{+}p expression at 29°C. No growth was observed for JY1109 at 23°C. At 29°C, expression levels of Ura4^{+}p in JY1109 were further reduced by the presence of thiamine, as indicated by smaller colony sizes.

Expression of Ura4^{ts}p from the pREP3X expression construct (JY1110) enabled growth at both 23°C and 29°C, but only in the absence of thiamine. Presumably the presence of thiamine repressed the expression of Ura4^{ts}p from the pREP3X expression construct to a level that was insufficient to sustain growth at

both temperatures. Expression of Ura4^{ts}p from pREP81X in JY1111 (resulting in lower levels of expression compared to the pREP3X vector) did not enable growth at 23°C or 29°C, regardless of the presence or absence of thiamine. This indicates that when expressed from pREP81X the level of Ura4^{ts}p expression was insufficient to sustain JY383 growth regardless of the presence or absence of thiamine.

These results reflect the different expression levels achieved with the pREP expression vectors, and illustrate how different levels of Ura4p activity can be qualitatively determined. The results indicate that at 23°C, thiamine repression of *ura4*^{ts} expression from pREP3X in JY383 reduced intracellular Ura4 activity to a level incapable of sustaining growth. Thiamine repression of wild-type Ura4^{+}p expression at 23°C slowed growth of JY383, but there was still sufficient Ura4 activity to sustain growth. The results demonstrate that Ura4^{ts}p retains some activity at 29°C, but that the protein must be overexpressed for this to be sufficient to support cell growth.

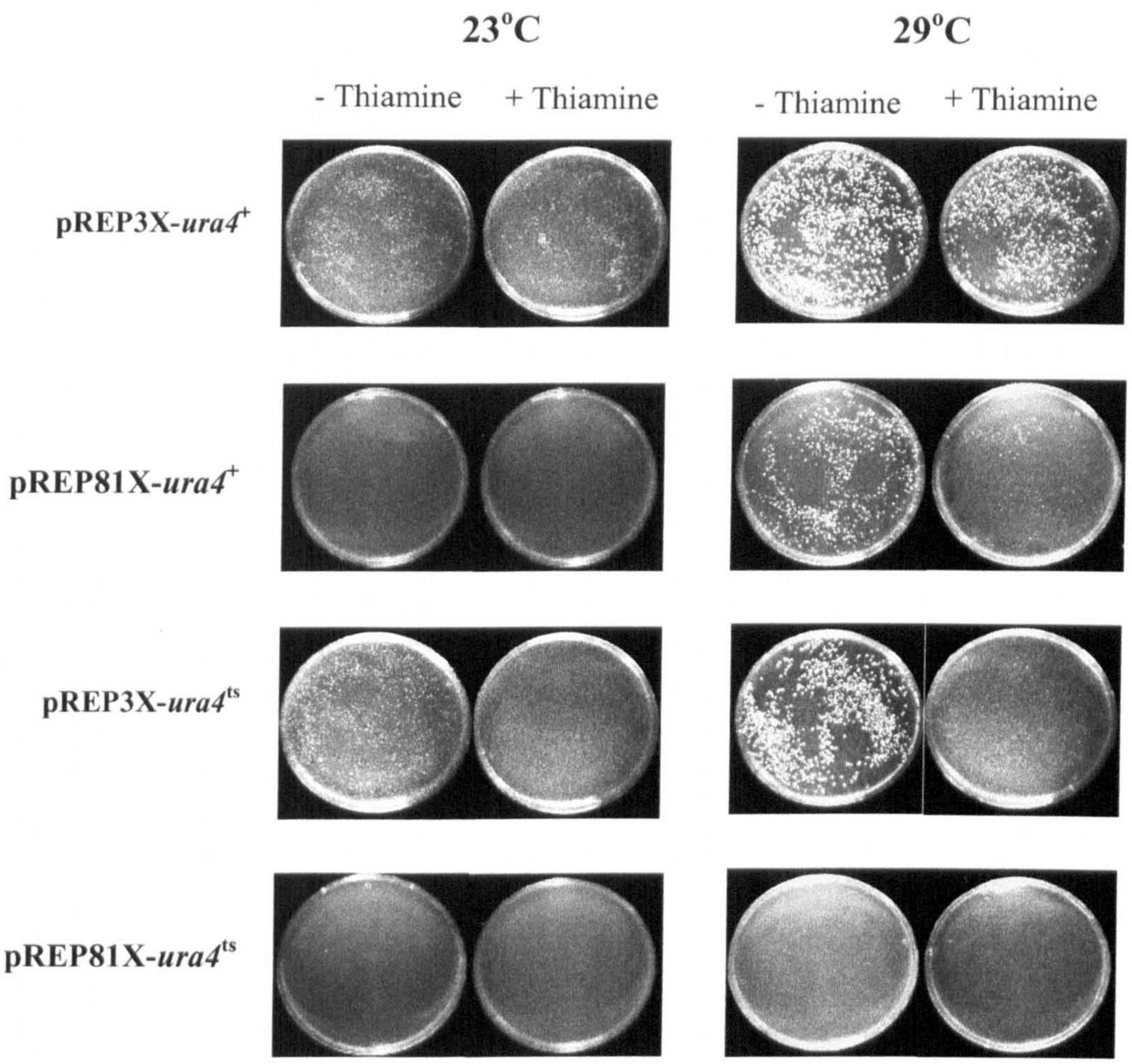


Figure 34. Ura4^{ts}p exhibits reduced activity compared to the wild-type Ura4p

JY1108 (pREP3x-*ura4*$^{+}$), JY1109 (pREP81x-*ura4*$^{+}$), JY1110 (pREP3x-*ura4*^{ts}) and JY1111 (pREP81x-*ura4*^{ts}) were plated onto AA plates lacking uracil and leucine and either containing or lacking thiamine. Strains were plated at 23°C or 29°C. The repressive effects of thiamine upon growth were more pronounced for the JY1110 (pREP3x-*ura4*^{ts}) than JY1108 (pREP3x-*ura4*$^{+}$), and JY1111 (pREP81x-*ura4*^{ts}) was incapable for forming colonies under any of the conditions. The results indicate that reduced Ura4 activity is reflected in a reduced capacity to form colonies, and that Ura4^{ts}p exhibits reduced activity compared to wild-type Ura4p.

3.7. Summary

A PCR-based random mutagenesis approach was used to isolate a temperature-sensitive *Sz. pombe* Ura4p protein. A single point mutation at nucleotide 782 in the open reading frame results in a leucine to proline substitution at residue 261 in the primary sequence of Ura4p.

The wild-type Ura4p supports growth at 37°C in the absence of uracil. When expressed from a single genomic copy, the reduced Ura4 activity of the *ura4^{ts}* allele prevented the sustained growth of strains on media lacking uracil at 37°C, while supporting growth at 23°C. The reduced activity of Ura4^{ts}p was demonstrated when the Ura4^{ts}p open reading frame was expressed from multicopy thiamine-repressible *Sz. pombe* expression vectors. These results reinforce the hypothesis that Ura4^{ts}p exhibits reduced activity relative to wild-type Ura4^{+}p, and that increased temperatures reduce Ura4^{ts}p activity.

This chapter illustrates how the *ura4^{ts}* cassette can be used to carry out multiple, sequential gene disruptions. The *ura4^{ts}* cassette can be used to disrupt a gene of choice by selecting for homologous integrants (*gene1*::*ura4^{ts}*) that can grow at low temperatures but are incapable of growth at high temperatures. The wild-type *ura4* cassette can then be used to disrupt a second gene of choice in the *gene1*::*ura4^{ts}* strain by selecting for transformants at the restrictive temperature. The Ura4^{ts}p marker simplifies discrimination between homologous integration events and maintenance of the *ura4^{ts}* gene on multi-copy, autonomously replicating plasmids, as multiple copies of the *ura4^{ts}* allele enable growth at high temperatures, whereas a single copy does not.

Chapter 4. Expression of mammalian RGS proteins in *Sz. pombe* reporter strains

Section I: Expression of RGS proteins

4.1. Introduction

The prototype member of the RGS (Regulators of G protein Signalling) family of proteins, SST2, was isolated during a genetic screen of *Saccharomyces cerevisiae* mutants hypersensitive to pheromone stimulation (Chan and Otte, 1982a; 1982b). Following isolation of *S. cerevisiae* SST2p, proteins in higher eukaryotes were identified that interact with or regulate G proteins and share sequence similarity with SST2. Such SST2 homologues are now known as RGS proteins. The budding yeast has since been used as a model system to assess the ability of heterogeneous RGS proteins to blunt the pheromone response pathway. The ability of human RGS1, RGS2, RGS3, RGS4, and GAIP to act as GAPs in the budding yeast has been shown for *S. cerevisiae* strains lacking SST2p (Druey *et al.*, 1996). The expression of these human RGS proteins partially complemented the pheromone-hypersensitive phenotype of an ΔSST2 strain.

The *Sz. pombe* pheromone communication pathway also presents a useful tool for the investigation of G protein-coupled signalling pathways. At present only one G_α-RGS pair has been identified in *Sz. pombe* (Watson *et al.*, 1999; Pereira and Jones, 2001), and it is involved in the regulation of the pheromone communication pathway. In *Sz. pombe* the activated G_α subunit propagates the pheromone signal to downstream second messengers. Two cognate G_α-RGS pairs have been identified in *S. cerevisiae* (Dohlman *et al.*, 1996; Versele *et al.*, 1999), and while they provide non-overlapping functions in *S. cerevisiae*, it is possible that heterogeneous signalling proteins expressed in this yeast may affect both G protein-coupled signalling pathways to varying extents. Another difference between the two yeasts lies in the subunit responsible for propagating the pheromone signal downstream of the G protein. In *S. cerevisiae* the $G_{\beta\gamma}$ subunit is primarily responsible for activating downstream effectors (Nakayama *et al.*, 1988; Blinder *et al.*, 1989; Whiteway *et al.*, 1989). The affinity of RGS proteins for the G_α subunits of the two yeasts may also differ, and as a consequence, *Sz. pombe* may offer a more suitable model system for studying the activity of specific RGS proteins, as well as different components comprising G protein-coupled signalling pathways.

4.2.1. *Sz. pombe sxa2::lacZ* reporter strains

Dr. Kevin Davis and Dr. Mark Didmon constructed *Sz. pombe* reporter strains in the Davey laboratory to aide the investigation of signalling through the *Sz. pombe* pheromone communication pathway. In these strains, the reporter proteins bacterial β–galactosidase (LacZ) and *Sz. pombe* orotidine-5'-monophosphate decarboxylase (Ura4) are utilised. The expression of these reporter proteins is directed by the promoter of a tightly regulated pheromone-inducible gene, *sxa2*, which encodes a serine carboxypeptidase (Imai and Yamamoto, 1994; Ladds *et al.*, 1996). Expression of the *sxa2* gene product is induced upon signalling through the pheromone communication pathway in *Sz. pombe* M cells, and aids desensitisation to pheromone stimulation by degrading extracellular P-factor pheromone. Cells lacking the Sxa2p function are hypersensitive to P-factor pheromone, as they are unable to degrade extracellular pheromone. Transcription of *sxa2* is strongly induced within 2h of P-factor stimulation of nitrogen-starved cells (Imai and Yamamoto, 1994), and is tightly repressed after the response (Imai and Yamamoto, 1994; Davey and Nielsen, 1994). Increasing the amount of P-factor causes *sxa2* to be induced more strongly, with the consequence that *sxa2* promoter activity can be modulated according to the pheromone concentration to which cells are exposed.

Additional genetic alterations are present in the reporter strains to aid the investigation of the pheromone signal transduction pathway. In wild-type *Sz. pombe* cells, mating processes are repressed in rich medium via a cAMP pathway, as a result of inhibition of the activity of a transcription factor, Ste11p. The activity of Ste11p is required for the induction of nitrogen-starvation responsive genes (Sugimoto *et al.*, 1991). Mating and sporulation processes are only induced upon nitrogen starvation, as a consequence of a decrease in the intracellular cAMP level (Maeda *et al.*, 1990). The reduction in intracellular cAMP concentration upon starvation can be mimicked by disruption of the *cyr1* gene, encoding adenylate cyclase (mutants defective in *cyr1* have no detectable cAMP [Maeda *et al.*, 1990; Kawamukai *et al.*, 1991]). Disruption of *cyr1* relieves the repression of mating processes normally present in wild-type cells cultured in rich medium, and enables *Sz. pombe* M cells to respond to P-factor stimulation even in the presence of nutrients

(Imai and Yamamoto, 1994; Davey and Nielsen, 1994). Disruption of the *cyr1* gene ensures that the reporter strains do not need to be starved of nutrients before they can become responsive to pheromone.

Mating type in wild-type *Sz. pombe* cells is determined by the information carried at the *mat1* locus. The *mat2* and *mat3* loci store P and M cell mating type information respectively, but this is not expressed. Wild-type *Sz. pombe* spontaneously switch mating type as a result of transposition of mating type information from the silent *mat2* and *mat3* loci to the active *mat1* locus (Kelly *et al.*, 1988). Disruption of the *mat2* and *mat3* loci in the *Sz. pombe* reporter strains ensures cells do not switch mating type, and the reporter strains are thus mating type stable.

In the *Sz. pombe sxa2*::*lacZ* reporter strains the bacterial *lacZ* ORF (encoding β-galactosidase) replaces that of *sxa2*, and LacZ is expressed as a result of signalling through the pheromone communication pathway. The *Sz. pombe* biosynthetic gene *ura4* encodes orotidine-5'-monophosphate decarboxylase (Grimm *et al.*, 1988), which is required for growth in the absence of uracil in growth media. In the *Sz. pombe sxa2*::*ura4* reporter strains, the *ura4* ORF replaces that of *sxa2*, and cells can only grow on media lacking uracil as a result of pheromone signalling.

4.2.2. The *Sz. pombe* Rgs1 protein is a desensitisation factor in the pheromone communication pathway

The *Sz. pombe* Rgs1 protein (szRgs1p) was first identified as a hypothetical ORF showing sequence similarity to proteins classified as RGS proteins (Tesmer *et al.*, 1997; Watson *et al.*, 1999). Disruption of *rgs1* in *Sz. pombe* M cells has been investigated using the *Sz. pombe sxa2*::*lacZ* reporter strains by Watson *et al.* (1999), in which the *lacZ* ORF is driven by *sxa2* promoter activity. Disruption of *rgs1* resulted in hypersensitivity to pheromone, with induction of LacZ expression occurring at a considerably lower pheromone concentration than in cells containing the endogenous *rgs1* gene (Section 4.2.4). Cells lacking *rgs1* also exhibited an increased level of pheromone-independent LacZ expression.

4.2.3. P-factor induces β-galactosidase expression in the *rgs1*$^+$ LacZ reporter strain

The LacZ reporter strain, JY544 (*rgs1*$^+$, *sxa2*::*lacZ*), possesses the genomic copy of the *Sz. pombe rgs1* gene. The *LacZ* ORF replaces that of *sxa2*, causing *LacZ* expression to come under the control of the pheromone-inducible *sxa2* promoter. Consequently, pheromone-induced *sxa2* promoter activity results in *lacZ* expression, which can be monitored by the use of chromogenic substrates. The pheromone-independent and pheromone-dependent β-galactosidase activity of the *rgs1*$^+$ *sxa2*::*lacZ* reporter strain (JY544) is shown in Figure 35.

The *rgs1*$^+$ LacZ reporter strain had a low pheromone-independent level of LacZ activity, indicating that there was a low level of signal transduction through the pheromone signalling pathway in the absence of extracellular P-factor. LacZ activity remained at the same low level up to 100U/ml P-factor, at which point *LacZ* expression was induced. The expression of *lacZ* was further increased at 1000U/ml P-factor, at which the maximum level of LacZ activity was achieved.

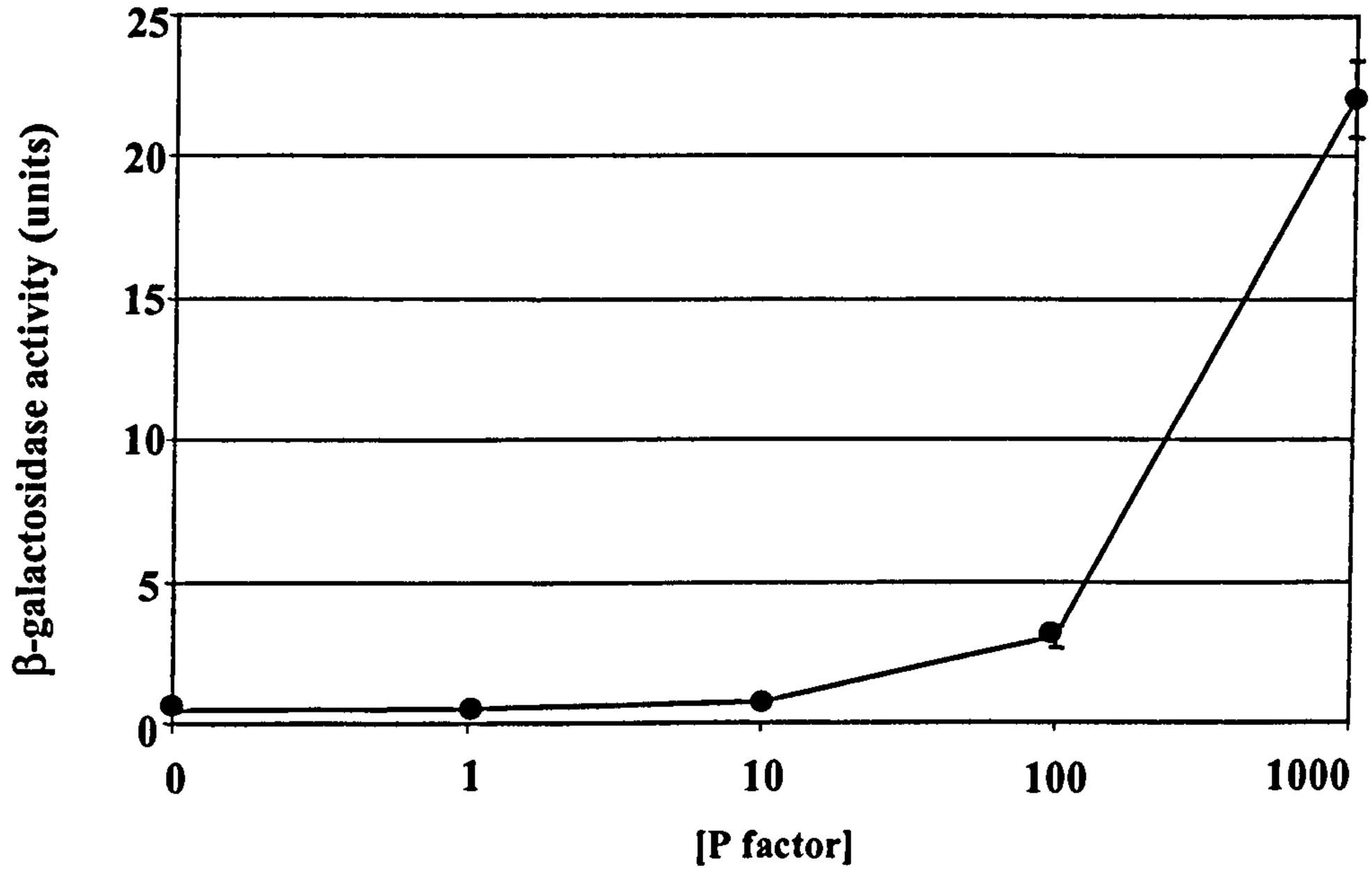


Figure 35. *sxa2::lacZ* locus induction in response to P-factor pheromone stimulation

The *rgs1*$^+$ *sxa2::lacZ* reporter strain, JY544, was assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Cells were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

4.2.4. Disruption of *rgs1* increases sensitivity to P-factor pheromone

Previously in the Davey laboratory, the *rgs1* locus in JY544 (*rgs1*$^+$, *sxa2*::*lacZ*) was disrupted with the *ura4* cassette by Dr. Peter Watson, creating the *rgs1*$^-$ *sxa2*::*lacZ* reporter strain JY629. In JY629 the *rgs1* ORF is replaced with the *ura4* cassette.

Figure 36 shows the β-galactosidase activities of the *rgs1*$^+$ (JY544) and *rgs1*$^-$ (JY629) LacZ reporter strains when stimulated with a range of P-factor concentrations. Disruption of the *rgs1* gene in the *rgs1*$^+$ LacZ reporter strain increased the level of pheromone-independent LacZ activity approximately 5-fold compared to that of the *rgs1*$^+$ reporter strain, reflecting a greater level of pheromone-independent signalling through the pheromone communication pathway. *Sz. pombe* Rgs1p was expressed constitutively, as pheromone-independent LacZ expression was increased following the disruption of the *rgs1* gene. This is possibly to prevent signalling through the pheromone communication pathway in the absence of extracellular P-factor as a consequence of spontaneous dissociation of GDP from G_α subunits.

The *rgs1*$^-$ reporter strain also had greater sensitivity to P-factor compared to the *rgs1*$^+$ strain, as LacZ activity was induced at a 100-fold lower concentration of P-factor. Induction of LacZ activity in the *rgs1*$^+$ LacZ reporter strain was only seen at 100U/ml P-factor compared to 1U/ml P factor for the *rgs1*$^-$ strain.

The β-galactosidase activity of the *rgs1*$^+$ strain was greater than that of the *rgs1*$^-$ strain at 1000U/ml P-factor. This indicates that when stimulated with 1000U/ml P-factor there was a greater degree of signalling through the pheromone communication pathway in the *rgs1*$^+$ reporter strain compared to the *rgs1*$^-$ reporter strain. The reason for this is unknown at present.

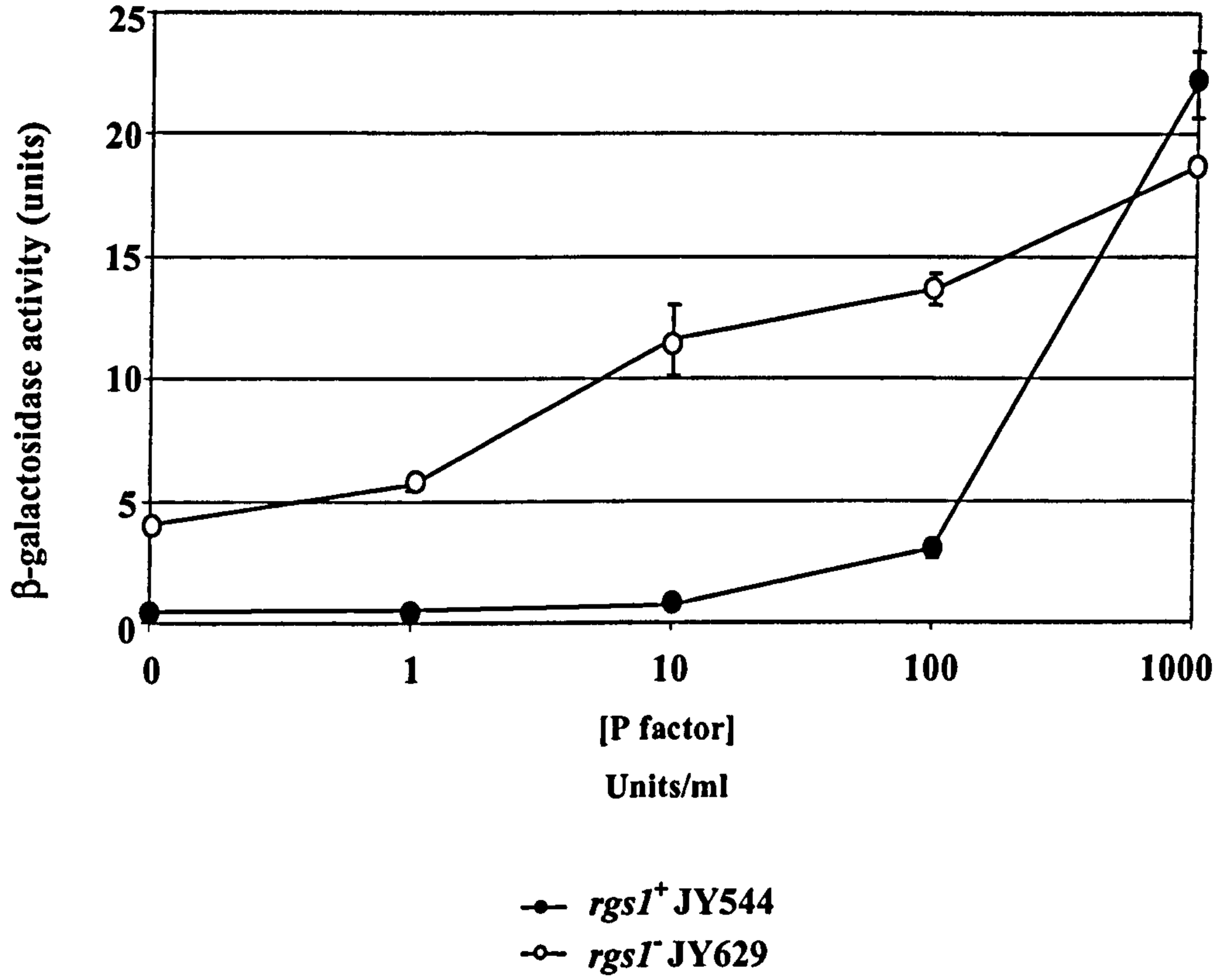


Figure 36. Disruption of *rgs1* increases sensitivity and response to P-factor pheromone

The *rgs1*$^+$ (JY544) and *rgs1*$^-$ (JY629) *sxa2::lacZ* reporter strains were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Reporter strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

One possible explanation for the crossing over of the *rgs1*$^+$ and *rgs1*$^-$ β-galactosidase traces at 1000U/ml P-factor, is that at high levels of P-factor stimulation the presence of szRgs1p in the *rgs1*$^+$ LacZ reporter strain may increase the proportion of GDP-bound G_α subunits available for re-activation in the cell compared to that in the *rgs1*$^-$ LacZ reporter strain. The absence of szRgs1p in the *rgs1*$^-$ LacZ reporter strain has the consequence that GTP bound to G_α subunits can only be hydrolysed as a result of the slow intrinsic GTPase activity of the G_α subunit. As a consequence, a proportion of the GTP-bound G_α subunits within the cell are slowly undergoing hydrolysis, and as a result are possibly inactive and refractory to receptor-stimulated re-activation. The GAP activity of szRgs1p in the *rgs1*$^+$ LacZ reporter strain would increase the intrinsic GTPase activity of the G_α subunits, and as a consequence, may increase the proportion of GDP-bound G_α subunits available for activation compared that in the *rgs1*$^-$ LacZ reporter strain. This phenomenon of RGS proteins increasing GTPase cycle kinetics has been observed in mammalian systems. RGS3, RGS4 and RGS8 caused an acceleration of the kinetics of activation of G protein-coupled inwardly-activating K^+ channels without compromising current amplitudes (Doupnik *et al.*, 1997; Bünnemann and Hosey, 1998).

It can be seen from Figure 36 that the absence of the negatively regulating szRgs1p in the *rgs1*$^-$ *sxa2*::*lacZ Sz. pombe* reporter stain increased pheromone-independent LacZ activity as well as increasing sensitivity and response to P-factor pheromone stimulation up to 100U/ml P-factor.

4.2.5. Expression of szRgs1p in the *sxa2*::*lacZ* reporter strains reduced sensitivity to P-factor pheromone

The ORF for *Sz. pombe* Rgs1 was previously cloned into the *Sz. pombe* expression vector pREP3X by Dr. Peter Watson in the Davey laboratory (generating the szRgs1p expression construct JD1013). JD1013 contains the *rgs1* ORF cloned immediately downstream of the repressible *nmt1* promoter in pREP3X. To investigate whether expressing szRgs1p from the pREP3X expression vector could affect LacZ expression in the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains, JD1013 was

introduced into the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains, and transformants selected on AA plates lacking leucine.

Figure 37 shows the β-galactosidase activity of JY544 (*rgs1*$^+$) containing the empty pREP3X vector (JY744), JY629 (*rgs1*$^-$) transformed with the empty pREP3X vector (JY762), JY544 transformed with the szRgs1p expression construct (JY746) and JY629 transformed with szRgs1p expression construct (JY764).

The *rgs1*$^-$ LacZ reporter strain containing the empty expression vector (JY762) had an increased level of pheromone-independent LacZ activity relative to the *rgs1*$^+$ strain containing the empty expression vector (JY744), and exhibited increased sensitivity and response to pheromone up to 100U/ml P-factor. The response profiles of the *rgs1*$^+$ and *rgs1*$^-$ *sxa2*::*lacZ* reporter strains transformed with the empty pREP3X vector resembled that seen for the strains lacking the empty vector (Figure 36), although the LacZ activity levels were lower at all P-factor concentrations. This is possibly due to the burden of replicating the multi-copy vector in the transformed strains.

Expression of szRgs1p (achieved via expression from pREP3X) in the *rgs1*$^+$ strain (JY746) reduced pheromone-dependent LacZ activity at all P-factor concentrations investigated, compared to the vector alone (JY744). The expression of szRgs1p from pREP3X did not completely repress pheromone-independent *sxa2* induction in the *rgs1*$^+$ reporter strain. Expression of szRgs1p in the *rgs1*$^-$ reporter strain (JY764) reduced pheromone-independent LacZ activity to below the level seen for the *rgs1*$^-$ strain containing vector alone. Pheromone-dependent LacZ activity in the *rgs1*$^-$ reporter strain was also reduced up to 1000 U/ml P-factor. Thus, expression of szRgs1p from a multicopy expression vector rescued the hypersensitive phenotype of the *rgs1*$^-$ LacZ reporter strain.

These results indicate that expression of szRgs1p in the *rgs1*$^+$ LacZ reporter strain did not decrease the level of pheromone-independent LacZ activity compared to a strain containing a single genomic copy of szRgs1p (JY744), but was capable of successfully reducing pheromone-dependent LacZ activity in this strain up to

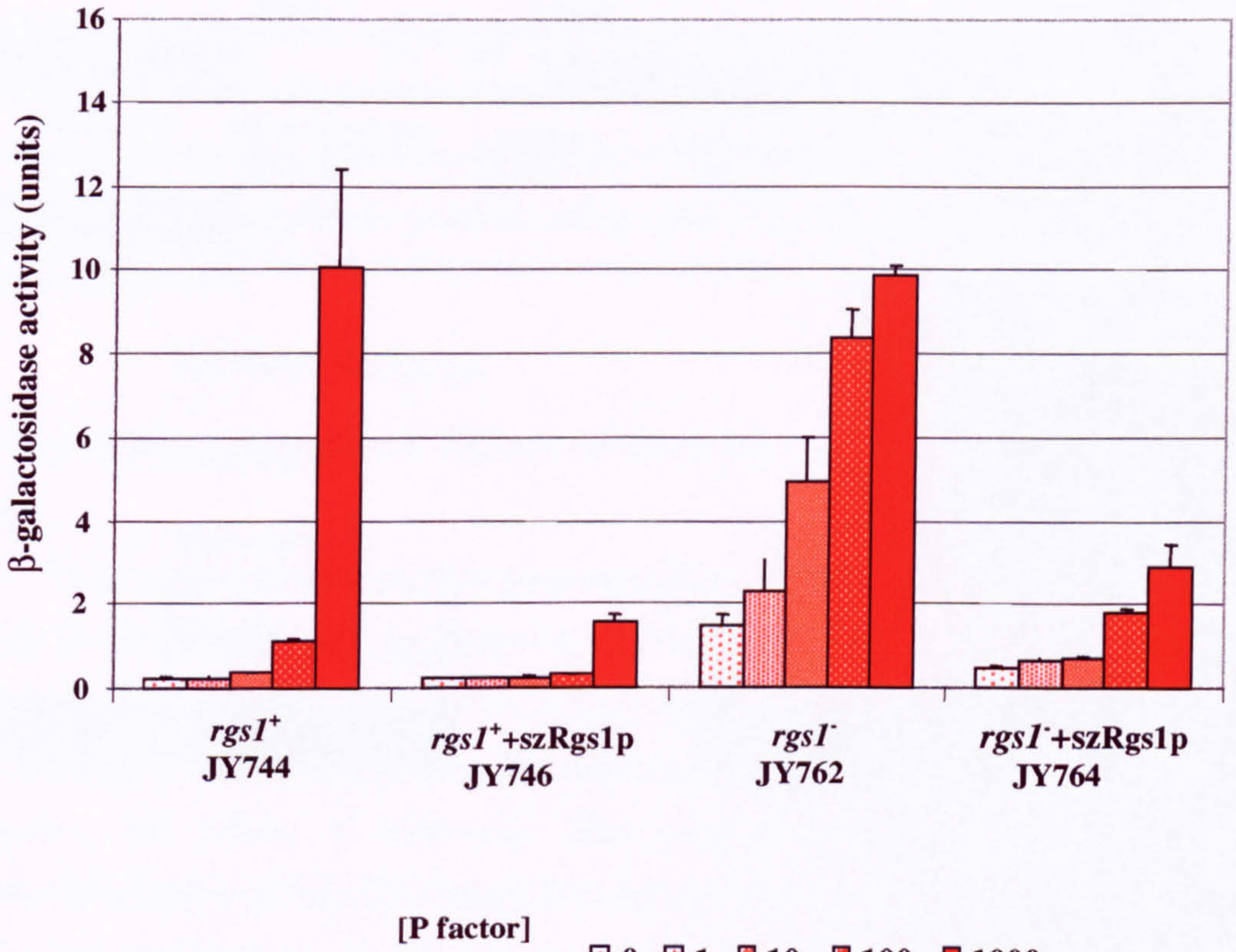


Figure 37. Expression of Rgs1p decreases sensitivity and response to P-factor pheromone in the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains

The *rgs1*$^+$ (JY744) and *rgs1*$^-$ (JY762) LacZ reporter strains expressing *Sz. pombe* Rgs1p (JY746 and JY764 respectively) were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

1000U/ml P-factor. Expression of szRgs1p in the *rgs1*⁻ strain was capable of reducing both pheromone-independent and pheromone-dependent LacZ activity. The data presented in Figure 37 indicate that increased GAP activity towards Gpa1 (the *Sz. pombe* G_α subunit), achieved via an expression construct, was capable of down-regulating signalling through the pheromone communication pathway, and could be assessed via measurement of LacZ activity within the LacZ reporter strains. Thus, the *Sz. pombe sxa2::lacZ* reporter strains present us with a relatively simple model system for investigating signalling through the pheromone communication pathway, and the effects of regulatory proteins acting upon this G protein-coupled signal transduction pathway.

4.3. Expression of mammalian RGS proteins in the *Sz. pombe* LacZ reporter strains

To investigate the effects of expressing mammalian RGS proteins upon LacZ activity in the parental *sxa2::lacZ* reporter strains, several mammalian RGS open reading frames were cloned into the *Sz. pombe* expression vector pREP3X, and the *rgs1*⁺ and *rgs1*⁻ *sxa2::lacZ* reporter strains transformed with the resulting expression constructs. The effects of expressing these mammalian RGS proteins upon pheromone-independent and pheromone-dependent β-galactosidase activities in the *rgs1*⁺ and *rgs1*⁻ LacZ reporter strains were determined using the β-galactosidase assay described in Chapter 2.

4.3.1. Expression of human RGS1 in the *rgs1*⁺ and *rgs1*⁻ LacZ reporter strains

Human RGS1 (huRGS1p) was originally identified through screens for B-lymphocyte-specific genes in chronic lymphocytic leukaemia cells (RGS1 mRNA is elevated in chronic lymphocytic leukaemia [Hong *et al.*, 1993]). RGS1 expression is specific to B-lymphocytes and is induced by mitogenic stimuli (Newton *et al.*, 1993; Moratz *et al.*, 2000).

The human *Rgs1* 591bp ORF was amplified from a lymphoblastoma cDNA plasmid library (a gift from AstraZeneca Pharmaceuticals, UK) using the oligonucleotide primers JO910 and JO911. The sense primer JO910 includes 23

bases complementary to the human *Rgs1* ORF 5' terminus and contains a terminal *Bam*HI site to facilitate cloning. The antisense primer JO911 contains 22 bases complementary to the human *Rgs1* ORF 3' terminus and a terminal *Bam*HI site. The amplified fragment was restricted with *Bam*HI and cloned into the *Bam*HI restriction site of pKS. The human *Rgs1* ORF *Bam*HI fragment was then sequenced, and sub-cloned into the *Bam*HI restriction site of pREP3X. The *rgs1*$^{+}$ and *rgs1*$^{-}$ *sxa2*::*lacZ* reporter strains were transformed with the huRGS1p expression construct, and transformants were selected on AA plates lacking leucine.

The parental LacZ reporter strains JY744 (*rgs1*$^{+}$, pREP3X) and JY762 (*rgs1*$^{-}$, pREP3X) exhibited the expected response profiles (Figure 38). The *rgs1*$^{-}$ LacZ reporter strain exhibited an increased level of pheromone-independent LacZ activity, and increased sensitivity to pheromone compared to the *rgs1*$^{+}$ LacZ reporter strain.

Expression of huRGS1p in the *rgs1*$^{+}$ strain (JY756) reduced pheromone-dependent LacZ activity to a level comparable to that of the pheromone-independent level of LacZ activity in the *rgs1*$^{+}$ LacZ reporter strain up to 100U/ml P-factor (Figure 38). Expression of huRGS1p in the *rgs1*$^{+}$ LacZ reporter strain also reduced pheromone-dependent LacZ activity to a greater degree compared to that seen for the expression of *Sz. pombe* Rgs1p from the pREP3X vector at 100U/ml and 1000U/ml P-factor.

Expression of huRGS1p in the *rgs1*$^{-}$ reporter strain (JY774) reduced both pheromone-independent and pheromone-dependent LacZ activity. This reduction in pheromone-independent and pheromone-dependent LacZ activity was greater than that seen for expression of szRgs1p from the pREP3X vector in the *rgs1*$^{-}$ LacZ reporter strain.

These results suggest that when expressed from a multicopy expression vector, huRGS1p successfully rescued the increased sensitivity and response of the *rgs1*$^{-}$ reporter strain to pheromone, and had a greater ability to reduce both pheromone-independent and pheromone-dependent LacZ activity in the *rgs1*$^{+}$ and *rgs1*$^{-}$ reporter strains compared to szRgs1p when expressed from the same multicopy expression vector. This is possibly a consequence of greater expression levels, or huRGS1p may have greater GAP activity towards Gpa1p compared to szRgs1p.

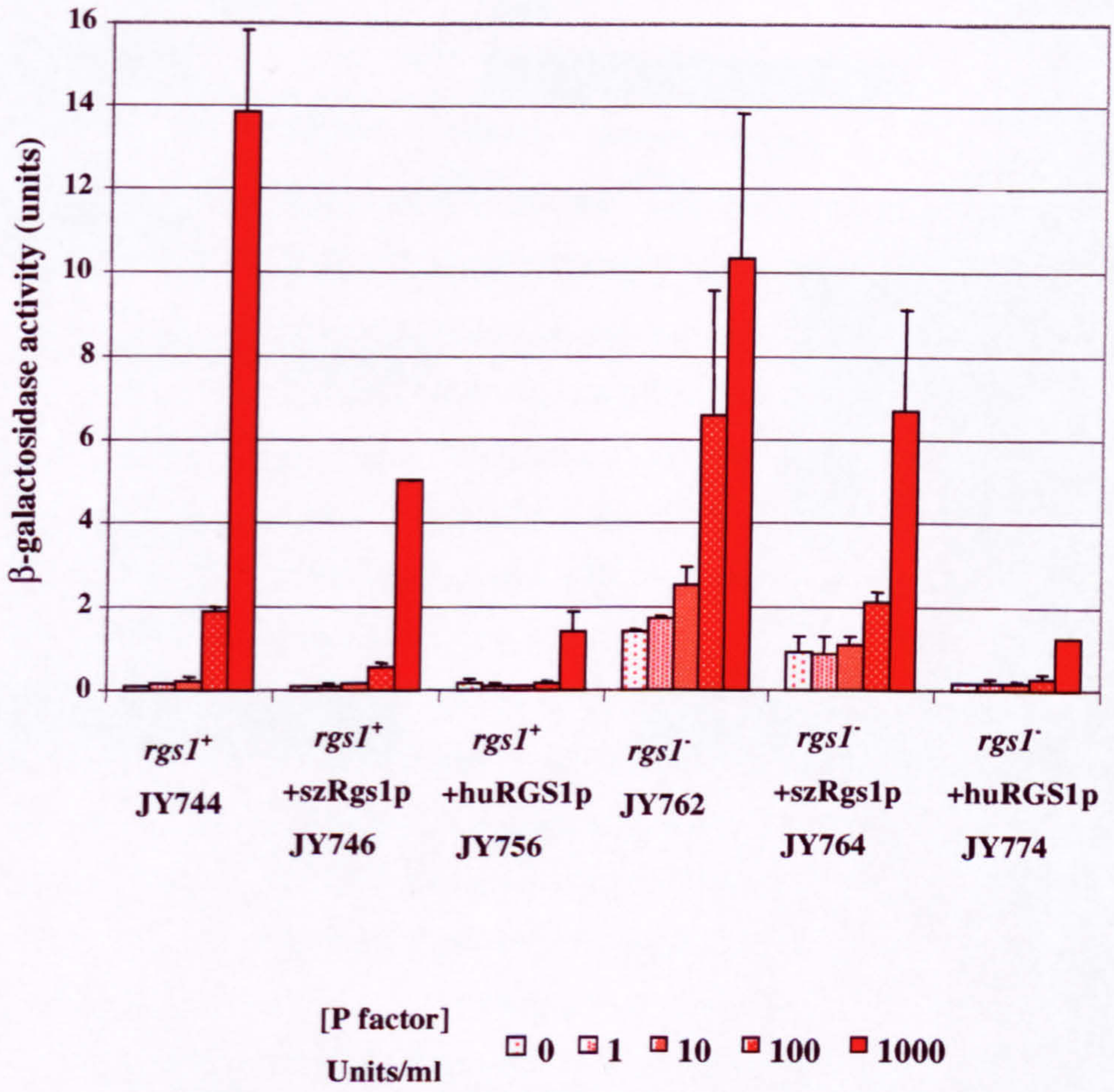


Figure 38. Expression of human RGS1p decreases sensitivity and response to P-factor pheromone in the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains

The *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains expressing huRGS1p (JY756 and JY774 respectively) were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

Human RGS1 has been shown to reduce pheromone sensitivity in *S. cerevisiae* Δ*SST2* mutants (Druey *et al.*, 1996) and has also been shown to inhibit MAPK activation in mammalian cells (Druey *et al.*, 1996). Human RGS1 is a $G_{i\alpha}$ and $G_{q\alpha}$ GAP and a potential $G_{12\alpha}$ effector antagonist (Moratz *et al.*, 2000), and in mammalian cells the activity of RGS1 is regulated by its expression being restricted to B-lymphocytes.

4.3.2. Expression of human RGS2 in the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains

RGS2 was isolated as a result of induced expression of its corresponding mRNA in cycloheximide-treated blood mononuclear cells (Siderovski *et al.*, 1994).

The human *Rgs2* 636bp ORF was amplified from a lymphoblastoma cDNA plasmid library (a gift from AstraZeneca Pharmaceuticals, UK) using the oligonucleotide primers JO912 and JO913. The sense primer JO912 includes 23 bases complementary to the human *Rgs2* ORF 5' terminus and a terminal *Bam*HI site. The anti-sense primer JO913 includes 23 bases complementary to the human *Rgs2* ORF 3' terminus and a terminal *Bam*HI site to facilitate cloning. The amplified product was digested with *BamHI* and cloned into the *Bam*HI restriction site of pKS. The human *Rgs2 Bam*HI fragment was sequenced and sub-cloned into *Bam*HI site of the expression vector pREP3X. The *rgs1*$^+$ and *rgs1*$^-$ *sxa2*::*lacZ* reporter strains were transformed with the huRGS2p expression construct, and transformants selected on AA plates lacking leucine.

Expression of huRGS2p from pREP3X in the *rgs1*$^+$ reporter strain (JY758) reduced neither pheromone-independent LacZ activity nor pheromone-dependent LacZ activity up to 1000 U/ml P-factor (Figure 39). However, expression of the huRGS2p protein in the *rgs1*$^-$ reporter strain (JY776) reduced both pheromone-independent and pheromone-dependent LacZ activity (up to 100U/ml P-factor) to a greater degree than szRgs1p, though to a lesser degree than that seen for huRGS1p (Figure 38).

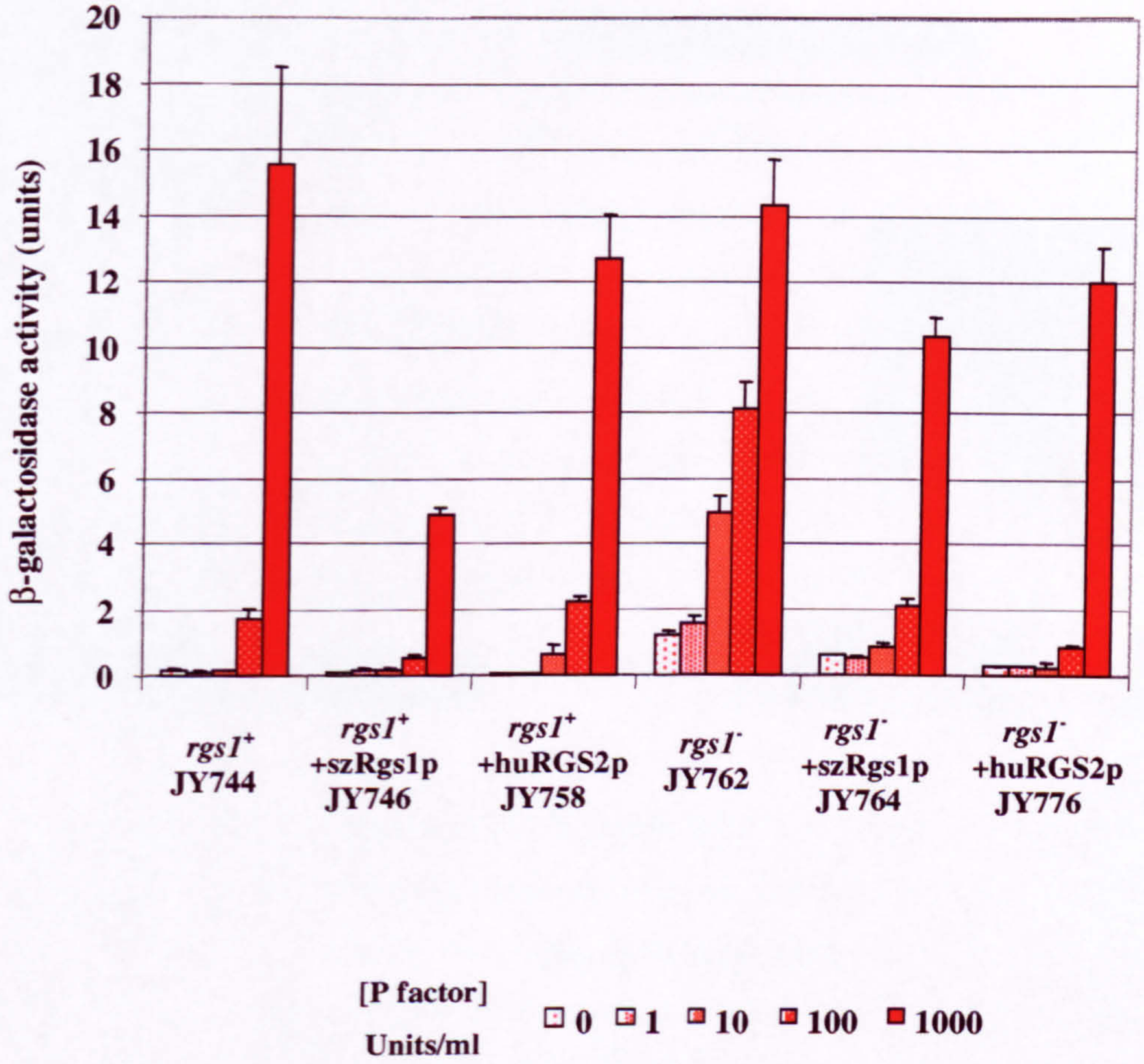


Figure 39. Expression of human RGS2p decreases sensitivity and response to P-factor pheromone in the *rgs1*⁻ LacZ reporter strain but not the *rgs1*⁺ LacZ reporter strain

The *rgs1*⁺ and *rgs1*⁻ LacZ reporter strains expressing huRGS2p (JY758 and JY776 respectively) were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

These results indicate that the expression of huRGS2p was incapable of reducing pheromone-independent and pheromone-dependent LacZ activity in the *rgs1*$^+$ reporter strain, but reduced pheromone-independent and pheromone-dependent LacZ activity (up to 1000U/ml P-factor) in the *rgs1*$^-$ LacZ reporter strain, albeit to a lesser extent than that seen for huRGS1p. This could be explained if huRGS2p had a low affinity for the *Sz. pombe* G_α subunit, resulting in its GAP activity only being observed in the absence of endogenous szRgs1p.

Human RGS2 has been shown to reduce pheromone sensitivity in *S. cerevisiae* Δ*SST2* mutants (Druey *et al.*, 1996) and to inhibit MAPK activation in mammalian cells. Work done by other groups suggests that huRGS2p is a more potent GAP towards $G_{\alpha q}$ than $G_{\alpha i}$ subunits (Heximer *et al.*, 1997; Song *et al.*, 1999), and this may be the reason for reduced ability of human RGS2 to reduce signalling through the pheromone communication pathway in *Sz. pombe* compared to huRGS1p. In mammalian cells the G_q specificity of RGS2 may be a means of regulating its activity. Low levels of RGS2 expression may be sufficient to attenuate $G_{\alpha q}$-coupled signalling pathways without affecting $G_{\alpha i}$-mediated responses, whereas higher levels of expression could attenuate both types of signalling pathway.

4.3.2.1. Human RGS2p is expressed in *Sz. pombe rgs1*$^-$ LacZ reporter strain

It is possible to predict that the observed reduction in RGS activity of huRGS2p compared to huRGS1p could be due to the inefficient expression of huRGS2p, as opposed to a reduction in its ability to modulate the pheromone communication pathway. To determine whether huRGS2p was expressed from pREP3X in the *Sz. pombe rgs1*$^-$ LacZ reporter strain, Western blot analysis was carried out.

Crude extracts were prepared (Section 2.2.10.1.) for the *rgs1*$^-$ LacZ reporter strain transformed with the empty pREP3X vector (JY762), the *rgs1*$^-$ LacZ reporter strain expressing szRgs1p from pREP3X (JY764) and the *rgs1*$^-$ LacZ reporter strain expressing huRGS2p from pREP3X (JY776). Yeast extracts were subjected to electrophoresis in a gel containing 12.5% (w/v) acrylamide. Proteins were transferred onto a nitocellulose filter and allowed to react with rabbit anti-RGS2 antibody. Immunoreactive bands were visualised with goat anti-rabbit antibody

coupled to horseradish peroxidase, and an ECL Western blotting system was used to stain the bands before exposure to x-ray films (Section 2.2.10.).

A band of approximately 35kD can be seen in all lanes (Figure 40). The greater intensity of the band in Lane 1 was a result of the overloading of crude yeast extract (determined by Silver staining, not shown). The identity of the 35kD band is at present unknown. A band of approximately 28kD can be seen in Lane 3 (*rgs1*$^-$, pREP3X-*Rgs2*). As the 28kD band seen in Lane 3 was only expressed in JY776 (the *rgs1*$^-$ *sxa2*::*lacZ* reporter strain transformed with the pREP3X-huRGS2p expression construct), it is reasonable to assume that this band represents huRGS2p. Human RGS2 has a published size of 24.4kD (Siderovski *et al.*, 1994). This increase in size is possibly due to covalent modification of huRGS2p in *Sz. pombe*. A number of RGS proteins are palmitoylated in mammalian cells (Srinivasa *et al.*, 1998a; DeVries *et al.*, 1995), and while palmitoylation has not been reported for huRGS2p, it may represent a mechanism of regulating huRGS2p activity. RGS2 is phosphorylated by protein kinase C (PKC) in mammalian cells (Cunningham *et al.*, 2001), resulting in a reduction in RGS activity.

These results indicate that huRGS2p was expressed in the *rgs1*$^-$ LacZ reporter strain from the pREP3X vector. This suggests that the reduced capacity of huRGS2p to inhibit expression of *lacZ* (Figure 39) compared to huRGS1p (Figure 38), may result from reduced GAP activity of huRGS2p against the *Sz. pombe* G_α subunit (Gpa1p). This is possibly due to its G_α subunit specificity (RGS2 has reported to preferentially regulate $G_{\alpha q}$ [Heximer *et al.*, 1999]). It also appears that human RGS2 may be covalently modified in *Sz. pombe*.

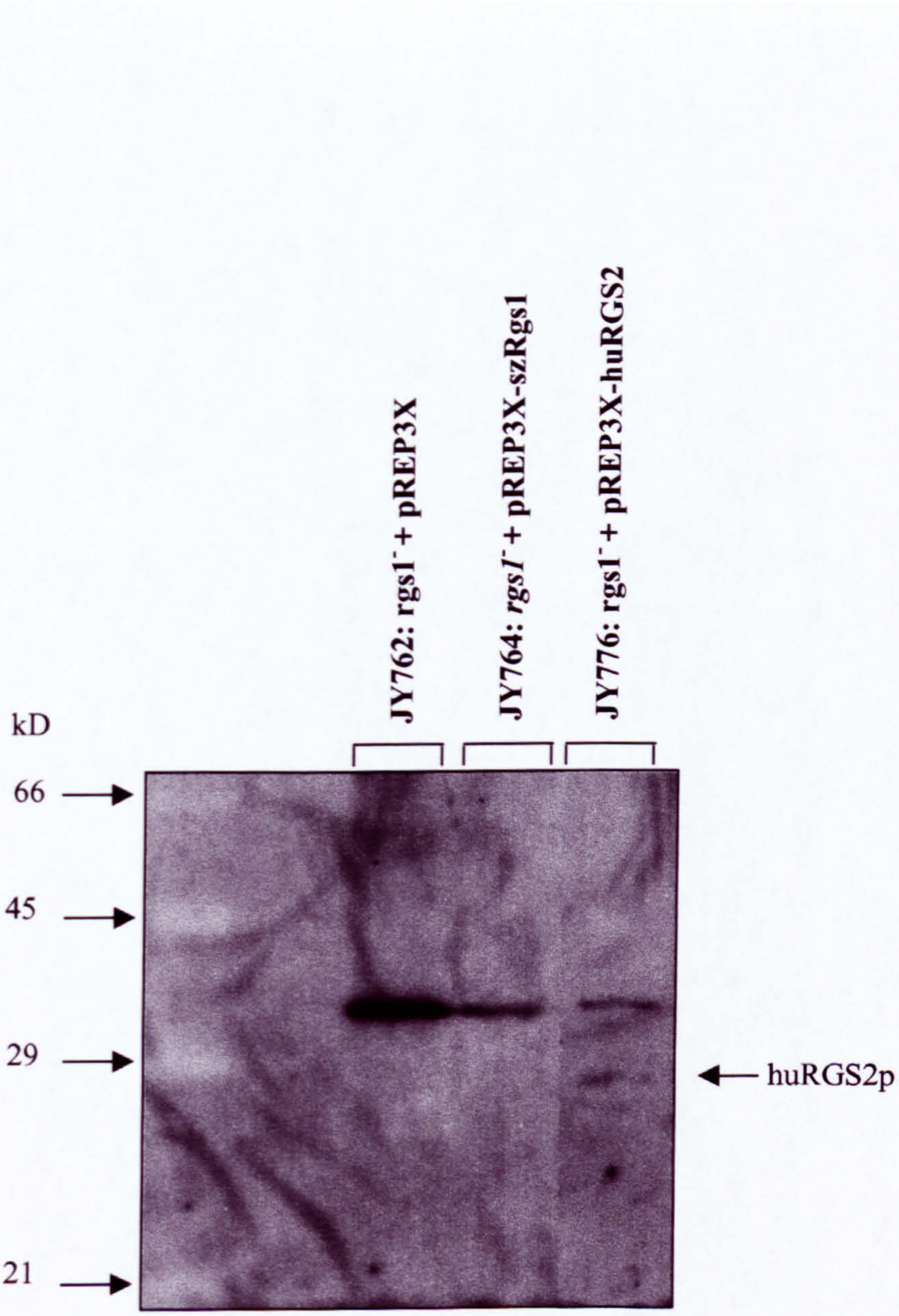


Figure 40. Human RGS2 is expressed from pREP3X in the *Sz. pombe rgs1*⁻ LacZ reporter strain

Crude extracts were prepared from the *rgs1*⁻ LacZ reporter JY762 (Lane 1), the *rgs1*⁻ LacZ reporter expressing szRgs1, JY764 (Lane 2), and the *rgs1*⁻ LacZ reporter expressing huRGS2p, JY776 (Lane 3). A 35kD band can be seen in all lanes, while a band of 28kD is only present in Lane 3. Extracts were subjected to electrophoresis, blotted onto a nitocellulose filter, and allowed to react with rabbit anti-RGS2 antibody. Immunoreactive bands were visualised with goat anti-rabbit antibody coupled to horseradish peroxidase, and an ECL Western blotting system was used to stain the bands before exposure to x-ray film.

4.3.3. Expression of murine RGS2 in the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strain

It has been shown that the expression of some human RGS proteins from expression constructs was capable of negatively modulating the pheromone signalling pathway in *Sz. pombe*. It was therefore of interest to determine whether a non-human mammalian RGS protein could also act as a Gpa1 GAP in the *Sz. pombe* LacZ reporter strains, and murine RGS2 was chosen for investigation. Murine RGS2 shares 95% overall identity with human RGS2, and has been shown to be involved in the regulation of T cell activation, anxiety and male aggression in mice (Oliveira-dos-Santos *et al.*, 2000).

The murine *Rgs2* ORF was amplified from a pKS-murineRGS2 construct (a gift from Sheng-Cai Lin [Chen *et al.*, 1997]) using the oligonucleotide primers JO912 and JO913. The sense primer JO912 includes 23 bases complementary to the murine *Rgs2* ORF 5' terminus and a terminal *Bam*HI site. The anti-sense primer JO913 includes 23 bases complementary to the murine *Rgs2* ORF 3' terminus and a terminal *Bam*HI site to facilitate cloning. The amplified product was cloned into the *Bam*HI restriction site of pKS, sequenced and sub-cloned into the *Bam*HI site of pREP3X. The *rgs1*$^+$ and *rgs1*$^-$ *sxa2::lacZ* reporter strains were transformed with the resulting expression construct, and transformants selected on AA plates lacking leucine.

Expression of muRGS2p in the *rgs1*$^+$ reporter strain (JY929) reduced pheromone-independent and pheromone-dependent LacZ activity up to 1000U/ml P-factor, but did not reduce pheromone-dependent LacZ activity at 1000U/ml P-factor to the level seen for expression of szRgs1p (Figure 41).

Expression of muRGS2p reduced both pheromone-independent and pheromone-dependent LacZ activity up to 1000U/ml P-factor in the *rgs1*$^-$ reporter strain, and appeared to have a greater reductive effect upon pheromone-dependent and pheromone-independent LacZ activity compared to expression of szRgs1p. JY764 (*rgs1*$^-$, pREP3X-szRgs1p) exhibited lower LacZ activity levels compared to

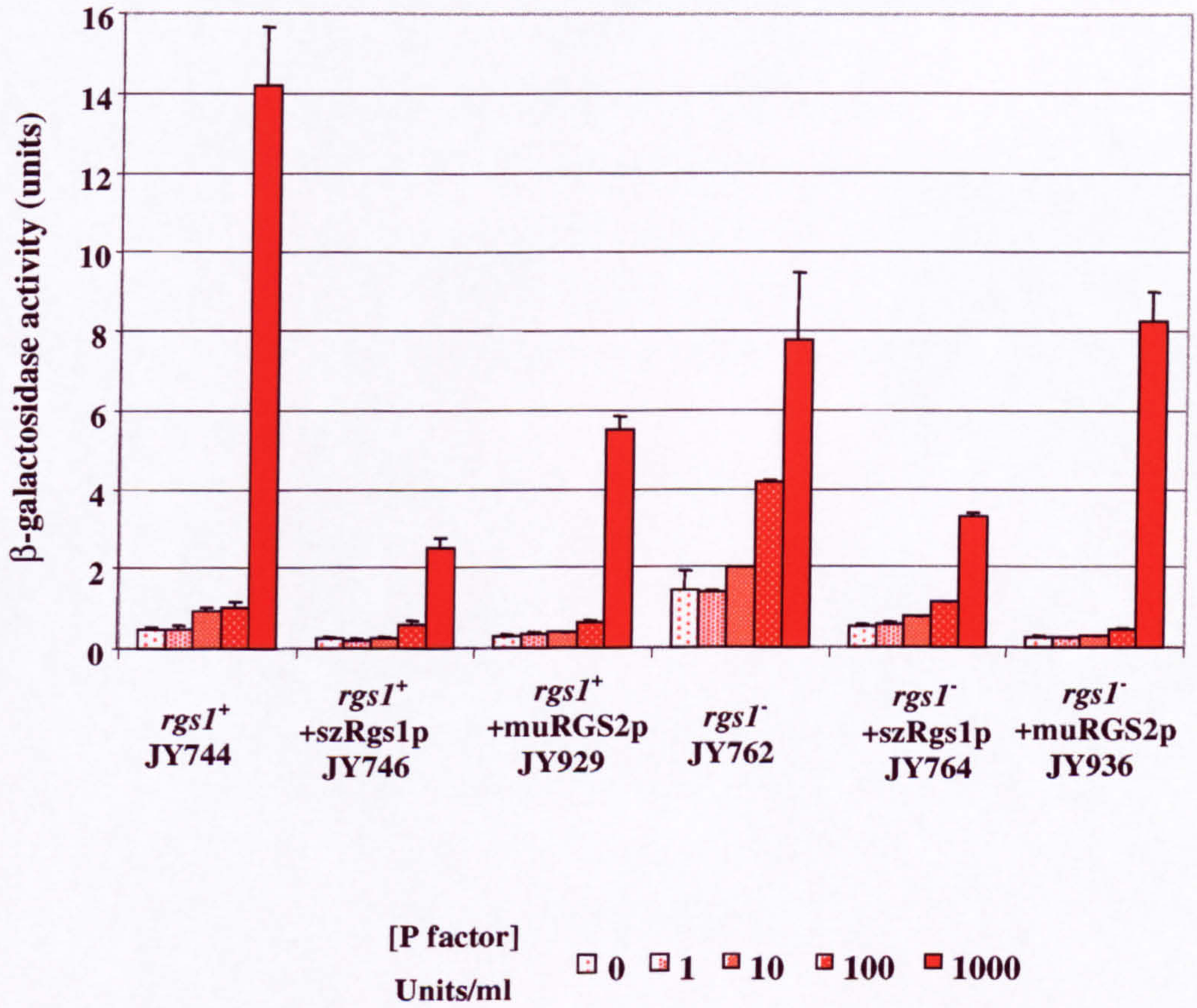


Figure 41. Expression of murine RGS2p decreases sensitivity and response to P-factor pheromone in the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains

The *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains expressing muRGS2p (JY929 and JY936 respectively) were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

the same strain in Figure 38 and Figure 39, and this may possibly result from variability between experiments.

These results indicate that muRGS2p was capable of negatively regulating the *Sz. pombe* pheromone communication pathway, reinforcing the suitability of the LacZ reporter strains for the investigation of mammalian RGS protein activity upon G protein signalling pathways.

4.3.4. Expression of human RGS3 in the *rgs1*$^+$ and *rgs1*$^+$ LacZ reporter strains

The screening of a B-cell cDNA library with an oligonucleotide probe corresponding to sequences conserved between RGS1 and RGS2 led to the identification of RGS3 (Druey *et al.*, 1996). It has been reported there are two major RGS3 transcripts present in multiple tissues (Chatterjee *et al.*, 1997; Dulin *et al.*, 2000; Mittman *et al.*, 2001).

The human *Rgs3* 1560bp ORF was amplified from a lymphoblastoma cDNA plasmid library (a gift from AstraZeneca Pharmaceuticals, UK) using the oligonucleotide primers JO914 and JO915. The sense primer JO914 includes 22 bases complementary to the human RGS3 ORF 5' terminus. The antisense primer JO915 includes 22 bases complementary to the human Rgs3 ORF 3' terminus. The product was cloned into the *Eco*RV restriction site of a pREP3X derivative and sequenced. The *rgs1*$^+$ and *rgs1*$^-$ *sxa2*::*lacZ* reporter strains were transformed with the huRGS3p expression construct, and transformants were selected on AA plates lacking leucine.

Expression of huRGS3p in the *rgs1*$^+$ reporter strain (JY971) reduced neither pheromone-independent LacZ activity nor pheromone-dependent LacZ activity up to 100U/ml P-factor (Figure 42). HuRGS3p was capable of down-regulating LacZ expression at 1000U/ml P-factor in the *rgs1*$^+$ LacZ reporter strain, but to a lesser extent than that seen for huRGS1p (Figure 38). Expression of huRGS3p in the *rgs1*$^-$ reporter strain (JY972) did not appear to have any reductive effect upon pheromone-independent or pheromone-dependent LacZ activity (up to 1000U/ml P-factor), and the JY972 LacZ response profile resembled that of the *rgs1*$^-$ LacZ reporter strain (JY762).

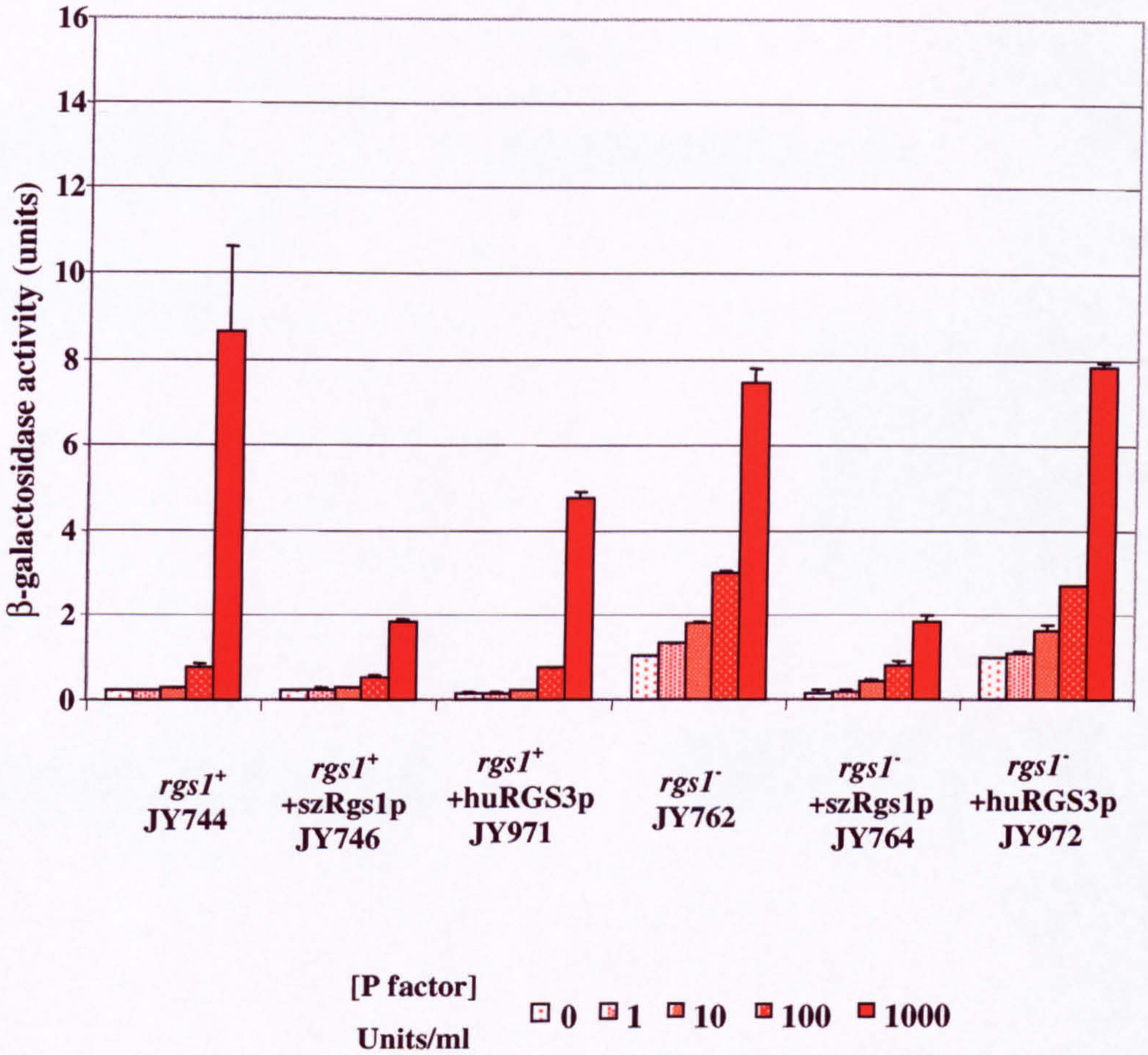


Figure 42. Expression of human RGS3p does not reduce sensitivity or response to P-factor pheromone in the *rgs1*$^+$ or *rgs1*$^-$ LacZ reporter strains

The *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains expressing huRGS3p (JY971 and JY972 respectively) were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

The fact that the expression of huRGS3p did not appear capable of reducing LacZ activity in the LacZ reporter strains indicates that this RGS protein was either not expressed in the reporter strains, or it was incapable of exerting RGS activity upon the *Sz. pombe* G_α subunit, Gpa1.

Human RGS3 has been shown to reduce pheromone sensitivity in *S. cerevisiae* Δ*SST2* mutants (Druey *et al.*, 1996), and has been shown to inhibit MAPK activation in mammalian cells (Druey *et al.*, 1996; Dulin *et al.*, 1999).

4.3.5. Expression of human RGS4 in the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains

RGS4 was identified by the screening of rat cDNAs, that when expressed in a *S. cerevisiae SST2* mutant, could stimulate recovery from pheromone-induced growth arrest, and partially block pheromone-induced gene transcription (Druey *et al.*, 1996). Human RGS4 displays 97% amino acid identity with rat RGS4, and most structural and biochemical information is known about this RGS protein.

The human *Rgs4* ORF (618bp) was amplified from a lymphoblastoma cDNA plasmid library (a gift from AstraZeneca Pharmaceuticals, UK) using the oligonucleotide primers JO917 and JO918. The sense primer JO917 includes 22 bases complementary to the human *Rgs4* ORF 5' terminus and a terminal *Bam*HI site. The anti-sense primer JO918 include 21 bases complementary to the human *Rgs4* ORF 3' terminus and a terminal *Bam*HI site. The amplified product was cloned into the *Bam*HI site in pKS, sequenced, and sub-cloned into the *Bam*HI site of pREP3X. The *rgs1*$^+$ and *rgs1*$^-$ *sxa2::lacZ* reporter strains were transformed with the resulting huRGS4p expression construct, and transformants were selected on AA plates lacking leucine .

Expression of huRGS4p from the pREP3X vector in the *rgs1*$^+$ reporter strain (JY760) did not completely inhibit pheromone-independent LacZ activity, but reduced pheromone-dependent LacZ activity above 10U/ml P-factor in this strain (Figure 43). HuRGS4p appeared capable of reducing pheromone-independent and pheromone-dependent LacZ activity in the *rgs1*$^+$ reporter strain to a greater degree than that seen for szRgs1p expressed from the pREP vector. However, due to the variability observed between strains and experiments, this may not be significant.

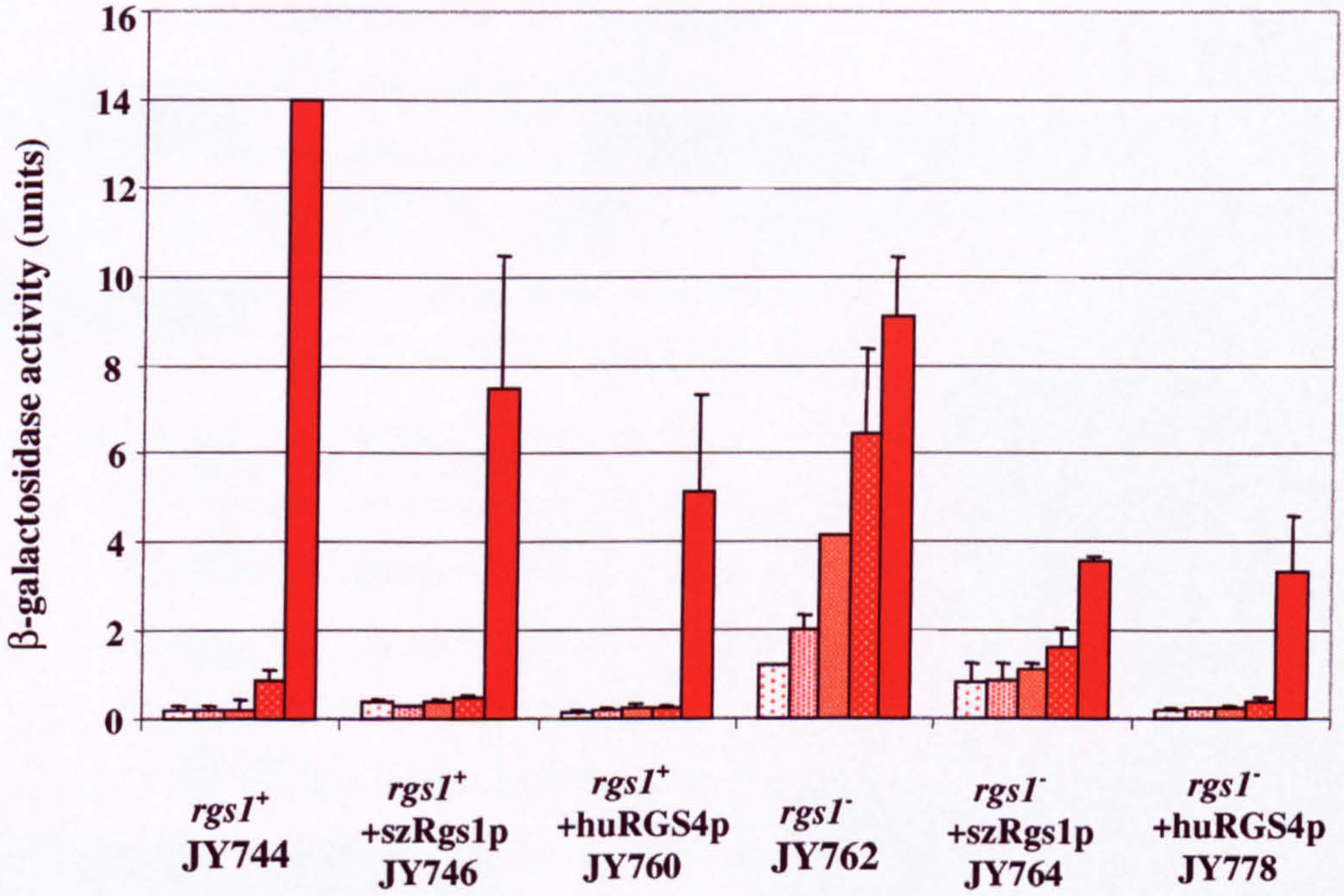


Figure 43. Expression of human RGS4p decreases sensitivity and response to P-factor in the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains

The *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains expressing huRGS4p (JY760 and JY778 respectively) were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

High levels of huRGS4p in the *rgs1*⁻ reporter strain (JY778) reduced both pheromone-independent and pheromone-dependent LacZ activity up to 1000U/ml P-factor, and appeared to exert a greater reductive effect upon pheromone-independent and pheromone-dependent LacZ activity up to 1000U/ml P-factor when compared to the expression of szRgs1p in the *rgs1*⁻ reporter strain. This is possibly due to variability between strains and/or experiments.

These results indicate that high expression levels of huRGS4p in the *rgs1*⁺ and *rgs1*⁻ reporter strains were capable of reducing pheromone-independent LacZ activity and sensitivity to pheromone stimulation in the *rgs1*⁺ and *rgs1*⁻ reporter strains, and appeared to have a greater efficacy in reducing both pheromone-independent and pheromone-dependent LacZ activity in the *rgs1*⁺ and *rgs1*⁻ reporter strains when compared to expression of szRgs1p.

Expression of human RGS4 has been shown to reduce pheromone sensitivity in *S. cerevisiae* Δ*SST2* mutants (Druey *et al.*, 1996). RGS4 was the most successful (followed by RGS1 and an RGS3 truncated variant, RGS3 and RGS2). Druey *et al.* (1996) also showed that RGS4 inhibits MAPK activation in mammalian cells. RGS4 has also been shown to complement an RGS2 mutant in *S. cerevisiae* (Versele *et al.*, 1999). RGS4 is a relatively promiscuous RGS protein, displaying little preference for different $G_{\alpha i}$ and $G_{\alpha q}$ subunits (Diversé-Pierluissi *et al.*, 1999; Cavalli *et al.*, 2000; Rogers *et al.*, 2001).

4.3.5.1. Human RGS4 is expressed in *Sz. pombe* LacZ reporter strains

Crude extracts were prepared (Section 2.2.10.1.) from the *rgs1*⁻ LacZ reporter strain transformed with the empty pREP3X vector (JY762) and the *rgs1*⁻ LacZ reporter strain expressing huRGS4p from pREP3X (JY778). Yeast extracts were subjected to electrophoresis in a gel containing 12.5% (w/v) acrylamide. Proteins were transferred onto nitocellulose filters and allowed to react with rabbit anti-RGS4 antibody. Immunoreactive bands were visualised with goat anti-rabbit antibody coupled to horseradish peroxidase and an ECL Western blotting system was used to stain the bands before exposure to x-ray films (See Section 2.2.10.).

A band of approximately 28kD can be seen in Lane 2 of Figure 44 (*rgs1*⁻, pREP3X-RGS4). This band was not seen in Lane 1. Human RGS4 has a

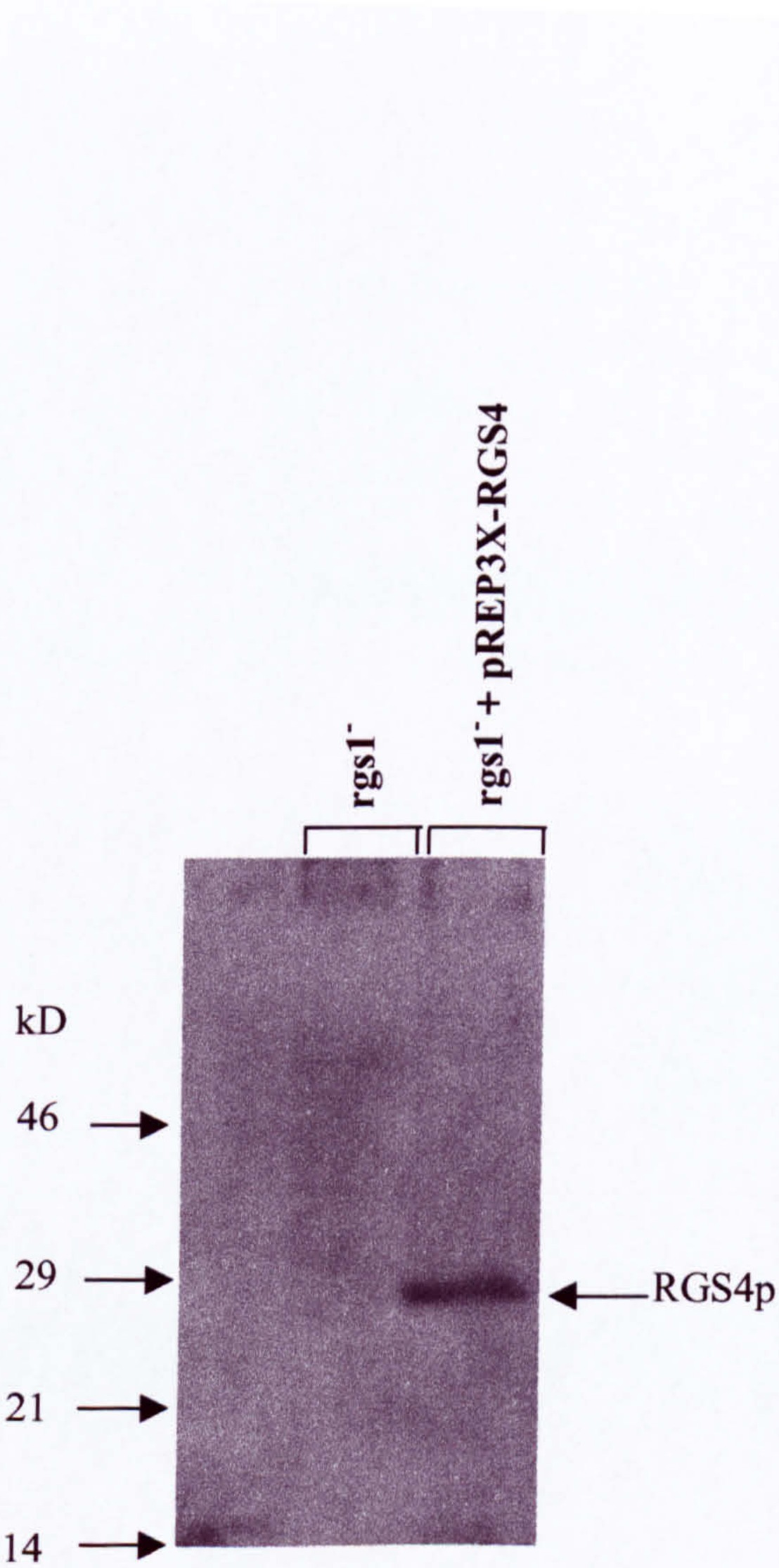


Figure 44. Human RGS4 is expressed from pREP3X in the *Sz. pombe rgs1*⁻ LacZ reporter strain

Crude yeast extracts were prepared from the *rgs1*⁻ LacZ reporter, JY762, (Lane1) and the *rgs1*⁻ LacZ reporter overexpressing huRGS4p, JY778 (Lane2). Extracts were subjected to electrophoresis, blotted onto a nitocellulose filter, and allowed to react with rabbit anti-RGS4 antibody. Immunoreactive bands were visualised with goat anti-rabbit antibody coupled to horseradish peroxidase, and an ECL Western blotting system was used to stain the bands before exposure to x-ray film. A band of 28kD can be seen in Lane 2.

published size of 24.2kD (Berman *et al.*, 1996), which is smaller than the band seen in Lane 2. As for huRGS2p expressed in the *rgs1*⁻ LacZ reporter strain, the apparent larger size of huRGS4p when expressed in *Sz. pombe* is possibly due to covalent modification of the protein. RGS4 is palmitoylated in mammalian cells, with Cys-2 and Cys-12 likely sites (Srinivasa *et al.*, 1998a). These results suggest that huRGS4p was successfully expressed in the *rgs1*⁻ LacZ reporter strain when expressed from the pREP3X expression vector. The 35kD band observed in Figure 40 was not visualised with the rabbit anti-RGS4 antibody.

4.3.6. Expression of human RGS9-2 in the *rgs1*⁺ and *rgs1*⁻ LacZ reporter strains

Major transcripts of the RGS9 gene are alternatively spliced into a retina-specific transcript (RGS9-1) and a brain-specific transcript (RGS9-2). RGS9-2 is a striatal enriched alternatively spliced product of the RGS9 gene. The RGS9-2 protein is 191 residues longer than the retinal form, and has a unique 3' non-coding region (Rahman *et al.*, 1999; Zhang *et al.*, 1999). Alternative splicing has also been observed for other RGS proteins (Snow *et al.*, 1998; Chatterjee and Fisher 2000).

The human *Rgs9-2* 2031bp ORF was amplified from a pKS-*Rgs9-2* construct (a gift from Stephen Gold, Rahman *et al.*, 1999) using the oligonucleotide primers JO1016 and JO1017. The sense primer JO1016 contains 27 bases complementary to the human *Rgs9-2* 5' terminus and a terminal *Bam*HI site. The anti-sense primer JO1017 includes 24 bases complementary to the human *Rgs9-2* ORF 3' terminus and a terminal *Bam*HI site. The amplified product was cloned into the *Bam*HI site of pKS, sequenced, and sub-cloned into the *Bam*HI site of pREP3X. The *rgs1*⁺ and *rgs1*⁻ *sxa2*::*lacZ* reporter strains were transformed with the huRGS9-2p expression construct, and transformants selected on AA plates lacking leucine.

Expression of human pRGS9-2 in the *rgs1*⁺ reporter strain (JY931) did not exert a reductive effect upon pheromone-independent LacZ activity (Figure 45). It was however capable of exerting a reductive effect upon pheromone-dependent LacZ activity at 100U/ml and 1000U/ml P-factor.

High expression levels of huRGS9-2p in the *rgs1*⁻ reporter strain reduced pheromone-independent LacZ activity, but to a smaller degree than that seen for expression of *Sz. pombe* Rgs1p. This reduction did not reach the low pheromone-

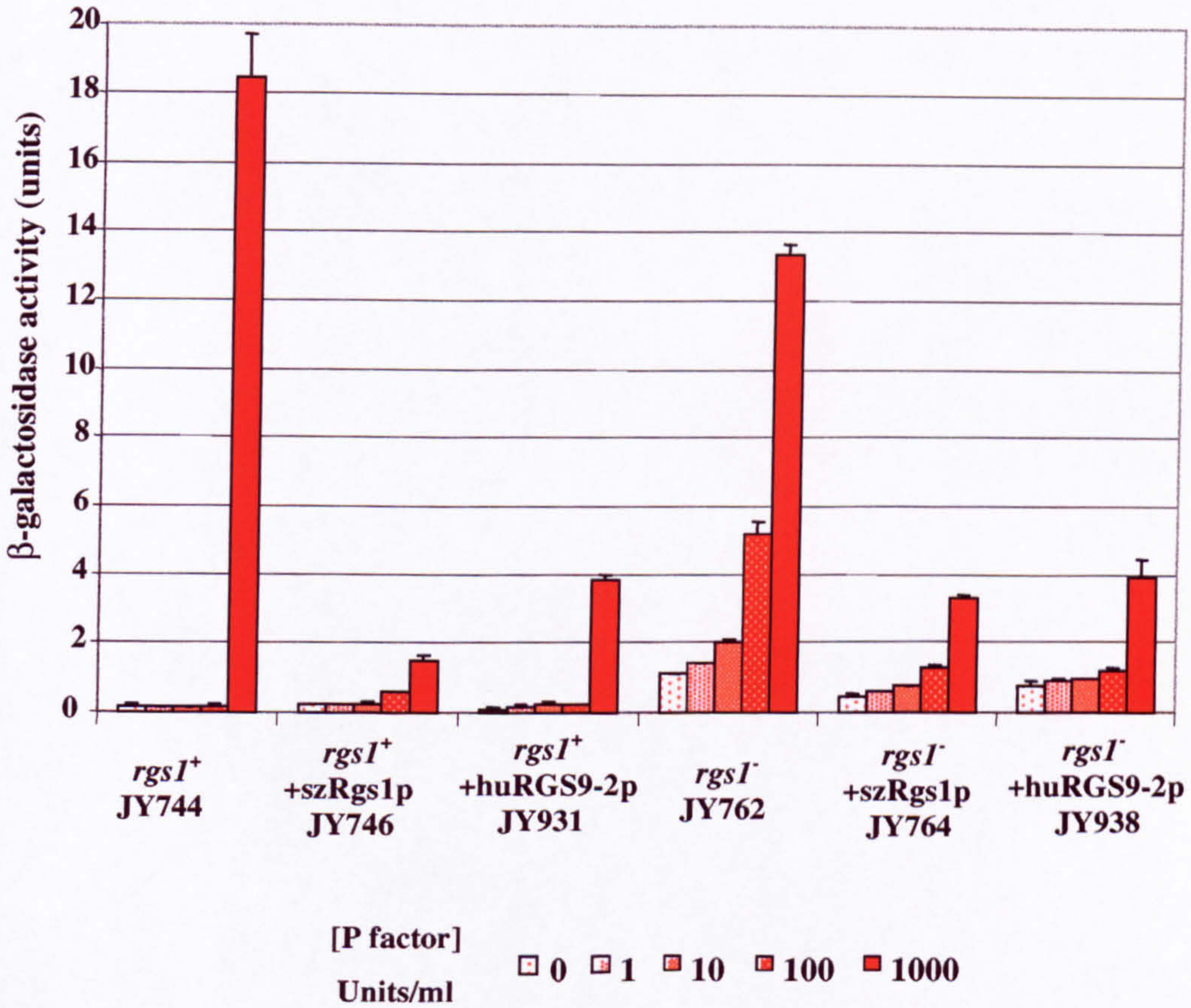


Figure 45. Expression of human RGS9-2p decreases sensitivity and response to P-factor pheromone in the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains

The *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains expressing huRGS9-2p (JY931 and JY938 respectively) were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

independent LacZ activity of the *rgs1*$^+$ reporter strain. Expression of huRGS9-2p reduced pheromone-dependent LacZ activity in the *rgs1*$^-$ reporter strain up to 1000U/ml P-factor, to a similar degree as *Sz. pombe* Rgs1p.

RGS9-2 is specifically expressed in the striatum, where it is involved in the desensitisation of $G_{\alpha i}/G_{\alpha o}$-coupled μ-opioid receptors (Rahman *et al.*, 1999; Granneman *et al.*, 1998). This G_α subunit specificity is possibly the basis behind the reduced ability of huRGS9-2p to modulate signalling through the *Sz. pombe* pheromone communication pathway compared to, for example, huRGS1p and huRGS4p.

4.3.7. Expression of *Sz. pombe* Rgs1 C-terminus in the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains

RGS proteins fall into two major size classes – those that encode the RGS box flanked by short N- and C-termini, and those which have the potential to encode additional information. The *Sz. pombe* Rgs1 protein falls into the latter category. In the *rgs1* ORF approximately 1kb of additional sequence lies upstream of its RGS domain-coding region. It was therefore of interest to determine whether expression of the C-terminus alone (incorporating the RGS domain without the additional information encoded at the *rgs1* N-terminus) could modulate signal transduction through the pheromone signalling pathway in the LacZ reporter strains. The RGS domain of RGS16 has been shown to be functional *in vitro*, but the flanking regions are required for activity *in vivo* (Chen and Lin, 1998). The szRgs1p C-terminus product initiates at an internal methionine residue at residue 274 in the full-length szRgs1p.

The *Sz. pombe* Rgs1 C-terminal RGS domain (627bp) was amplified from JD912 (containing the full-length *Sz. pombe rgs1* ORF cloned into pKS) using the oligonucleotide primers JO923 and JO600 (Figure 46). The sense primer JO923 contains 24 bases complementary to the *rgs1* ORF initiating at nucleotide position +820 relative to the *rgs1* initiator codon (this corresponds to an internal Met residue at residue 274 in the szRgs1p primary sequence). The anti-sense primer JO600 includes 22 bases complementary to the *Sz. pombe rgs1* 3' terminus initiating at

nucleotide position +1448 relative to the *rgs1* initiator codon and contains a terminal *Eco*RV site. The 627bp product was cloned into the *Eco*RV restriction site of a pREP3X derivative and sequenced. The *rgs1*$^{+}$ and *rgs1*$^{-}$ *sxa2*::*lacZ* reporter strains were transformed with the resulting expression construct, and transformants were selected on AA plates lacking leucine.

Expression of the *Sz. pombe* Rgs1 C-terminus in the *rgs1*$^{+}$ reporter strain (JY750) did not reduce pheromone-independent LacZ activity (Figure 47). A small reduction in LacZ activity at 1000U/ml P-factor was seen when the *Sz. pombe* Rgs1 C-terminus was expressed in the *rgs1*$^{+}$ reporter strain, although this reductive effect was not as great as the reductive effect seen for the full length szRgs1p.

Expression of the *Sz. pombe* Rgs1 C-terminus in the *rgs1*$^{-}$ reporter strain (JY768) reduced pheromone-independent LacZ activity and pheromone-dependent LacZ activity up to 10U/ml P-factor to a similar degree as that seen for the full-length szRgs1p. However, while the *Sz. pombe* Rgs1 C-terminus reduced LacZ activity in the *rgs1*$^{-}$ reporter strain at 100U/ml P factor to a small degree, it did not appear to be as effective as the full-length szRgs1p in reducing LacZ activity in the *rgs1*$^{-}$ reporter strain stimulated with 100U/ml or 1000U/ml P-factor.

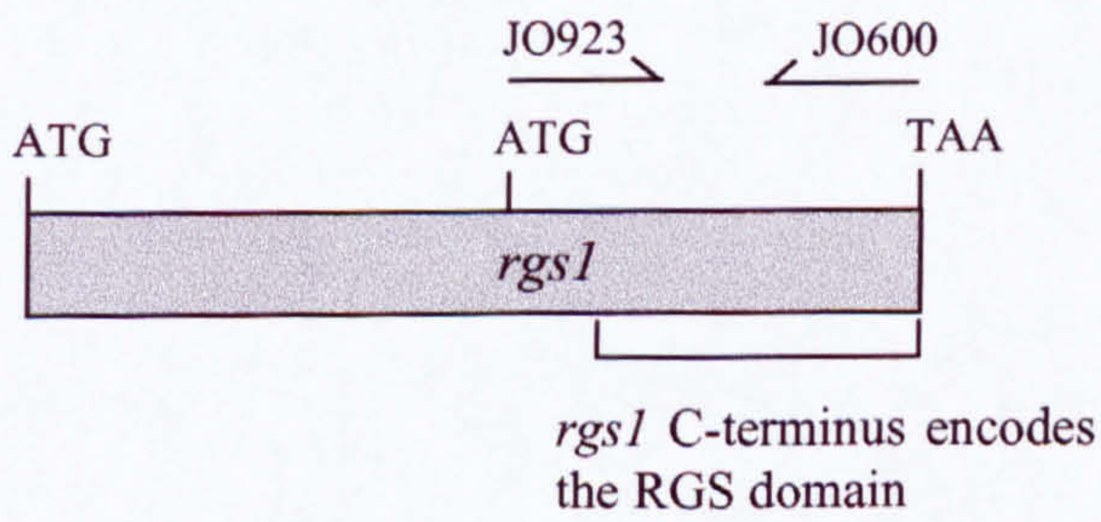


Figure 46. Amplification of the *Sz. pombe rgs1* C-terminus

The C-terminal 627bp of *Sz. pombe rgs1* were amplified with the oligonucleotides JO923 and JO600. JO923 initiates at an ATG codon encoding an internal Met residue at amino acid position 274 in the full-length szRgs1p

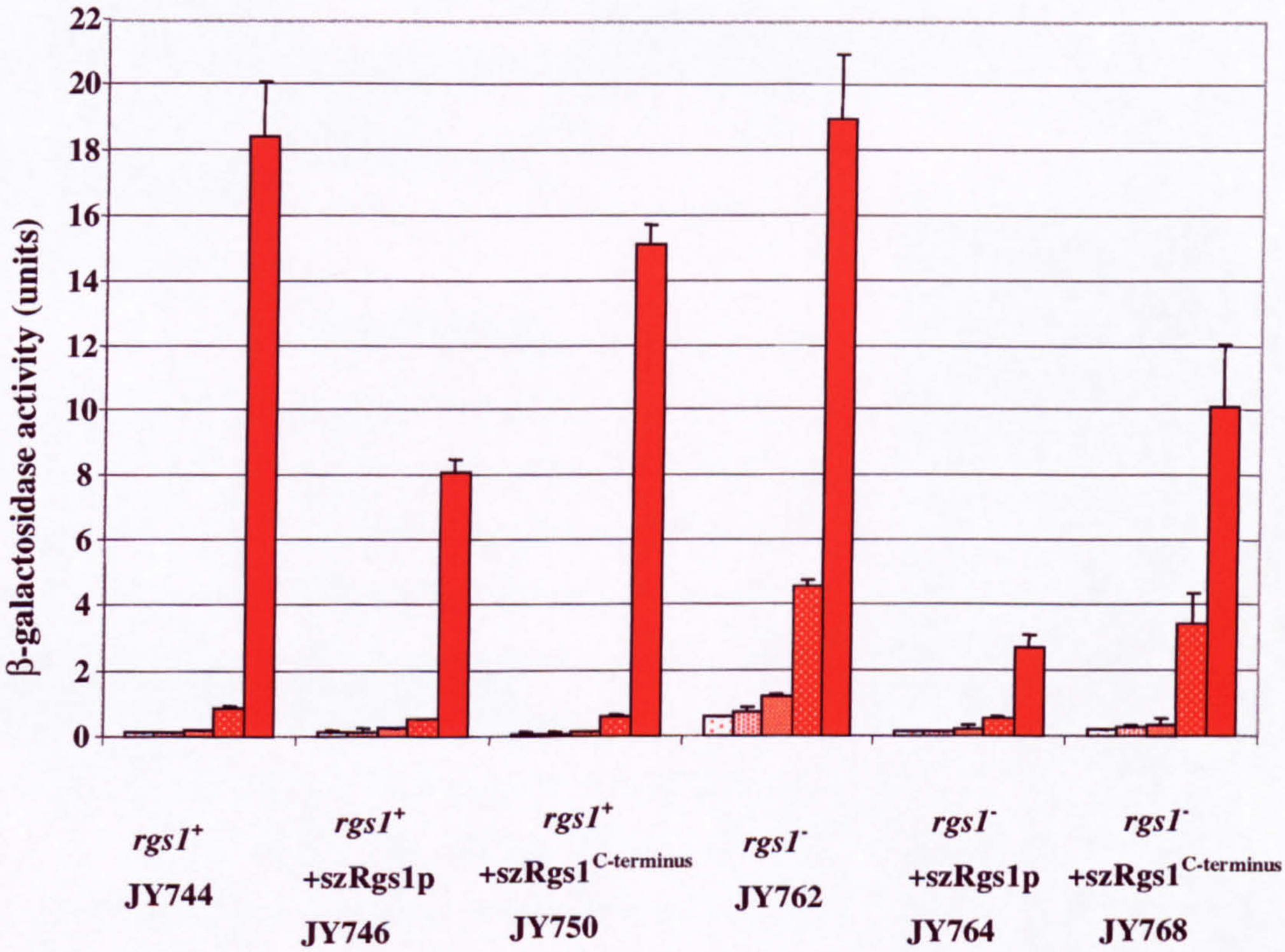


Figure 47. Expression of *Sz. pombe* Rgs1p C-terminus decreases sensitivity and response to P-factor pheromone at low pheromone concentrations

The *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains expressing the szRgs1p C terminus (JY750 and JY768 respectively) were assayed for β-galactosidase activity following exposure to a range P factor pheromone concentrations. Strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

These results suggest that while the Rgs1 C-terminus retained some ability to reduce LacZ activity in the *rgs1*$^{+}$ and *rgs1*$^{-}$ LacZ reporter strains, this ability was similar to that of the full-length szRgs1p only at low levels of signalling through the pheromone communication pathway, indicating that the N-terminus of szRgs1p is required for full RGS activity.

Section II: Expression of human RGS1 and human RGS4 in the *Sz. pombe* LacZ reporter strain from single copy

4.4.1. Introduction

The disadvantage of using multicopy expression vectors to direct the expression of RGS proteins is that they can result in variable expression levels. In the search for RGS mutants with increased GAP activity, a different approach was therefore required. I decided that the integration of a single copy of a human RGS ORF at the *rgs1* locus would result in reproducible levels of expression. This would be important for future mutational studies, as the variable copy number and expression levels of pREP expression vectors could potentially lead to ambiguous results in the search for RGS proteins with altered activities.

Human RGS1 and human RGS4 were chosen to continue with their characterisation, as these two human RGS proteins had the greatest ability to reduce LacZ activity when expressed in the *Sz. pombe rgs1*$^{+}$ and *rgs1*$^{-}$ LacZ reporter strains from a multicopy expression vector (Section 4.3).

Integration cassettes were constructed and introduced into the *rgs1*$^{-}$ LacZ reporter strain genome, so that the human *Rgs1* ORF or human *Rgs4* ORF exactly replaced the genomic *rgs1* ORF. Correct integration of the human *Rgs1* and human *Rgs4* ORFs result in the expression of these human RGS proteins being under the direction of the *rgs1* promoter, and consequently expression should be induced as a result of signalling through the pheromone communication pathway. This integration strategy made use of the fact that the loss of Ura4p activity from the *rgs1*$^{-}$ LacZ reporter strain (the *ura4* cassette replaces the *rgs1* ORF in this reporter strain) could be positively selected for with the use of 5-FOA in selective plates.

4.4.2. Integration of the human *Rgs1* ORF in the *rgs1⁻ sxa2::lacZ* reporter strain

Briefly, the human *Rgs1* open reading frame was cloned between the *Sz. pombe rgs1* 5' and 3' non-coding regions by Dr. Stuart Allen in the laboratory. The human *Rgs1* ORF was amplified using the oligonucleotides JO1119 and JO1120 from the pKS-huRGS1 construct described in Section 2.3. The sense primer JO1119 contains 54 bases complementary to the *Sz. pombe rgs1* 5' non-coding region (nucleotide positions –53 to -1 relative to the *rgs1* initiator codon) followed by 23 bases complementary to the first N-terminal 23 bases of the human *Rgs1* ORF sequence (positions +1 to +23 relative to the human *Rgs1* initiator codon). The antisense primer JO1120 contains 21 bases complementary to the C-terminal human *Rgs1* ORF sequence (positions +588 to +568 relative to the human *Rgs1* initiator codon) followed by 61 bases complementary to the *Sz. pombe rgs1* 3' non-coding region (nucleotide positions +1504 to +1444 relative to the *rgs1* initiator codon). JO1119 and JO1120 amplify a fragment containing the human *Rgs1* ORF flanked with *Sz. pombe* 5' and 3' non-coding sequences.

The fragment amplified by JO1119 and JO1120 was cloned into a construct generated to provide additional *Sz. pombe rgs1* 5' and 3' non-coding sequences to aid homologous integration. The sense primer JO1268 contains 21 bases complementary to the *Sz. pombe rgs1* 3' non-coding region, initiating at nucleotide position +1505 relative to the *rgs1* initiator codon. The antisense primer JO1267 contains 18 bases complementary to the *Sz. pombe rgs1* 5' non-coding region initiating at nucleotide position -58 relative to the *rgs1* initiator codon. JO1267 and JO1268 were used in conjunction with a construct containing the *Sz. pombe rgs1* 5' and 3' non-coding regions (JD1394) to generate a linear DNA fragment with which the human *Rgs1* ORF fragment amplified by JO1119 and JO1120 could be ligated, so that the human *Rgs1* ORF was situated between the *Sz. pombe rgs1* 5' and 3' non-coding regions (Figure 48).

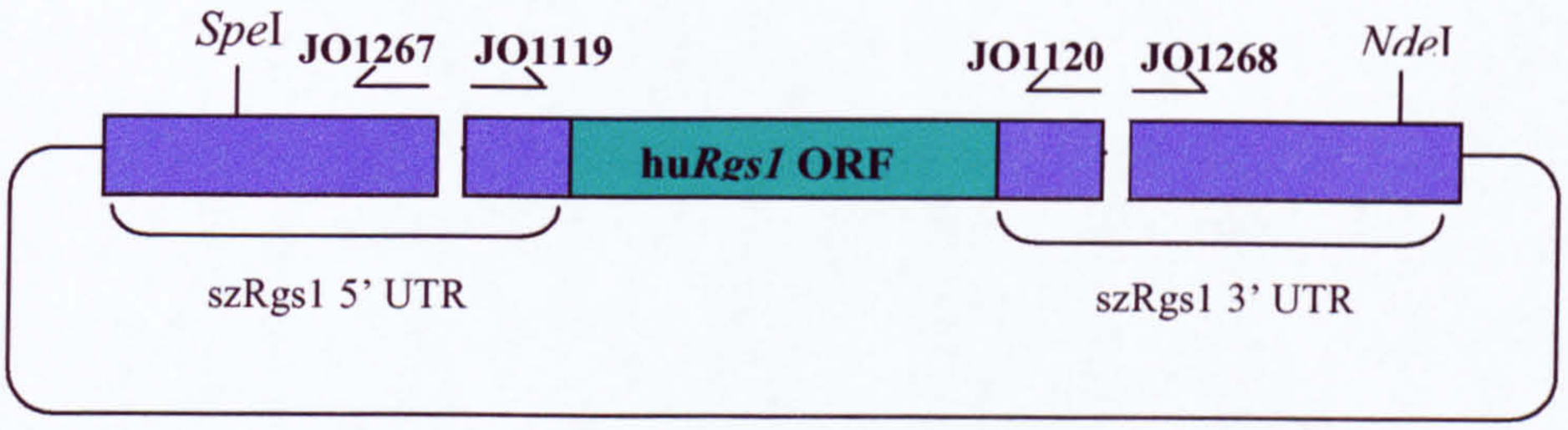
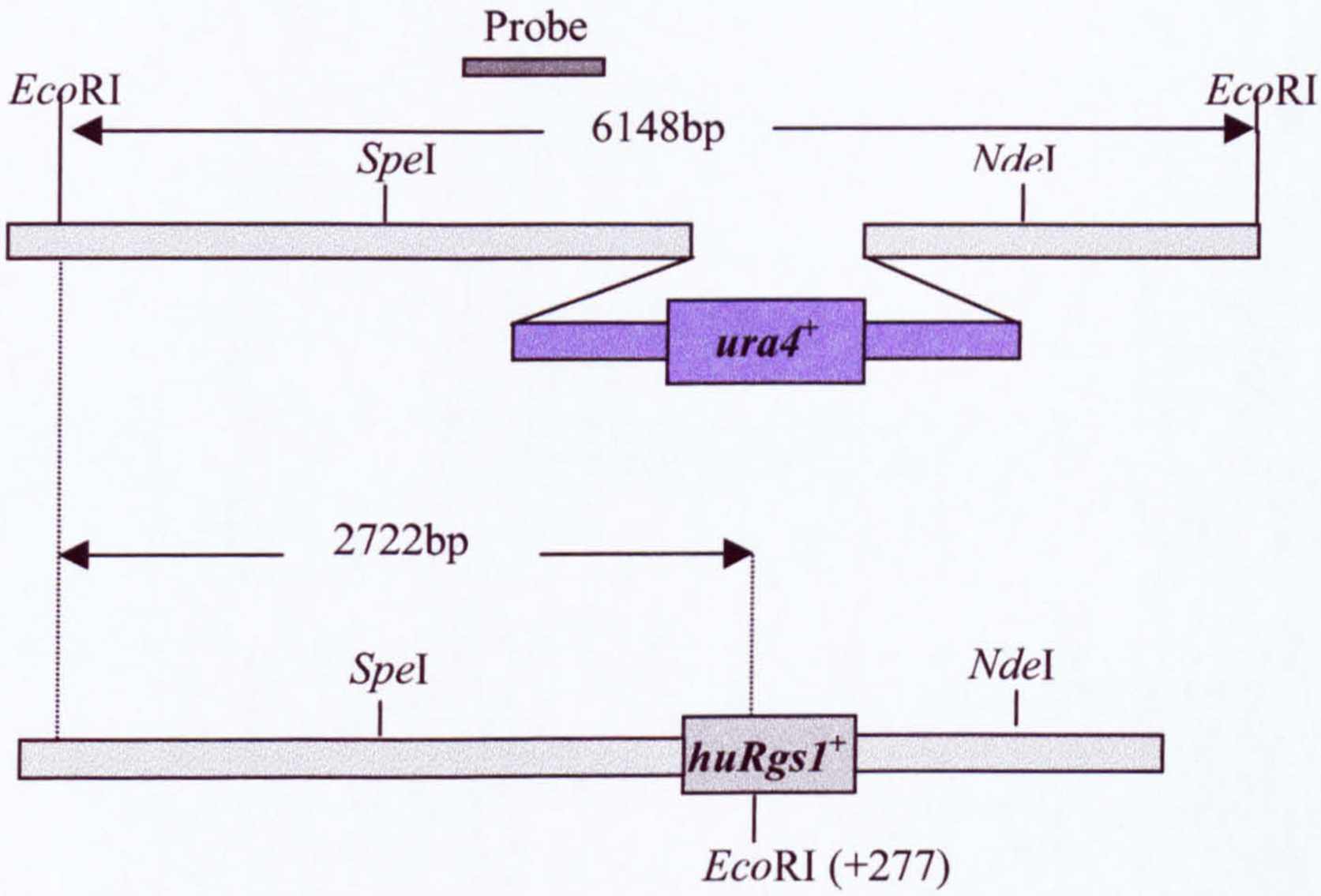


Figure 48. Creation of the human RGS1 integration construct

JO1119 and JO1120 were used to amplify the human *Rgs1* ORF flanked with *Sz. pombe rgs1* 5' and 3' non-coding regions. This fragment was cloned into a fragment amplified with JO1267 and JO1268, which provided additional *Sz. pombe rgs1* 5' and 3' non-coding regions.

The *rgs1*⁻ *sxa2*::*lacZ* reporter strain JY629 was transformed with the human *Rgs1* ORF integration fragment (liberated via *Spe*I/*Nde*I restriction from the *rgs1*::*Rgs1* integration construct). Cells were cultured in YEALU medium until they reached stationary phase (approximately 48h) to enable recovery of cells and exhaustion of intracellular uracil stores. Cells were plated onto AA plates containing 5-FOA and uracil, and incubated for 5d at 29°C. 5-FOA selects for the absence of Ura4 activity, as Ura4 converts it into a compound toxic to *Sz. pombe*. In the *rgs1*⁻ *sxa2*::*lacZ* reporter strain the presence of the *ura4* cassette at the *rgs1* locus results in Ura4 expression, with the consequence that cells are unable to grow on media containing 5-FOA. Thus, replacing the *ura4* cassette at the *rgs1* locus with the human *Rgs1* ORF enables cells to grow on media containing 5-FOA in the absence of pheromone signalling. Transformants were patched onto AA plates containing 5-FOA and uracil, and screened via PCR amplification for the presence of the human *Rgs1* ORF at the *rgs1* locus. This was carried out using oligonucleotide primers complementary to the *Sz. pombe* 5' non-coding region and the human *Rgs1* ORF.

Genomic DNA was prepared for strains positive by PCR, and the DNA digested with *Eco*RI. Digested DNA was separated on a 1% agarose gel, blotted onto a nitrocellulose filter, and probed with a DNA fragment amplified from the *rgs1* 5' sequence (Figure 49). One strain confirmed by Southern blot analysis was chosen to determine its pheromone-independent and pheromone-dependent β-galactosidase activity (JY1193).



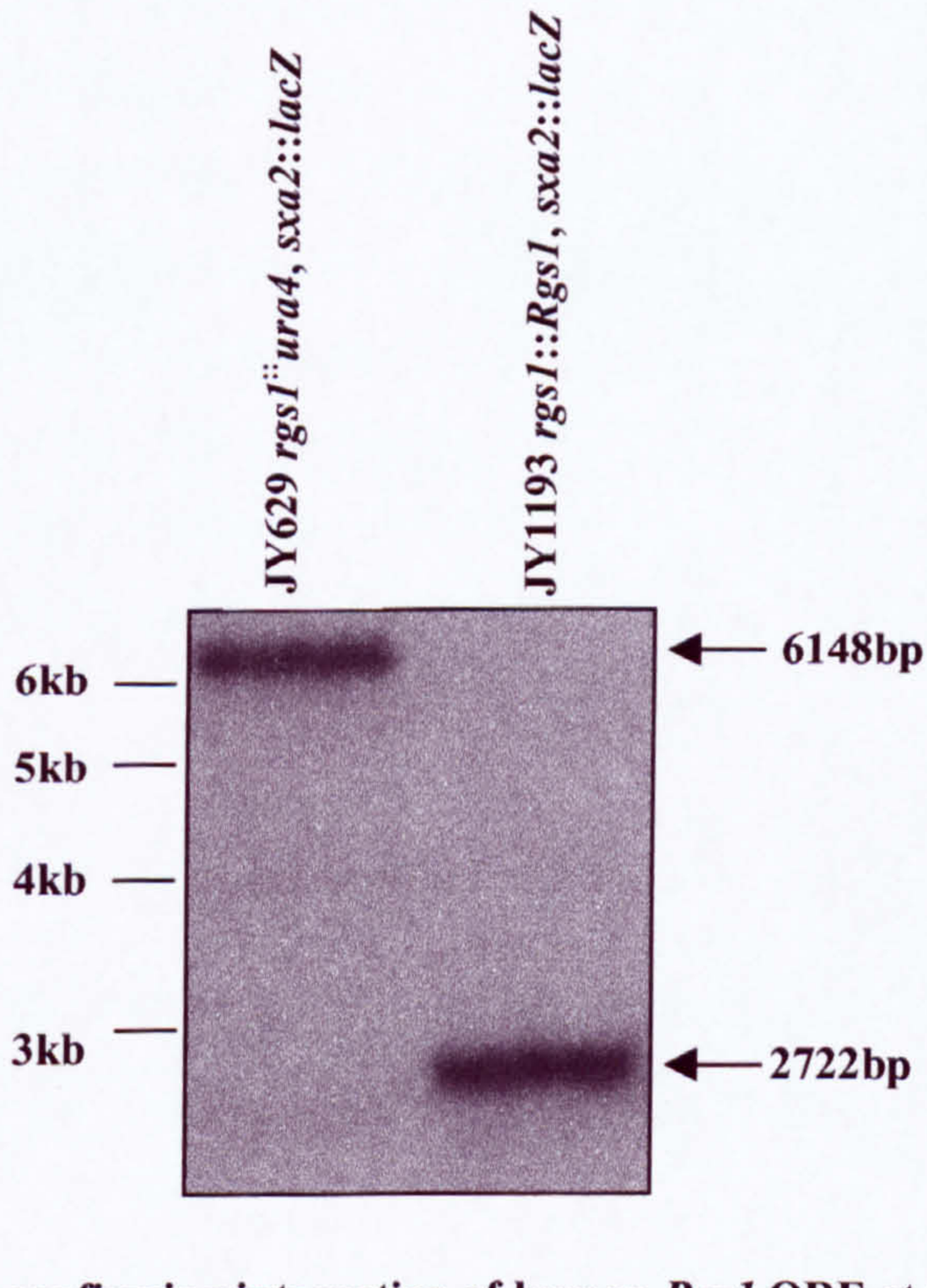


Figure 49. Southern blot confirming integration of human *Rgs1* ORF at the *rgs1* locus in the *rgs1*⁻ *sxa2*::*lacZ* reporter strain

Genomic DNA was prepared for JY629 (*rgs1*⁻, *sxa2*::*lacZ*) and JY1193 (*rgs1*::*Rgs1*, *sxa2*::*lacZ*), and the DNA digested with *Eco*RI. Integration of the human *Rgs1* ORF at the *rgs1* locus is indicated by a band of 2722bp, whilst non-integration of the *Rgs1* ORF is indicated by a band of 6148bp.

4.4.3. β-galactosidase activity of the *rgs1::Rgs1* LacZ reporter strain

JY1193 (*rgs1::Rgs1, sza2::lacZ*) exhibited comparable pheromone-independent β-galactosidase activity to the *rgs1*$^{+}$ LacZ reporter (JY544) (Figure 50). JY1193 also exhibited comparable pheromone-dependent β-galactosidase activity to JY544 up to 10U/ml P-factor. At 100U/ml and 1000U/ml P-factor the expression of huRGS1p from a single genomic copy did not reduce LacZ expression to a similar degree as szRgs1p, although pheromone-dependent LacZ activity of the *rgs1::Rgs1* strain is less than that of the *rgs1*$^{-}$ LacZ reporter strain at 100U/ml P-factor.

The results suggest that the presence of a single copy of the human *Rgs1* ORF at the *rgs1* locus in the *rgs1*$^{-}$ LacZ reporter strain was capable of reducing sensitivity and response to P-factor up to 10U/ml to a level seen for the *rgs1*$^{+}$ LacZ reporter strain, which contains a single copy of the endogenous *rgs1* ORF. However, a single copy of the human *Rgs1* ORF did not seem capable of reducing sensitivity or response to P-factor above a concentration of 10U/ml to the level seen for the *rgs1*$^{+}$ LacZ reporter strain. It is possible that at low P-factor concentrations the RGS activity of huRGS1p towards the *Sz. pombe* G_{α} subunit was sufficient to inhibit pheromone signalling to a comparable degree as szRgs1p. At high P-factor concentrations, the number of GTP-bound G_{α} subunits may have increased to such a level that huRGS1p activity was insufficient to inhibit the pheromone signalling pathway to the same degree as szRgs1p. This may have been due to lower affinity of huRgs1p for the *Sz. pombe* G_{α} subunit compared to szRgs1p.

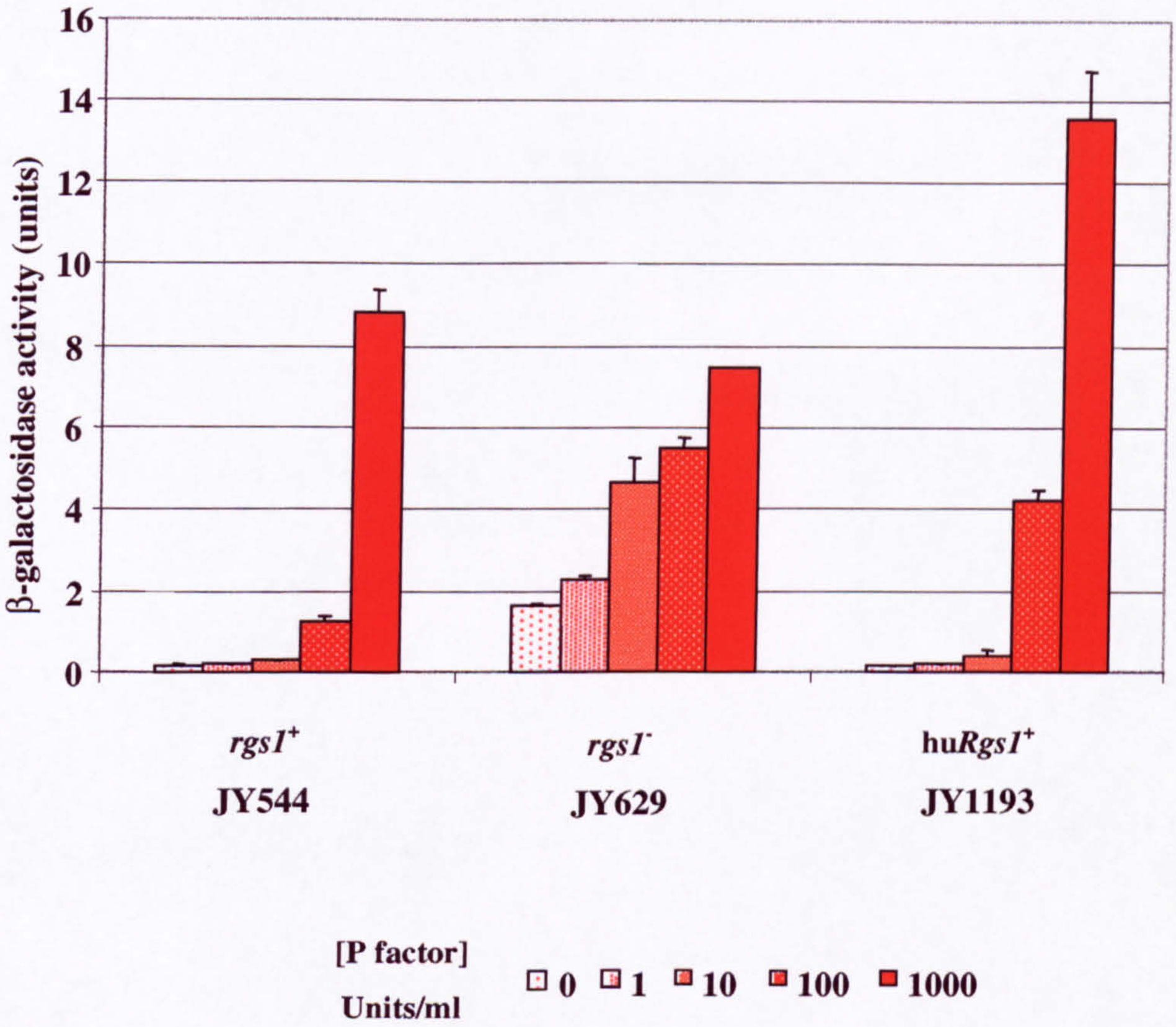


Figure 50. Integration of human Rgs1 ORF at *Sz. pombe rgs1* locus decreases sensitivity and response to P-factor pheromone at low P-factor concentrations

The *rgs1*$^{+}$ (JY544), *rgs1*$^{-}$ (JY629) and *Rgs1*$^{+}$ (JY1193; human *Rgs1* ORF replaces that of *Sz. pombe rgs1*) LacZ reporter strains were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Strains were cultured to a density of $5x10^{5}$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

4.4.4. Integration of the *Rgs4* ORF in the *rgs1*⁻ LacZ reporter strain

In order to generate an *rgs1*::*Rgs4* integration construct the human *Rgs4* open reading frame was cloned into *Sz. pombe rgs1* 5' and 3' non-coding regions. The human *Rgs4* ORF was amplified using the oligonucleotides JO1121 and JO1122 from the pKS-*Rgs4* construct described in Section 4.3.4. The sense primer JO1121 contains 57 bases complementary to the *Sz. pombe rgs1* 5' non-coding region (initiating at position –57 relative to the *rgs1* initiator codon) followed by 23 bases complementary to the first N-terminal 23 bases of the human *Rgs4* ORF. The antisense primer JO1122 contains 61 bases complementary to the *Sz. pombe rgs1* 3' non-coding region (initiating at position +1504 relative to the *rgs1* initiator codon) followed by 23 bases complementary to the C-terminal 3' human *Rgs4* ORF sequence. JO1121 and JO1122 were used to amplify a fragment containing the human *Rgs4* ORF flanked with *Sz. pombe* 5' and 3' non-coding sequences.

The fragment amplified by JO1121 and JO1122 was cloned into a construct generated to provide additional *Sz. pombe rgs1* 5' and 3' non-coding sequences to aid homologous integration. The sense primer JO1268 contains 21 bases complementary to the *Sz. pombe rgs1* 3' non-coding region, initiating at nucleotide position +1505 relative to the *rgs1* initiator codon. The antisense primer JO1267 contains 18 bases complementary to the *Sz. pombe rgs1* 5' non-coding region initiating at nucleotide position -58 relative to the *rgs1* initiator codon.

JO1267 and JO1268 were used in conjunction with a construct containing the *Sz. pombe rgs1* 5' and 3' non-coding regions (JD1394) to generate a linear DNA fragment with which the *rgs1*::*Rgs4* fragment amplified by JO1121 and JO1122 could be ligated, so that the human *Rgs4* ORF is situated flush between the *Sz. pombe rgs1* 5' and 3' non-coding regions (Figure 51).

The *rgs1*⁻ *sxa2*::*lacZ* reporter strain JY629 was transformed with the human *Rgs4* ORF integration fragment liberated via *Spe*I/*Nde*I restriction from the *rgs1*::*Rgs4* integration construct. Cells were cultured in YEALU medium until they reached stationary phase (approximately 48h), plated onto AA plates containing 5-

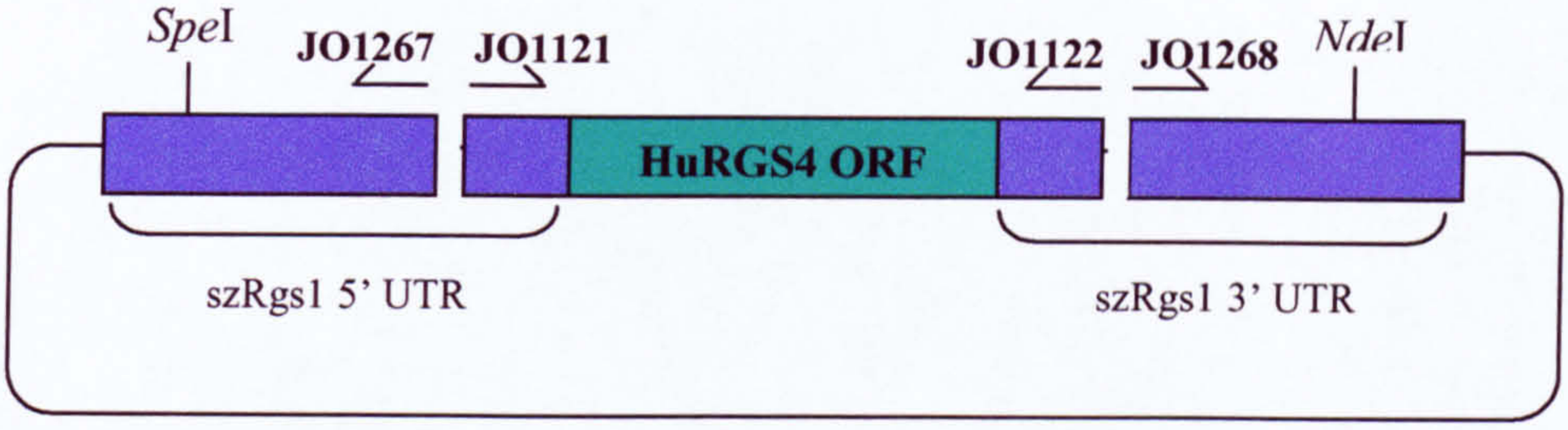


Figure 51. Creation of the human RGS4 integration construct

JO1121 and JO1122 were used to amplify the human *Rgs4* ORF flanked with *Sz. pombe rgs1* 5' and 3' non-coding regions. This fragment was cloned into a fragment amplified with JO1267 and JO1268, which provided additional *Sz. pombe rgs1* 5' and 3' non-coding regions.

FOA and uracil, and incubated for 5d at 29°C. Colonies were patched onto AA plates containing 5-FOA and uracil, and screened via PCR amplification for the presence of the human *Rgs4* ORF at the *rgs1* locus. This was carried out using oligonucleotide primers complementary to the *Sz. pombe rgs1* 5' non-coding region and the human *Rgs4* ORF.

Genomic DNA was prepared for strains positive by PCR, and the DNA digested with *Eco*RI. Digested DNA was separated on a 1% agarose gel, blotted onto a nitrocellulose filter, and probed with a fragment amplified from the *rgs1* 5' non-coding region (Figure 52). One strain confirmed by Southern blot analysis was chosen to characterise its pheromone-independent and pheromone-dependent β-galactosidase activity (JY1153).

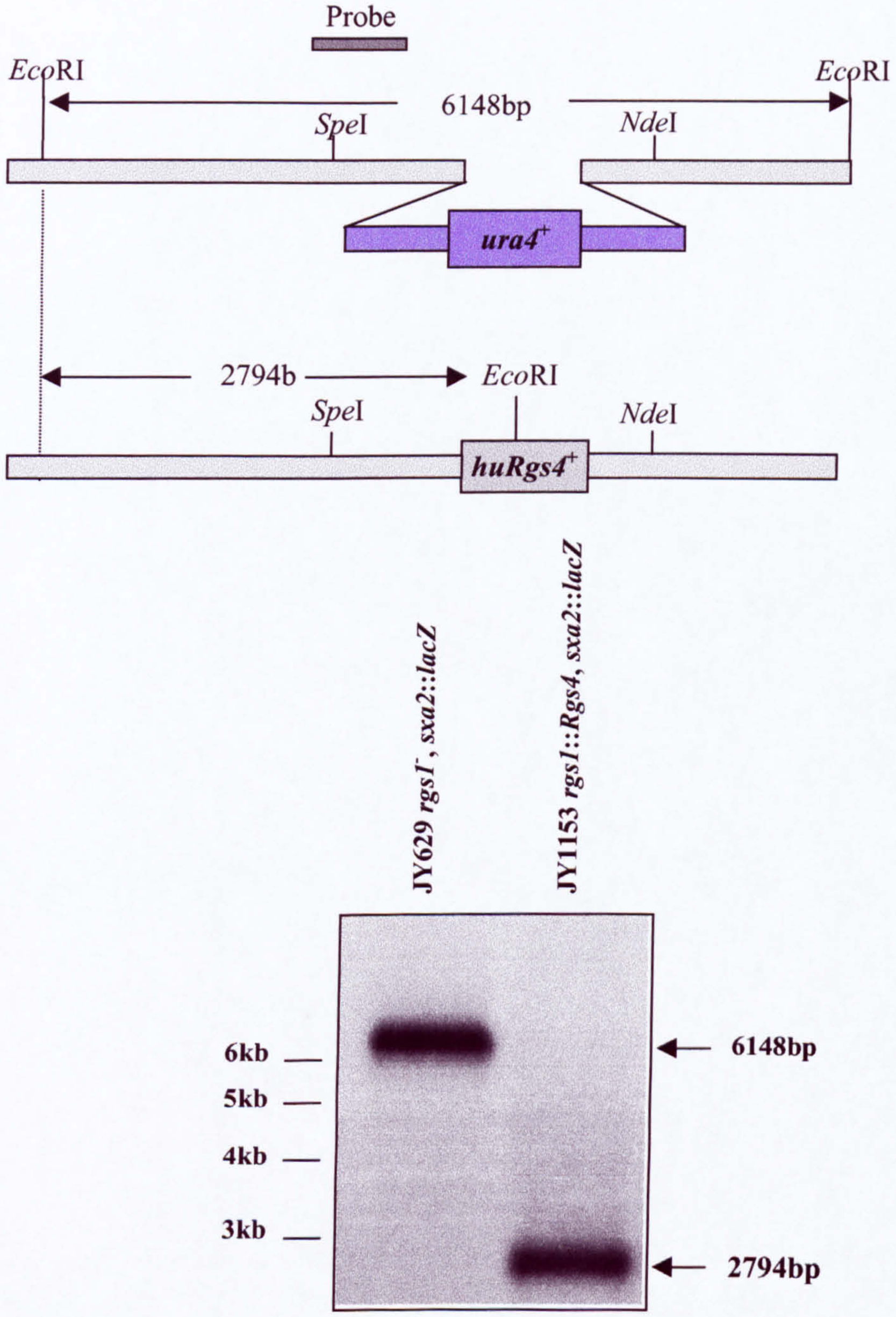


Figure 52. Southern blot confirming integration of human *Rgs4* ORF at the *rgs1* locus in the *rgs1*⁻ *sxa2::lacZ* reporter strain

Genomic DNA was prepared for JY629 (*rgs1*⁻, *sxa2::lacZ*) and JY1153 (*rgs1::Rgs4*, *sxa2::lacZ*), and the DNA digested with *Eco*RI. Integration of the *Rgs4* ORF at the *rgs1* locus is indicated by a band of 2794bp, whilst non-integration of the *Rgs1* ORF is indicated by a band of 6148bp.

4.4.5. β-galactosidase activity of the *rgs1*::*Rgs4* LacZ reporter strain

Once confirmed by Southern blot, the pheromone-independent and pheromone-dependent β-galactosidase activity of the *rgs1*::*Rgs4 sxa2*::*lacZ* strain (JY1153) in response to P-factor stimulation was determined (Figure 53).

The presence of a single copy of the human *Rgs4* ORF at the *Sz. pombe rgs1* locus in the *rgs1*$^-$ LacZ reporter strain slightly reduced LacZ activity compared the *rgs1*$^-$ LacZ reporter strain (this was possibly due to strain and experiment variation), but the level of signalling in the RGS4 integrant was not reduced to that of the *rgs1*$^+$ LacZ reporter strain. The *rgs1*::*Rgs4* LacZ reporter strain had an increased pheromone-independent LacZ activity and increased sensitivity and response to P-factor stimulation compared to the *rgs1*$^+$ LacZ reporter strain. These results suggest that the presence of a single copy of the human *Rgs4* ORF at the *rgs1* locus was not capable of rescuing the hypersensitive phenotype of the *rgs1*$^-$ LacZ reporter strain. This could have been due to the G_α subunit specificity of human RGS4, or due to reduced expression of RGS4 compared to that of szRgs1p in the *rgs1*$^+$ LacZ reporter strain. RGS4 successfully reduced LacZ expression in the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains when expressed from a multicopy expression vector (Section 4.3.4). If huRGS4p had a lower affinity for the *Sz. pombe* G_α subunit compared to endogenous szRgs1p, it may have been unable to reduce signalling through the pheromone communication pathway when expressed from a single genomic copy.

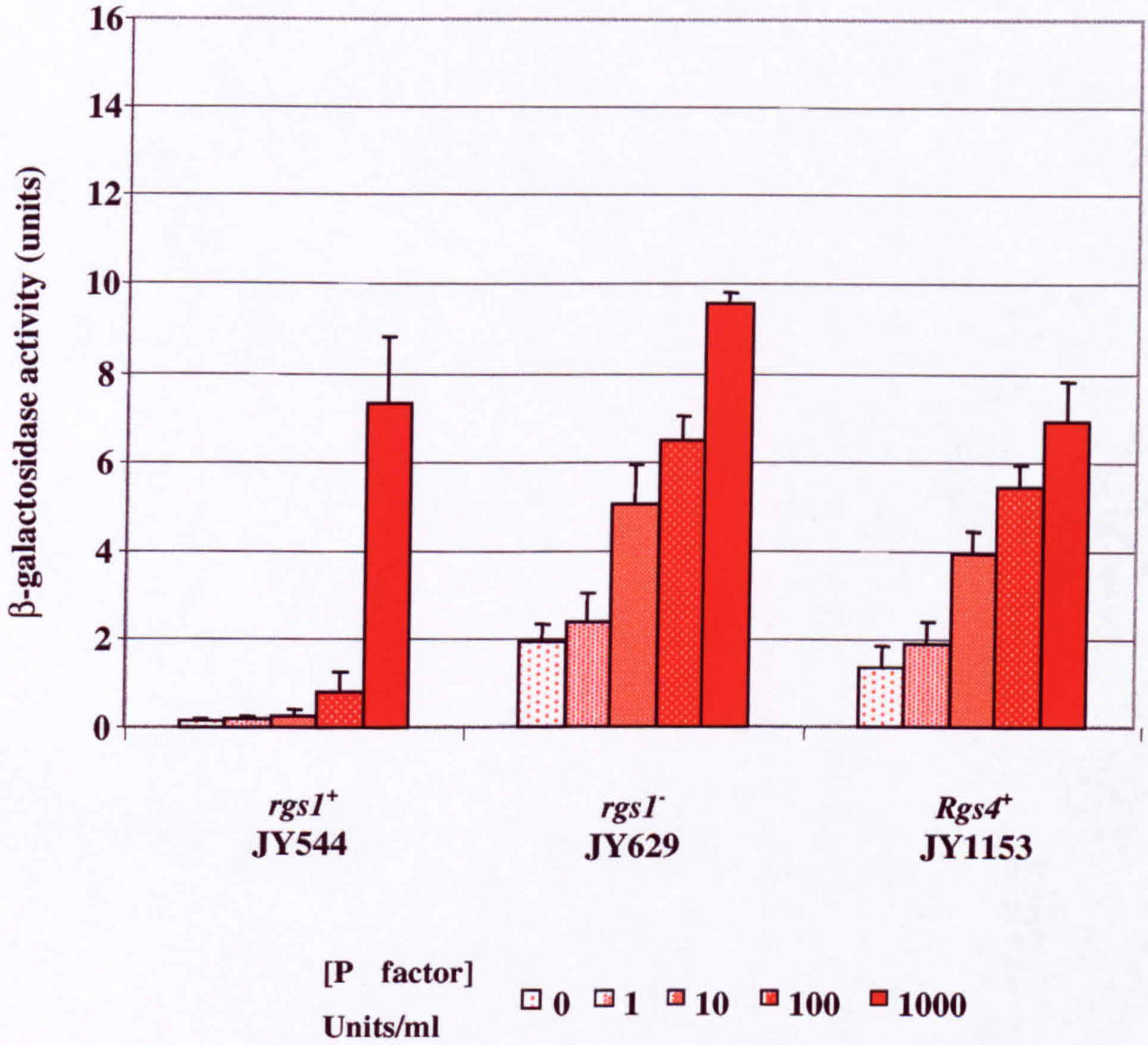


Figure 53. Integration of human *Rgs4* ORF at the *rgs1* locus does not decreases sensitivity and response to P-factor pheromone at low pheromone concentrations

The *rgs1*$^+$ (JY544), *rgs1*$^-$ (JY629) and *Rgs4*$^+$ (JY1153; human Rgs4 ORF replaces that of *Sz. pombe rgs1*) LacZ reporter strains were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

4.4.6. Growth characteristics of *rgs1*::*Rgs4* LacZ reporter strain

The *rgs1*::*Rgs4* LacZ reporter strain does not contain the *ura4* gene within its genome, unlike the *rgs1*$^-$ LacZ reporter strain, and it is possible that a reduced growth rate resulting from the absence of the *ura4* gene in the *rgs1*::*Rgs4* LacZ reporter strain could affect the determination of its β-galactosidase activity when compared to that of the *rgs1*$^-$ LacZ reporter strain. To determine whether these two reporter strains exhibited different growth rates (which could influence the calculation of β-galactosidase activities), their generation times in AA media containing uracil were determined.

Growth characteristics of the *rgs1*$^-$ and *rgs1*::*Rgs4* strains were determined in AA media containing alanine, leucine and uracil at 29°C. Briefly, 50ml medium were inoculated with cells to obtain a cell density of $2x10^5$ cells/ml, and cells were incubated in a shaking incubator at 29°C. Once strains reached a density of approximately $1x10^6$ cells/ml cell counts were determined hourly via a Coulter Channelyser.

As can be seen from Figure 54, the *rgs1*$^-$ *sxa2*::*lacZ* reporter strain JY629 (*ura4*$^+$) had a slightly faster doubling time (4h) compared to the *rgs1*::*Rgs4*, *sxa2*::*lacZ* reporter strain JY1153 (*ura4*$^-$) (4½h). As the generation times of the two strains did not differ greatly, it is unlikely that the absence of the *ura4* gene in the *rgs1*::*Rgs4* LacZ reporter strain affected the determination of its β-galactosidase activity.

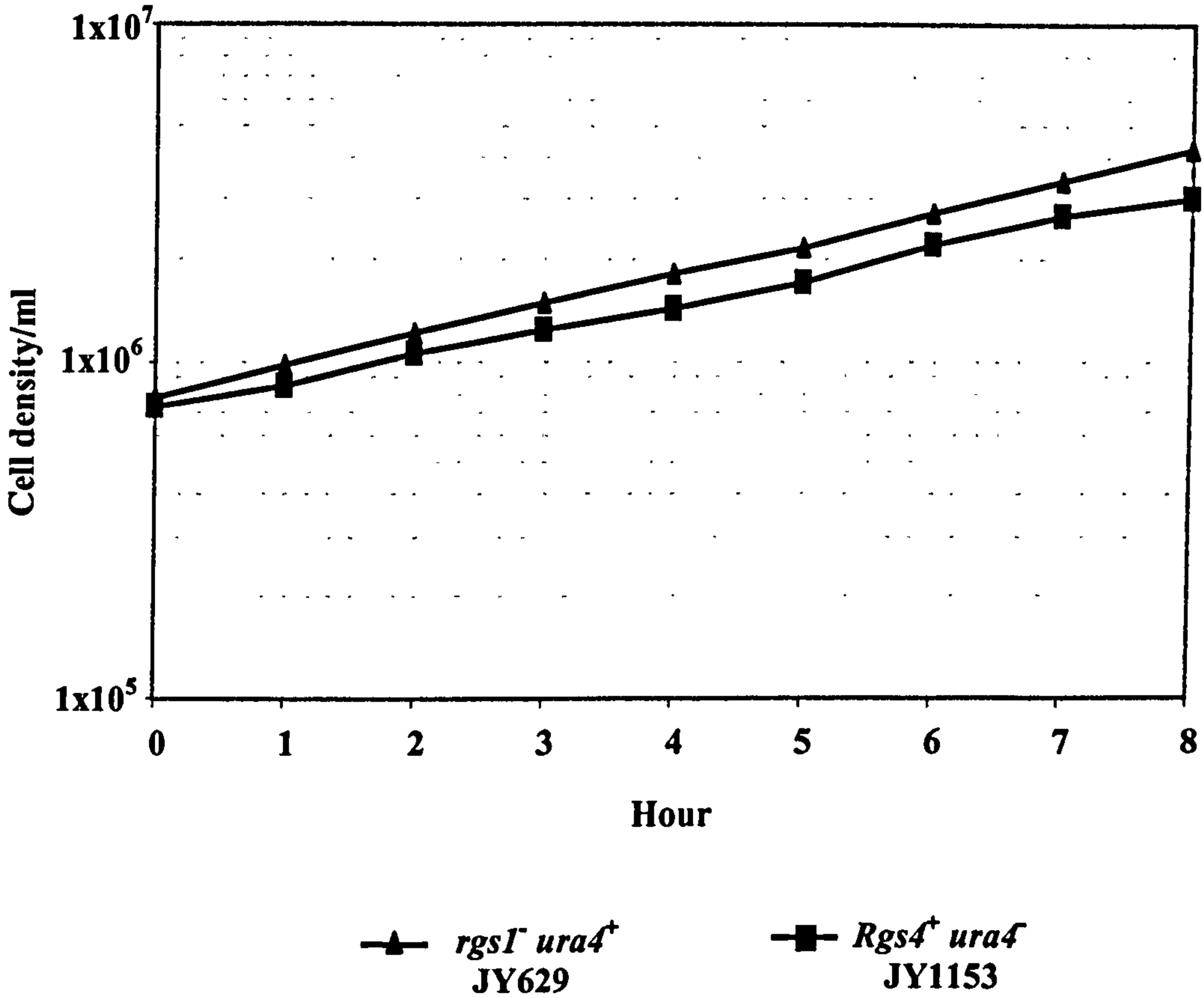


Figure 54. The *rgs1*⁻ LacZ reporter strain exhibits a faster generation time than the *rgs1*::*Rgs4* LacZ reporter strain

Growth characteristics of the *rgs1*⁻ (JY629) and *rgs1*::*Rgs4* (JY1153) LacZ reporter strains were observed in AA media containing alanine, leucine and uracil at 29°C. 50ml medium were inoculated with $2x10^5$ cells/ml, and cells were cultured in a shaking incubator at 29°C. JY629 had a generation time of 4h, while JY1153 had a slightly longer generation time of 4½h.

4.4.7. Expression of human RGS4 from single copy

It is possible that the increased sensitivity and response to pheromone observed for the *rgs1*::*Rgs4* LacZ reporter strain compared to the *rgs1*$^{+}$ LacZ reporter strain was due to a reduced level of huRGS4p expression compared to that of szRgs1p in the *rgs1*$^{+}$ LacZ reporter strain.

To determine whether huRGS4p was expressed in the *rgs1*::*rgs4 sxa2*::*lacZ* strain, Western blot analysis was carried out. Crude yeast extracts were prepared for JY762 (*rgs1*$^{-}$ *sxa2*::*lacZ* pREP3X), JY778 (*rgs1*$^{-}$ *sxa2*::*lacZ* pREP3X-*Rgs4*), JY629 (*rgs1*$^{-}$, *sxa2*::*lacZ*) and JY1153 (*rgs1*::*Rgs4*, *sxa2*::*lacZ*). A separate isolate of JY1153 stimulated with 1000U/ml P-factor for 16h, to determine whether this would increase huRGS4p expression levels.

From Figure 55 it can be seen that a band of approximately 28kD in size was present in Lane 2 (*rgs1*$^{-}$, *sxa2*::*lacZ*, pREP3X-*Rgs4*). This band was not seen in Lane 1, which represents the *rgs1*$^{-}$ LacZ reporter strain transformed with the empty pREP3X expression vector. A band of 28kD was also seen in Lane 4 (*rgs1*::*Rgs4*, *sxa2*::*lacZ*), which indicates that human RGS4 is expressed when the human *Rgs4* ORF was present as a single genomic copy. Stimulation of the *rgs1*::*Rgs4* LacZ reporter strain with 1000U/ml P-factor for 16hr did not appear to up-regulate human RGS4 expression. The presence of the 28kD band in Lane 4 and its absence in Lane 5 was due to overloading of crude extract in Lane 4 (determined by silver staining, not shown).

As seen for expression of huRGS4p from a multicopy expression vector (Figure 44), the protein visualised in Figure 55 was larger than the published size of the human RGS4 protein. This is possibly due to covalent modification of the RGS4 protein in *Sz. pombe*. RGS4 is palmitoylated, with Cys-2 and Cys-12 the likely sites of palmitoylation (Srinivasa *et al.*, 1998a). Mutation of cysteine residues within the N-terminal domain does not affect plasma membrane localisation in yeast or its ability to inhibit signalling (Srinivasa *et al.*, 1998a). Plasma localisation is required for RGS4 function, and so features of its N-terminal

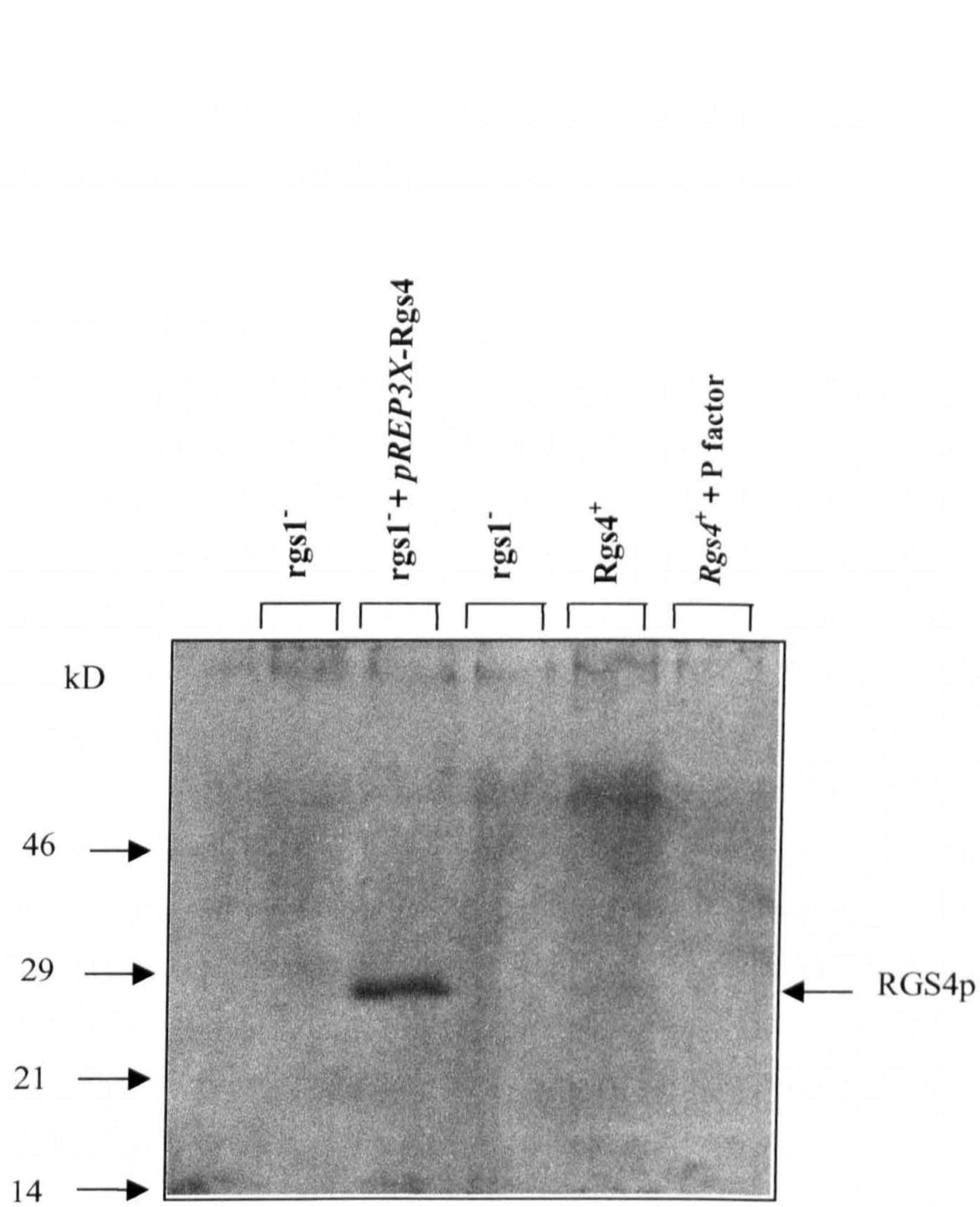


Figure 55. Human RGS4 is expressed in the *Sz. pombe rgs1*::*rgs4* LacZ reporter strain

Crude yeast extracts were prepared from the *rgs1*⁻ LacZ reporter strain transformed with pREP3X (Lane 1), the *rgs1*⁻ LacZ reporter strain overexpressing human RGS4 (Lane 2), the *rgs1*⁻ LacZ reporter strain (Lane 3) and the *Rgs4*⁺ LacZ reporter strain (Lane 4). A separate isolate of the *Rgs4*⁺ LacZ reporter strain (Lane 5) was incubated with 1000U/ml P-factor for 16h prior to extract preparation. Extracts were subjected to electrophoresis, blotted onto a nitocellulose filter, and allowed to react with rabbit anti-RGS4 antibody. Immunoreactive bands were visualised with goat anti-rabbit antibody coupled to horseradish peroxidase, and an ECL Western blotting system was used to stain the bands before exposure to x-ray film. A 28kD band can be seen in Lane 2 (*rgs1*⁻, pREP3X-huRGS4) and Lane 4 (*rgs1*::*Rgs4*, *sxa2*::*lacZ*).

domain other than palmitoylation are responsible for the plasma membrane association of RGS4 and its ability to inhibit pheromone response in yeast.

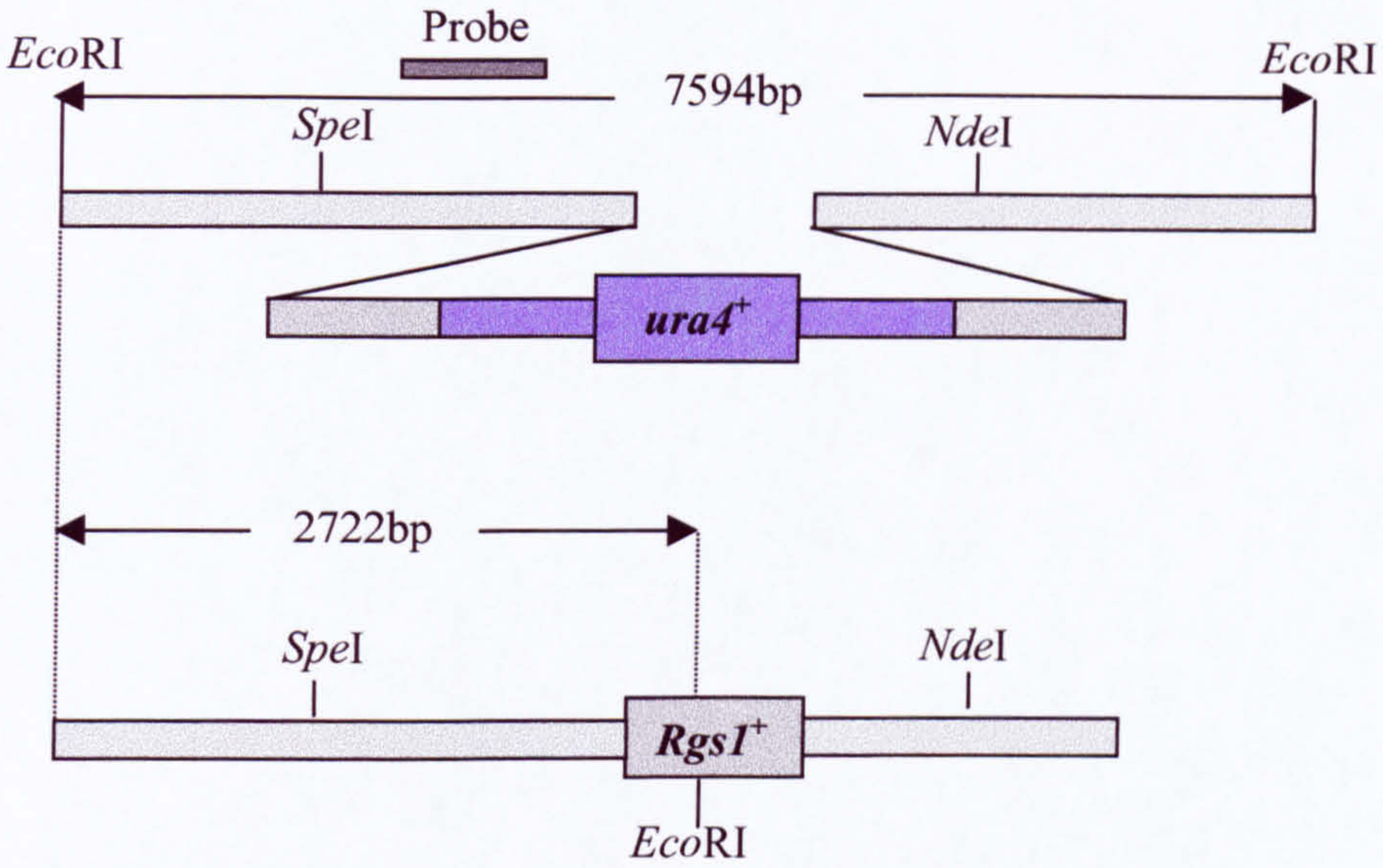
4.5. The *sxa2*::*ura4* reporter strain

In the *sxa2*::*ura4* reporter strains the *ura4* ORF replaces the *sxa2* ORF at the *sxa2* locus, and as a result Ura4 expression is directed by the pheromone-regulated *sxa2* promoter. Induction of *sxa2* by P-factor stimulation results in Ura4 expression, which can be detected by cell viability on media lacking uracil. The advantage of this reporter strain is that inhibition of the pheromone communication pathway can be detected by the use of the compound 5-FOA. 5-FOA is converted into a toxic compound by Ura4, and inhibition of the pheromone signalling pathway in *sxa2*::*ura4* reporter strains enables cells to grow in the presence of 5-FOA. Plates containing 5-FOA and a range of P-factor concentrations can be used to qualitatively determine the degree of pathway inhibition. P-factor stimulation induces *sxa2* induction in a dose-dependent manner: the greater the P-factor concentration, the greater the induction of *sxa2*, and the greater the Ura4 expression. The ability of *sxa2*::*ura4* reporter strains to grow at high P-factor concentrations in the presence of 5-FOA indicates inhibition of the pheromone communication pathway.

4.5.1. Integration of the human *Rgs1* ORF in the *sxa2*::*ura4* reporter strain

The *rgs1*::*Rgs1* integration construct described in section 4.4.2 was used to replace the endogenous *rgs1* ORF with that of human *Rgs1* in an *rgs1*::*ura4*, *sxa2*::*ura4* reporter strain. The *rgs1*::*ura4*, *sxa2*::*ura4* reporter strain JY727 was transformed with the *rgs1*::*Rgs1* integration fragment, liberated via *Spe*I/*Nde*I restriction. In JY727 the *rgs1* ORF is disrupted with the *ura4* cassette at an internal *Bam*HI site. Cells were cultured in YEALU medium until they reached stationary phase (approximately 48h) to enable recovery of cells and exhaustion of intracellular uracil stores. Cells were plated onto AA plates containing 5-FOA and uracil and incubated for 5d at 29°C. Transformants were patched onto AA plates containing 5-FOA and uracil, and screened via PCR amplification for the presence of the human *Rgs1* ORF at the *Sz. pombe rgs1* locus using oligonucleotide primers specific for the

rgs1 5' non-coding region and the human *Rgs1* ORF. Strains positive by PCR were analysed by Southern blot. Genomic DNA was digested with *Eco*RI and the digested DNA separated on a 1% agarose gel, blotted onto a nitrocellulose filter, and probed with a fragment amplified from the *rgs1* 5' non-coding region (Figure 56). One correct isolate (JY1194) was chosen to determine pheromone-independent and pheromone-dependent Ura4 expression.



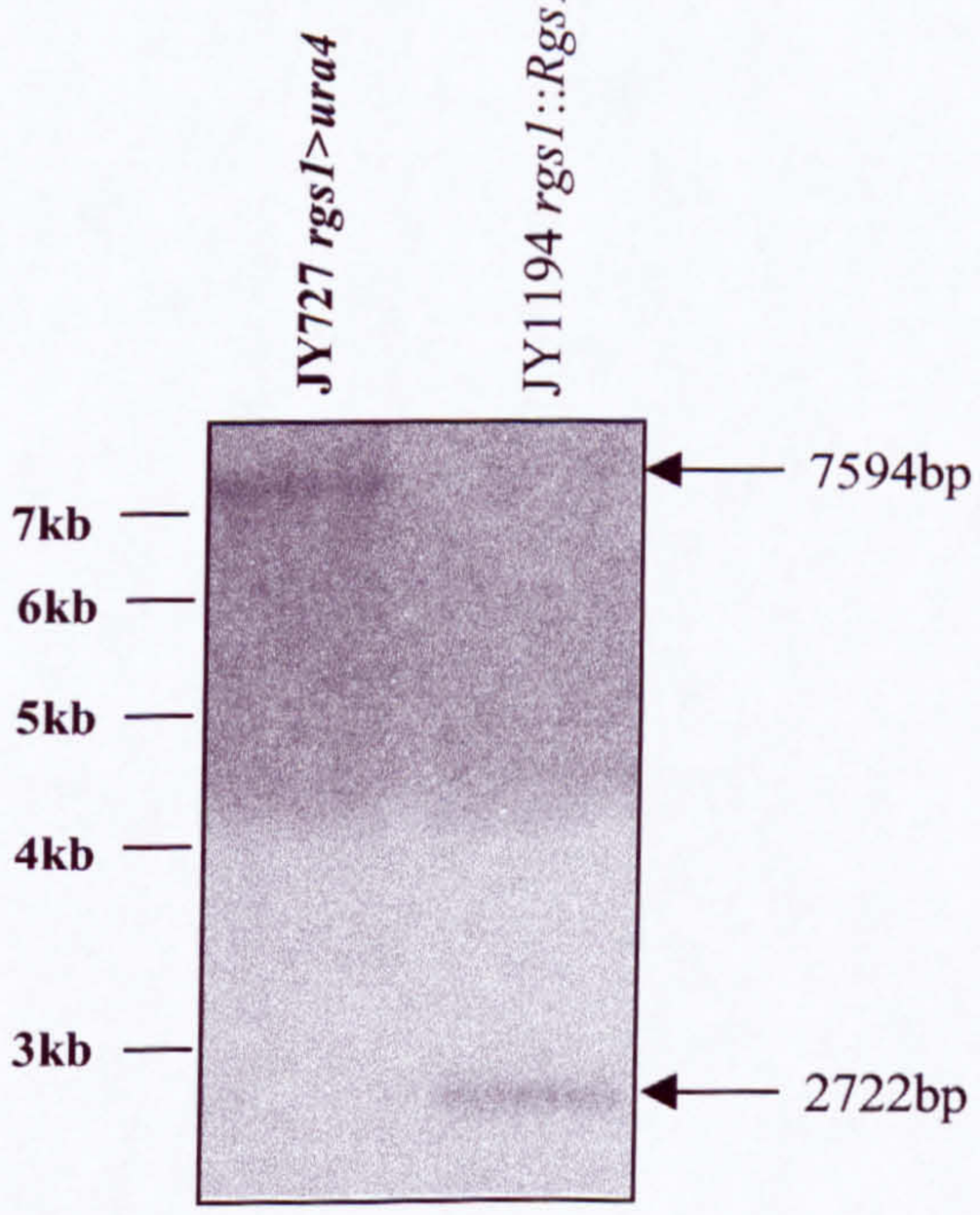


Figure 56. Southern blot confirming integration of the human *Rgs1* ORF at the *rgs1* locus in the *sxa2*::*ura4* reporter strain

Genomic DNA of JY727 (*rgs1*>*ura4*, *sxa2*::*ura4*) and JY1194 (*rgs1*::*Rgs1*, *sxa2*::*ura4*) were digested with *Eco*RI. Correct integration of the human *Rgs1* ORF at the *rgs1* locus is indicated by a band of 2722bp, whilst non-integration of the human *Rgs1* ORF is indicated by a band of 7594bp.

4.5.2. The *rgs1*::*Rgs1 sxa2*::*ura4* reporter strain is viable on AA plates containing 5-FOA and uracil up to 1U/ml P-factor

The *rgs1*$^{+}$ *sxa2*::*ura4* reporter strain JY603 (*rgs1*$^{+}$, *sxa2*::*ura4*) and JY1194 (*rgs1*::*Rgs1*, *sxa2*::*ura4*) were cultured overnight in AA media lacking uracil and $5x10^{4}$ cells plated onto AA plates containing 5-FOA, uracil, and a range of P-factor concentrations. Plates were incubated for 3d at 29°C.

The *rgs1*$^{+}$ *sxa2*::*ura4* reporter strain (JY603) was capable of growth up to 1U/ml P-factor (Figure 57). Integration of the human *Rgs1* ORF at the *rgs1* locus appeared to reduce signalling to a similar degree to that seen for the *rgs1*$^{+}$ Ura4 reporter strain up to 1U/ml P-factor. The *rgs1*$^{-}$ *sxa2*::*ura4* reporter strain is incapable of growth on AA plates containing 5-FOA and uracil in the absence or presence of pheromone, due to the increased pheromone-independent level of signalling and increased sensitivity to pheromone compared to the *rgs1*$^{+}$ Ura4 reporter strain. The *rgs1*::*Rgs1* Ura4 reporter strain does not present us with information concerning the degree of pathway inhibition, but indicates the P-factor concentration at which pheromone signalling in this strain resulted in sufficient Ura4 expression to prevent growth on 5-FOA plates.

These results corroborate those seen for the *rgs1*::*Rgs1 sxa2*::*lacZ* reporter strain (Figure 50). Expression of huRGS1p from a single genomic copy was capable of negatively regulating the pheromone communication pathway at low levels of P-factor (up to 10U/ml P-factor in the LacZ reporter strain and up to 1U/ml P-factor in the Ura4 reporter strain), but not at high levels of P-factor. The *rgs1*$^{+}$ *sxa2*::*ura4* reporter strain, JY603, was also capable of colony formation on 5-FOA plates up to 10U/ml P-factor.

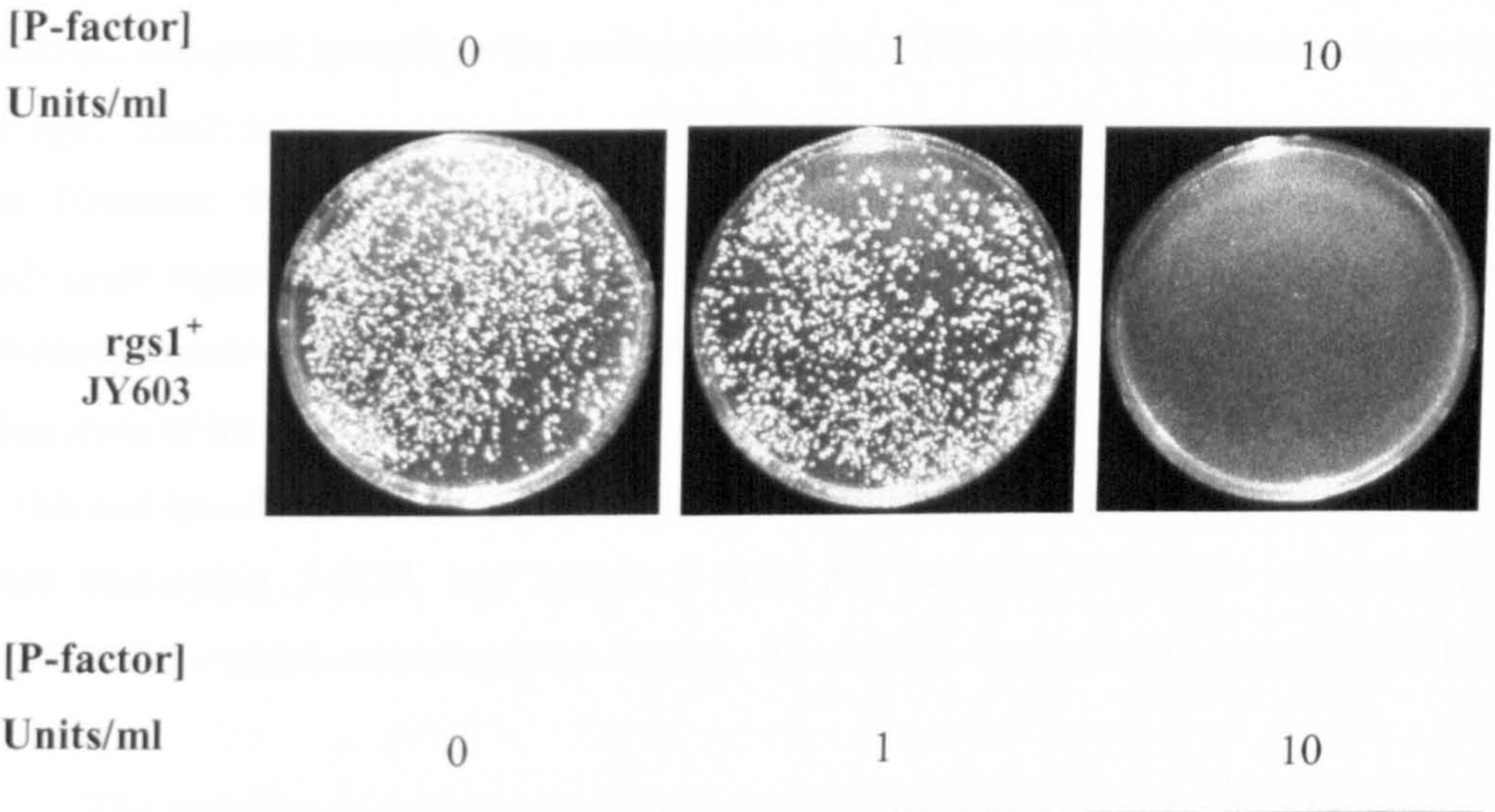


Figure 57. The *rgs1*::*Rgs1* Ura4 reporter strain displays similar sensitivity to P-factor compared to the *rgs1*$^+$ Ura4 reporter strain when cultured on AA plates containing 5-FOA and uracil

JY603 (*rgs1*$^+$, *sxa2*::*ura4*) and JY1194 (*rgs1*::*Rgs1*, *sxa2*::*ura4*) were plated onto AA plates containing 5-FOA and uracil and a range of P-factor concentrations. Plates were incubated for 3d at 29°C. JY603 and JY1194 were able to form colonies up to 1U/ml P-factor.

4.5.3. Integration of human *Rgs4* ORF in the *rgs1*⁻ *sxa2*::*ura4* reporter strain

The construct described in section 4.4.4 was used to generate an integration construct designed to replace the endogenous *rgs1* ORF with that of human *Rgs4* in the *rgs1*⁻ *sxa2*::*ura4* reporter strain (JY727). The *rgs1*::*Rgs4* integration fragment was liberated via *Spe*I/*Nde*I restriction, and transformed into the *rgs1*>*ura4*, *sxa2*::*ura4* reporter strain JY727. Cells were cultured in YEALU medium until stationary phase was reached (approximately 48h) to enable recovery of cells and exhaustion of intracellular uracil stores. Cells were plated onto AA plates containing 5-FOA and uracil and incubated for 5d at 29°C. Colonies were then patched onto AA plates containing 5-FOA and uracil. It was not possible to isolate *rgs1*::*Rgs4* transformants which contained the human *Rgs4* ORF at the *rgs1* locus by PCR screening.

The inability to isolate *rgs1*::*Rgs4*, *sxa2*::*ura4* transformants was probably due to increased pheromone-independent *sxa2* activity, which was observed in the *rgs1*::*Rgs4*, *sxa2*::*lacZ* reporter strain (Figure 53).

4.6. Summary

Disruption of the *rgs1* gene in the *Sz. pombe* LacZ reporter strain increased the pheromone-independent level of LacZ activity, and increased sensitivity and response to pheromone. This hypersensitive response to pheromone was rescued by expressing szRgs1p from a multicopy expression plasmid.

Expression of heterologous RGS proteins from expression constructs in the LacZ reporter strains negatively regulated the *Sz. pombe* pheromone communication pathway to varying extents. This variability of RGS activity was probably due to the G_α subunit specificity of specific RGS proteins. Out of the mammalian RGS proteins investigated, expression of huRGS1p and huRGS4p in the LacZ reporter strains had the greatest reductive effect upon signalling through the pheromone communication pathway. The data presented in Chapter 4 indicate that the activity of mammalian RGS proteins can be easily studied using the *Sz. pombe* LacZ reporter strains. However, the fact that heterogeneous RGS proteins can function in the *Sz. pombe* system, which is not physically relevant, means that conclusions about RGS function and regulation based solely upon data obtained from the reporter strains should be viewed with some caution. The ability of mammalian RGS proteins to function as GAPs in the *Sz. pombe* system illustrates the promiscuity of RGS proteins for different G_α subunits.

It appears that huRGS2p and huRGS4p could be covalently modified in *Sz. pombe*, and while palmitoylation has been observed for RGS4 (Srinivasa *et al.*, 1998a), there is at present no evidence for the palmitoylation of huRGS2p.

Chapter 5. The search for gain-of-function RGS mutants

5.1. Introduction

The resolution of the crystal structure of rat RGS4 complexed with AlF_4^--activated $G_{i\alpha1}$ (Tesmer *et al.*, 1997) provided significant structural information concerning the mechanism of RGS4 action and important residues within the RGS4 RGS domain. This study also confirmed the primary mechanism of RGS4 action upon the activated $G_{i\alpha1}$ subunit consisting of the stabilisation of the switch regions of $G_{i\alpha1}$ that undergo considerable conformational change during the GTPase cycle. The mechanism of RGS4 GAP activity has been further confirmed by other investigators (Berman *et al.*, 1996; Moy *et al.*, 1999; Sowa *et al.*, 2001). Numerous mutational studies have further characterised RGS4 activity and identified inactive RGS4 mutants, RGS4 mutants with decreased GAP activity and dominant-negative mutants (Natochin *et al.*, 1998; Druey and Kehrl, 1997; Srinivasa *et al.*, 1998b). At present there has been no report of a mammalian RGS protein with increased GAP activity towards G_α subunits. A dominant gain-of-function SST2 (an *S. cerevisiae* RGS protein) mutant has been reported (Dohlman *et al.*, 1995). However, the mutation conferring the hyperactivated phenotype resides in the non-conserved domain of SST2.

As I had previously determined that certain human RGS proteins (huRGS1p and huRGS4p) were capable of down-regulating the *Sz. pombe* pheromone signalling pathway when expressed from an expression vector, I considered the possibility of identifying huRGS4p gain-of-function mutants via random mutagenesis of the human *Rgs4* ORF via hydroxylamine treatment followed by the screening of mutants in the *rgs1⁻ sxa2::ura4 Sz. pombe* reporter strain. This approach would require integration of the human *Rgs4* ORF into the *Sz. pombe* genome, to ensure increased RGS activity within cells was not a result of indiscriminate expression from the expression vector (described in Section 4.5.1.). This mutagenic strategy had previously been utilised by Dr. Peter Watson in the Davey laboratory to isolate potential szRgs1p gain-of-function mutants, and I decided to use this approach in the isolation huRGS4p gain-of-function mutants.

5.2. Generation of potential *Sz. pombe* Rgs1 GOF mutants

5.2.1. Introduction

Prior to the mutagenesis of the human *Rgs4* ORF, Dr. Peter Watson undertook research in the Davey laboratory to isolate szRgs1p gain-of-function mutants. The *Sz. pombe rgs1* ORF was mutagenised via hydroxylamine treatment, and two potential gain-of-function mutations were identified in the *rgs1* ORF. The two mutations identified (A512→G and G913→A) resulted in a Histidine to Arginine substitution at amino acid residue 171 (A512→G) and a Valine to Isoleucine substitution at amino acid residue 305 (G913→A) in the *Sz. pombe* Rgs1p primary amino acid sequence. I recreated these mutations via site-directed PCR amplification to determine their effect upon szRgs1p activity in the *sxa2::lacZ* and *sxa2::ura4 Sz. pombe* reporter strains.

The *sxa2::lacZ* reporter strain has been described in Section 4.2.1. The *sxa2::ura4* reporter strain was described in Section 4.5.1. Briefly, in the *Sz. pombe* reporter strains the expression of a reporter protein (LacZ or *Sz. pombe* Ura4) is directed by the activity of the strictly-regulated, pheromone-dependent *sxa2* promoter. Signalling through the pheromone communication pathway following pheromone stimulation results in induction of *sxa2* promoter activity, and subsequent expression of the reporter protein. β-galactosidase activity can be assayed through the use of chromogenic substrates in liquid assays, and Ura4 activity can be assessed by determining strain viability on selective media plates.

5.2.2. Amplification of *Sz. pombe* Rgs1 gain-of-function site-directed mutations

For the Histidine to Arginine substitution at residue 171 in szRgs1p, the oligonucleotide primers JO1249 and JO1250 (Figure 58) were used in conjunction with a DNA construct (JD1394) containing the *rgs1* ORF flanked by its 5' and 3' non-coding regions (previously constructed by Dr. Peter Watson). The sense primer JO1249 contains 22 bases complementary to the *rgs1* ORF initiating at nucleotide position +513 relative to the *rgs1* initiator codon. The antisense primer JO1250 contains 21 bases complementary to the *rgs1* ORF initiating at nucleotide position +512 relative to the *rgs1* initiator codon, and introduces a guanine nucleotide at

nucleotide position +512 in the resulting *rgs1* ORF (replacing adenine in the wild-type *rgs1* ORF). Subsequent ligation circularised the amplified fragment, creating the A→G mutation at nucleotide position +512 in the *rgs1* ORF. Nucleotide substitution was confirmed by sequencing.

For the Valine to Isoleucine substitution at amino acid residue 305 in szRgs1p the oligonucleotide primers JO1247 and JO1248 were used with a DNA construct (JD1394) containing the *rgs1* ORF flanked by its 5' and 3' non-coding regions (Figure 59). The sense primer JO1247 contains 18 bases complementary to the *rgs1* ORF initiating at nucleotide position +914 relative to the *rgs1* initiator codon. The antisense primer JO1248 contains 20 bases complementary to the *rgs1* ORF initiating at nucleotide position +913 relative to the *rgs1* initiator codon, and introduces an adenine nucleotide at nucleotide position 913 in the resulting *rgs1* ORF (replacing guanine in the wild-type *rgs1* ORF). Subsequent ligation circularised the amplified fragment, creating the G→A mutation at nucleotide +913 in the *rgs1* ORF. Nucleotide substitution was confirmed by sequencing.

Generation of the Rgs1$^{His171 \rightarrow Arg}$ site-directed mutation

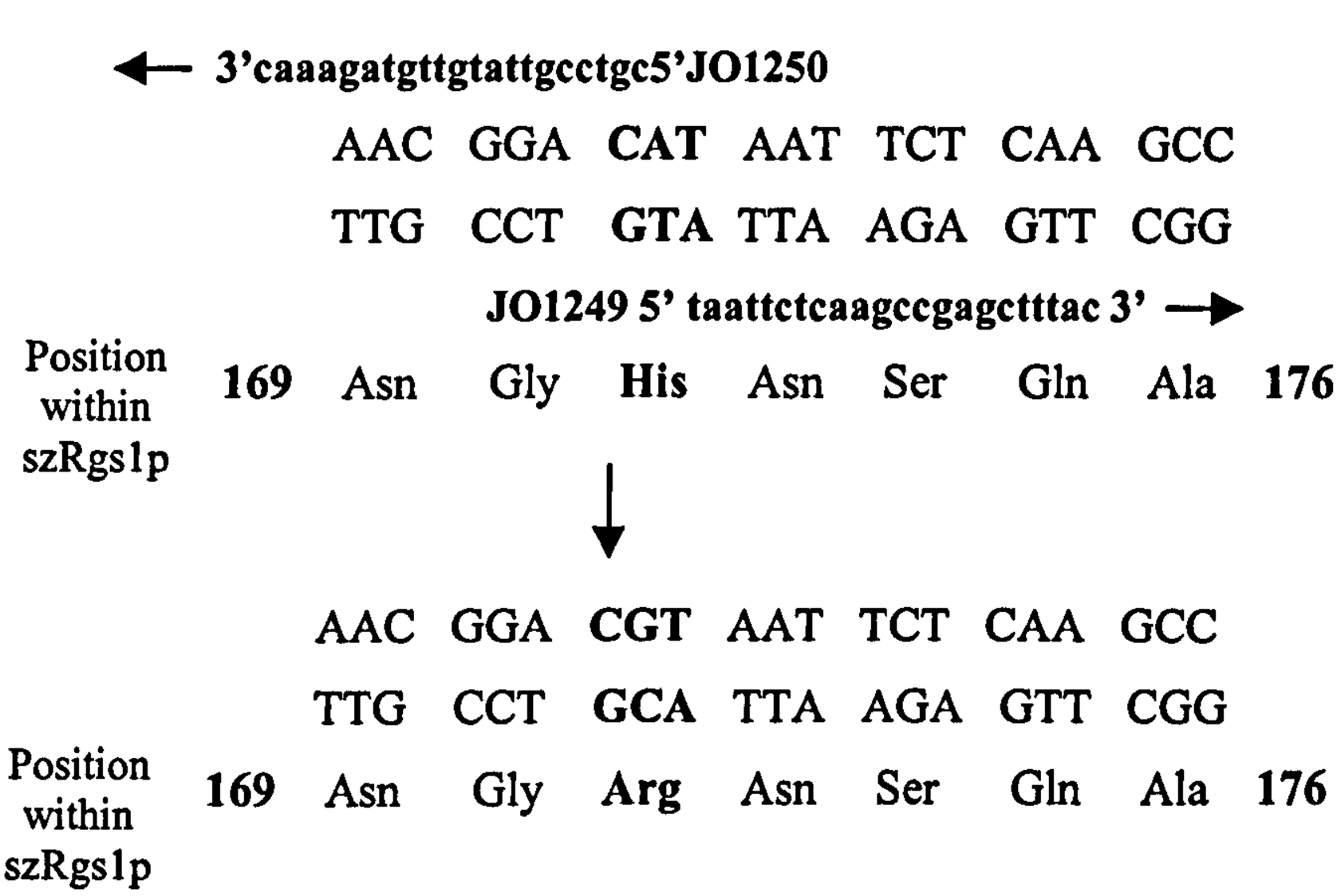


Figure 58. Recreation of the *Sz. pombe* Rgs1$^{His171 \rightarrow Arg}$ site-directed mutant

The oligonucleotide primers JO1249 and JO1250 were used in conjunction with a DNA template containing the *rgs1* ORF flanked by its 5' and 3' non-coding regions to generate the A→G mutation at nucleotide position +512 in the *Sz. pombe rgs1* ORF (relative to the initiator codon). JO1250 introduces a guanine nucleotide at position 512 in the resulting *rgs1* ORF (replacing adenine in the wild-type *rgs1* ORF).

Generation of the Rgs1$^{Val305\rightarrow Ile}$ site-directed mutation

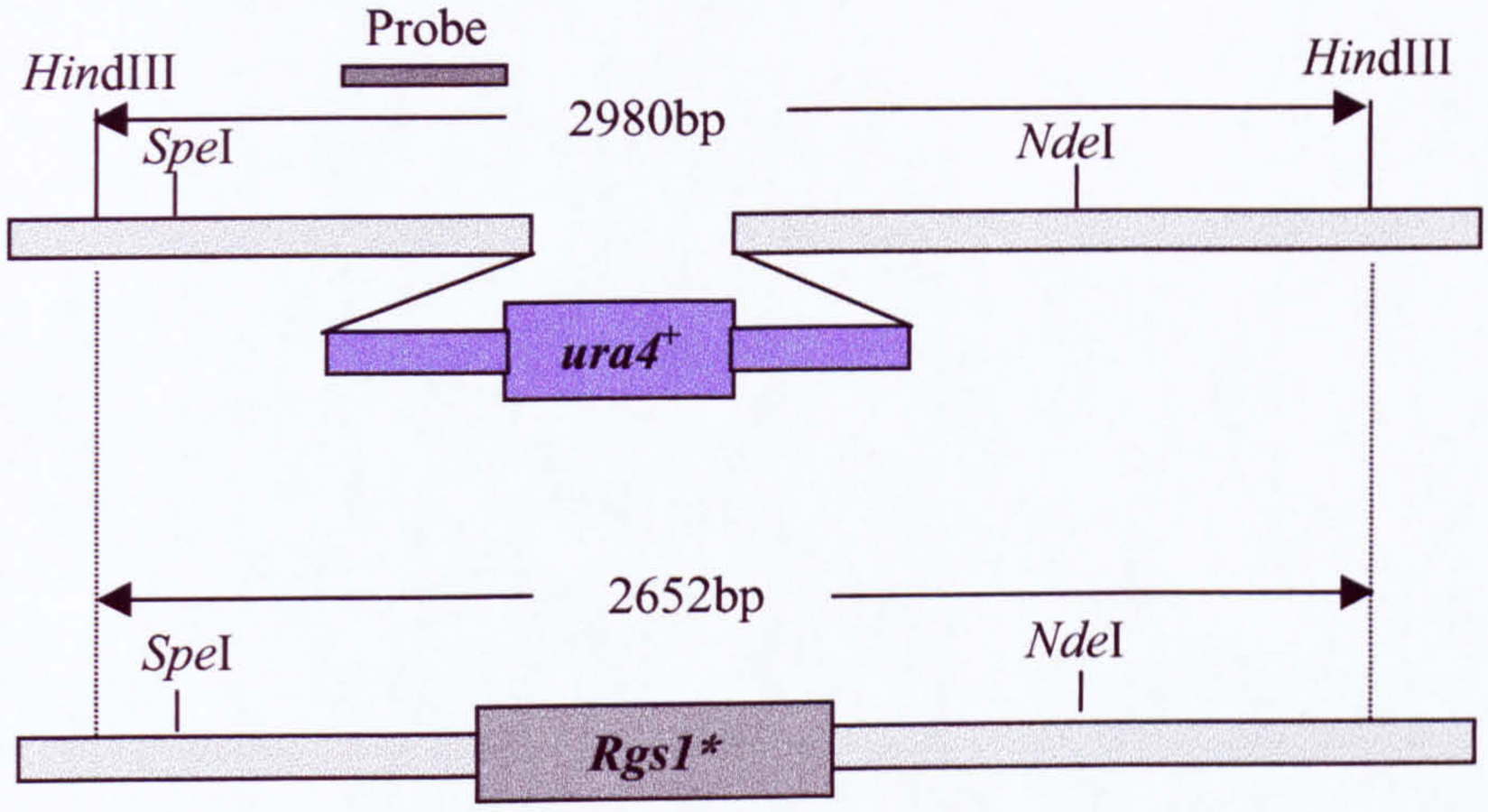
Figure 59. Recreation of the *Sz. pombe* Rgs1$^{Val305\rightarrow Ile}$ site-directed mutant

The oligonucleotide primers JO1247 and JO1248 were used in conjunction with a DNA template containing the *rgs1* ORF flanked by its 5' and 3' non-coding regions to generate the G→A mutation at nucleotide +913 in the *Sz. pombe rgs1* ORF (relative to the initiator codon). JO1248 introduces an adenine nucleotide at position 913 in the resulting *rgs1* ORF (replacing guanine in the wild-type *rgs1* ORF).

5.2.3. Integration of *rgs1*$^{His171\rightarrow Arg}$ and *rgs1*$^{Val305\rightarrow Ile}$ ORFs at the *rgs1* locus in the *rgs1*$^{-}$ *sxa2*::*lacZ* reporter strain

To determine whether the mutations described above conferred increased RGS activity upon szRgs1p, the mutant *rgs1* ORFs were integrated at the *rgs1* locus in the *rgs1*$^{-}$ LacZ reporter strain. The site-directed PCR amplification of the two mutant *rgs1* ORFs described in Section 5.2.2 created constructs in which the *rgs1*$^{His171\rightarrow Arg}$ and *rgs1*$^{Val305\rightarrow Ile}$ ORFs are flanked with the *rgs1* 5' and 3' non-coding regions. Integration fragments for the two mutant *rgs1* ORFs were liberated via *Spe*I/*Nde*I restriction, and the *rgs1*$^{-}$ LacZ reporter strain JY629 transformed with the integration fragments. Cells were grown to stationary phase in YEALU medium (approximately 48h), plated onto AA plates containing 5-FOA and uracil, and incubated for 5d at 29°C. Transformants were patched onto AA plates containing 5-FOA and uracil, and screened via PCR amplification using oligonucleotides specific for the *rgs1* 5' non-coding region and the *rgs1* ORF for the presence of the mutated *Sz. pombe rgs1* ORF at the *rgs1* locus.

Genomic DNA was prepared for strains positive by PCR, and the DNA digested with *Hin*dIII. Digested DNA was separated on a 1% agarose gel, blotted onto a nitrocellulose filter, and probed with a fragment amplified from the *rgs1* 5' non-coding region (Figure 60). Correct isolates were chosen for determination of β-galactosidase activity following P-factor stimulation (JY1190 [Rgs1$^{His171\rightarrow Arg}$] and JY1189 [Rgs1$^{Val305\rightarrow Ile}$]).



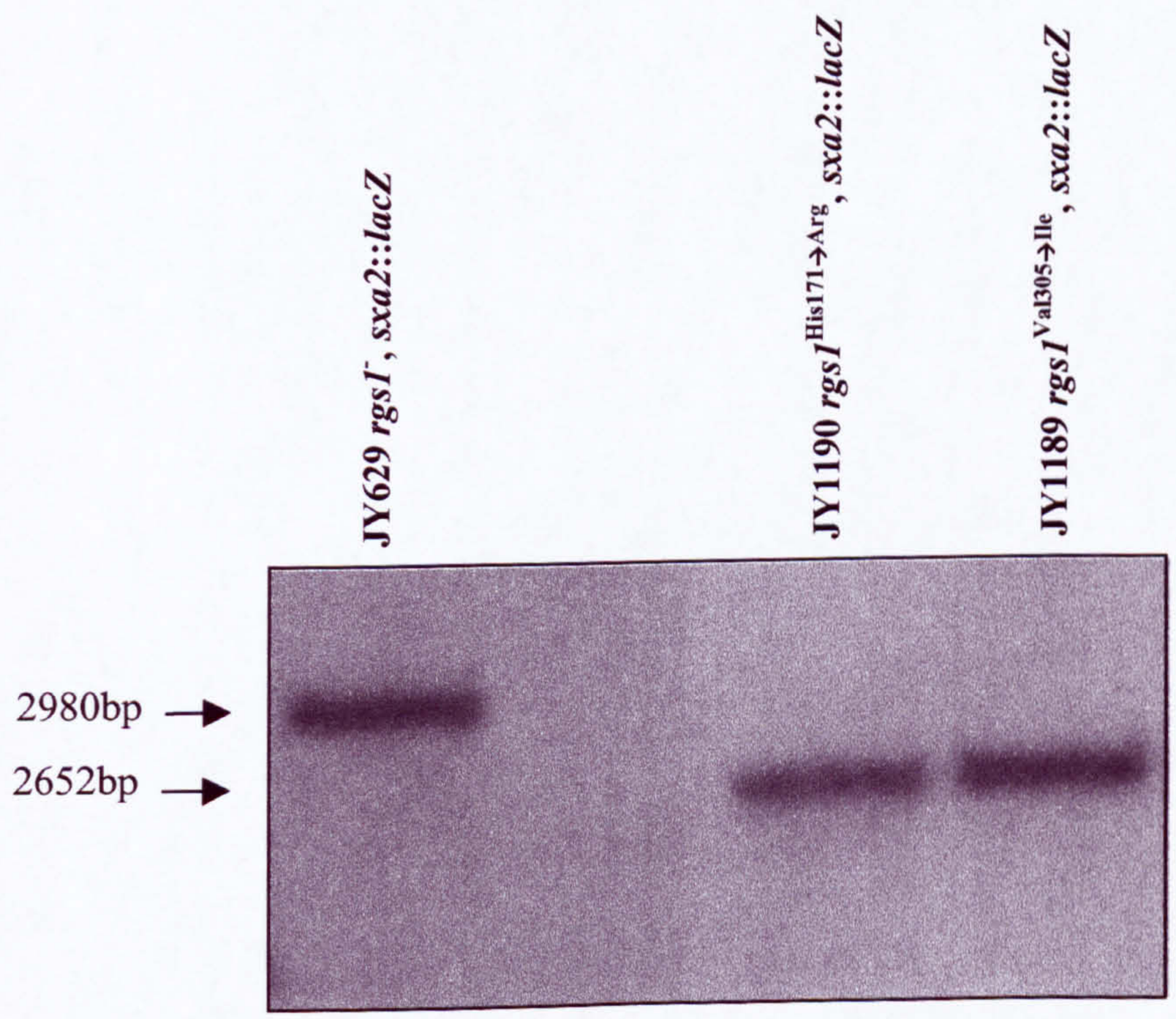


Figure 60. Southern blot analysis confirming integration of *rgs1*$^{His171\rightarrow Arg}$ and *rgs1*$^{Val305\rightarrow Ile}$ ORFs at the *rgs1* locus in the *rgs1*$^{-}$ *sxa2::lacZ* reporter strain

Genomic DNA was prepared for JY629, JY1190 and JY1189, and the DNA digested with *Hin*dIII. Digested DNA was subjected to Southern blot analysis. Correct integration of the *rgs1*$^{His171\rightarrow Arg}$ and *rgs1*$^{Val305\rightarrow Ile}$ ORFs is indicated by bands of 2652bp. Non-integration of the mutant *rgs1* ORFs is indicated a band of 2980bp (indicating the presence of the *ura4* cassette).

5.2.4. β-galactosidase activity of the Rgs1$^{His171\rightarrow Arg}$ and Rgs1$^{Val305\rightarrow Ile}$ LacZ reporter strains

Once confirmed by Southern blot analysis, the β-galactosidase activity of JY1190 (Rgs1$^{His171\rightarrow Arg}$) and JY1189 (Rgs1$^{Val305\rightarrow Ile}$) was determined following stimulation with a range of P-factor concentrations (Figures 61 and 62).

Integration of the *rgs1*$^{His171\rightarrow Arg}$ ORF at the *rgs1* locus in the *rgs1*$^{-}$ LacZ reporter strain reduced pheromone-independent LacZ activity down to the level seen for the *rgs1*$^{+}$ LacZ reporter strain JY544 (Figure 61). The Rgs1$^{His171\rightarrow Arg}$ protein also appeared capable of reducing pheromone-dependent LacZ activity down to those levels seen for the *rgs1*$^{+}$ LacZ reporter strain, up to 100U/ml P-factor. Thus, the Rgs1$^{His171\rightarrow Arg}$ LacZ reporter strain exhibited a similar response profile to the *rgs1*$^{+}$ LacZ reporter strain upon P-factor stimulation. These results indicate that in the *Sz. pombe* LacZ reporter strain the *Sz. pombe* Rgs1$^{His171\rightarrow Arg}$ mutation did not exhibit a phenotype consistent with the mutation conferring increased RGS activity compared to wild-type *Sz. pombe* Rgs1p. The His171→Arg mutation did not appear to adversely affect szRgs1p activity. The His171 residue lies outside the predicted RGS domain of szRgs1p.

Integration of the *rgs1*$^{Val305\rightarrow Ile}$ ORF at the *rgs1* locus in the *rgs1*$^{-}$ LacZ reporter strain (JY1189) reduced pheromone-independent LacZ activity down to the level seen for the *rgs1*$^{+}$ LacZ reporter strain JY544 (Figure 62), as seen for the Rgs1$^{His171\rightarrow Arg}$ mutation. The Rgs1$^{Val305\rightarrow Ile}$ mutant protein also appeared capable of reducing pheromone-dependent LacZ activity down to similar levels seen for the *rgs1*$^{+}$ LacZ reporter strain, up to 100U/ml P factor. As with the Rgs1$^{His171\rightarrow Arg}$ mutation, the Rgs1$^{Val305\rightarrow Ile}$ mutation did not appear to adversely affect szRgs1p activity. The Val305 residue lies outside the predicted RGS domain of szRgs1p.

These results indicate that in the *Sz. pombe* LacZ reporter strain the Rgs1$^{His171\rightarrow Arg}$ and Rgs1$^{Val305\rightarrow Ile}$ mutations did not exhibit a phenotype consistent with the mutations conferring increased RGS activity compared to the wild-type *Sz. pombe* Rgs1p.

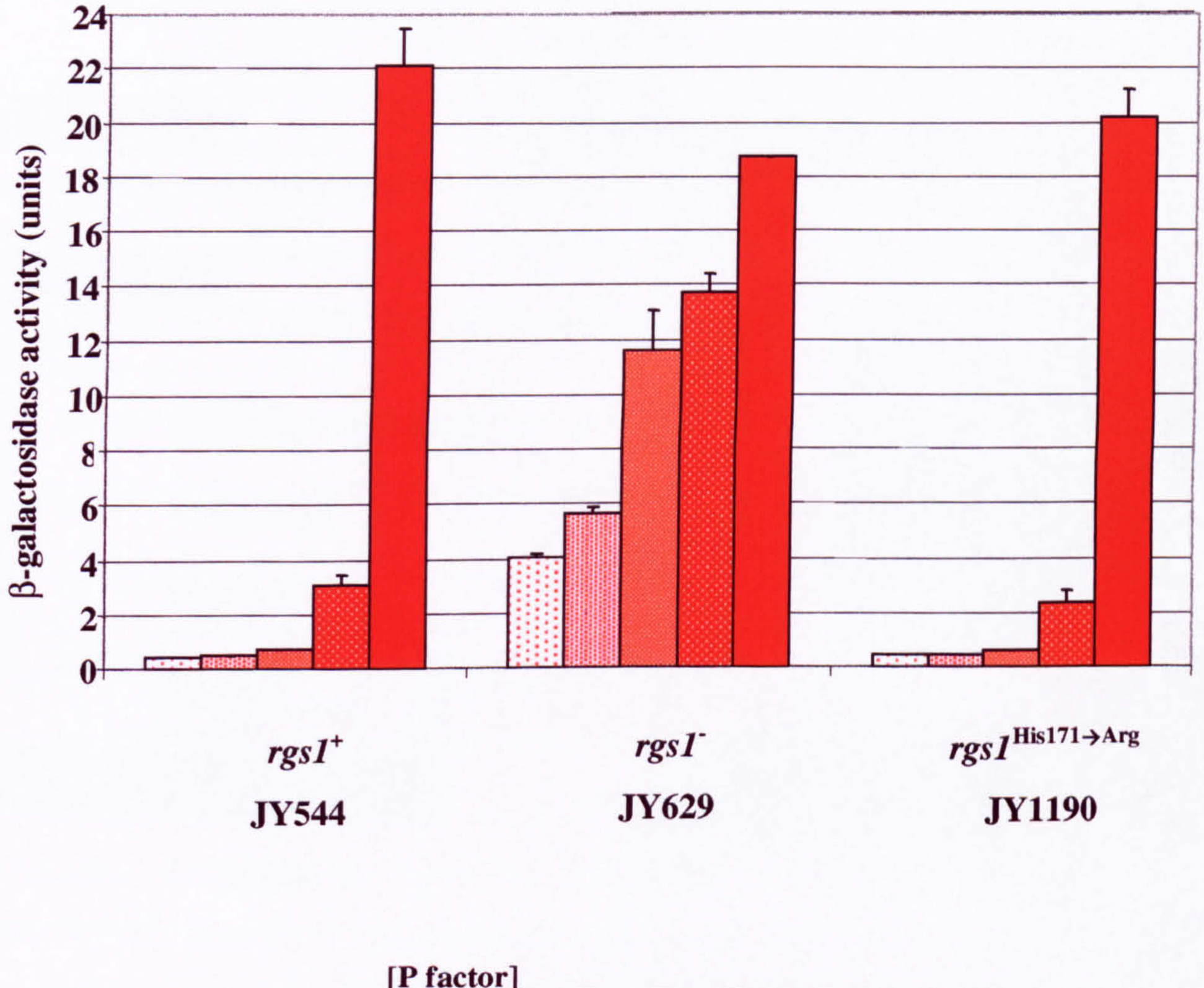


Figure 61. The *rgs1*$^{His171\rightarrow Arg}$ LacZ reporter strain exhibits similar pheromone independent and pheromone dependent β-galactosidase activity as the *rgs1*$^{+}$ LacZ reporter strain

The *rgs1*$^{+}$ (JY544), *rgs1*$^{-}$ (JY629) and *rgs1*$^{His171\rightarrow Arg}$ (JY1190) LacZ reporter strains were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Reporter strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

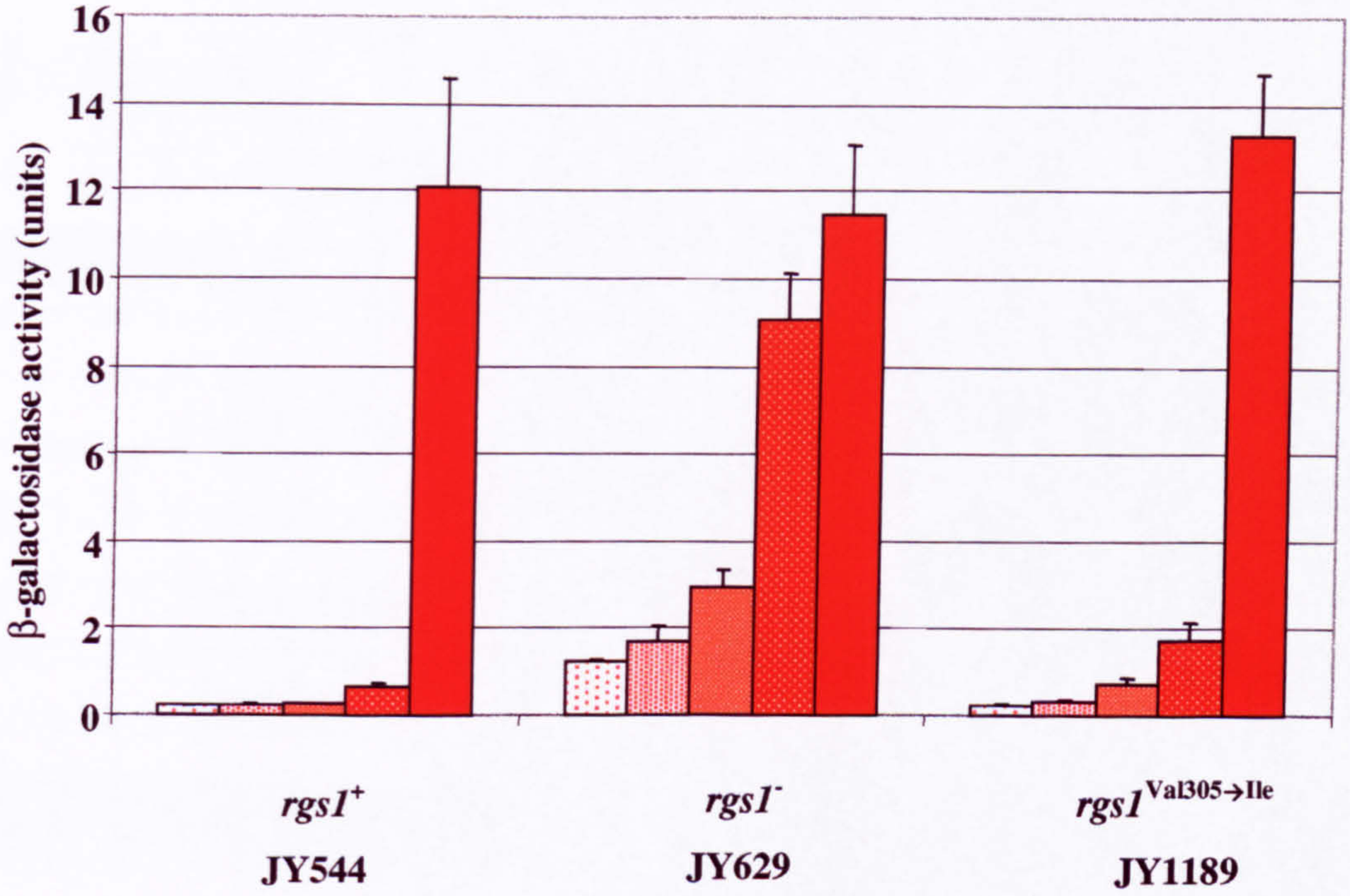


Figure 62. The *rgs1*$^{Val305\rightarrow Ile}$ LacZ reporter strain exhibits similar pheromone independent and pheromone dependent β-galactosidase activity as the *rgs1*$^{+}$ LacZ reporter strain

The *rgs1*$^{+}$ (JY544), *rgs1*$^{-}$ (JY629) and *rgs1*$^{Val305\rightarrow Ile}$ (JY1189) LacZ reporter strains were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Reporter strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

5.2.5. Integration of *rgs1*$^{His171\rightarrow Arg}$ and *rgs1*$^{Val305\rightarrow Ile}$ ORFs at the *rgs1* locus in the *sxa2*::*ura4* reporter strain

As the two *Sz. pombe* Rgs1p mutants were originally isolated from a screen using the *rgs1*$^{-}$ *sxa2*::*ura4* reporter strain, the re-created mutant *rgs1* ORFs were integrated at the *rgs1* locus in the *sxa2*::*ura4* reporter strain. In the *rgs1*$^{-}$ *sxa2*::*ura4* reporter strain the ORF for the reporter protein Ura4 is placed under the direction of the *sxa2* promoter (as is the LacZ ORF in the *sxa2*::*lacZ* reporter strains). As a consequence, Ura4 expression is only induced as a result of signalling through the pheromone communication pathway.

The *rgs1*::*rgs1*$^{His171\rightarrow Arg}$ and *rgs1*::*rgs1*$^{Val305\rightarrow Ile}$ integration fragments described in Section 6.2.3 were introduced into the *rgs1*::*ura4* *sxa2*::*ura4* reporter strain JY727 (in this *sxa2*::*ura4* reporter strain the *rgs1* ORF is disrupted with the *ura4* cassette at an internal *Bam*HI site). Cells were grown to stationary phase in YEALU medium (approximately 48h), plated onto AA plates containing uracil, 5-FOA and 100U/ml P-factor and incubated for 5d at 29°C. Colonies were patched onto AA plates containing uracil, 5-FOA and 100U/ml P-factor. 5-FOA selects for the absence of Ura4 activity. The absence of RGS activity in the *sxa2*::*ura4* reporter strain JY727 results in an increased level of pheromone-independent signal transduction through the pheromone signalling pathway, inducing Ura4 expression to such a level that cells are unable to grow on growth media containing 5-FOA. Any genetic change that results in reduced signalling though the pathway will enable cells to grow on media containing 5-FOA and P-factor pheromone (the greater the reduction in signalling through the pathway the greater the P-factor concentration on which cells can grow). The transformants were plated onto 100U/ml P-factor, as at this concentration the presence of the wild-type szRgs1p is incapable of inhibiting signalling to a sufficient degree to suppress Ura4 expression from the *sxa2* locus. Thus at 100U/ml P-factor, presumably only those strains in which there is a much reduced level of signalling will be viable. This could theoretically be achieved by the presence of a gain-of-function RGS protein.

Transformants were screened via PCR amplification using oligonucleotides complementary to the *rgs1* 5' non-coding region and the *rgs1* ORF downstream of the *ura4* cassette for the presence of the *Sz. pombe rgs1* ORF at the *rgs1* locus.

Genomic DNA was prepared for strains positive by PCR, and the DNA digested with *Hin*dIII. Digested DNA was separated on a 1% agarose gel, blotted onto a nitrocellulose filter, and probed with a fragment amplified from the *rgs1* 5' non-coding region (Figure 63). Correct isolates were chosen for characterisation of Ura4 expression on AA plates containing 5-FOA, uracil and P-factor pheromone (JY1192 [Rgs1$^{His171\rightarrow Arg}$] and JY1191 [Rgs1$^{Val305\rightarrow Ile}$]).

5.2.6. Rgs1$^{His171\rightarrow Arg}$ and Rgs1$^{Val305\rightarrow Ile}$ *sxa2::ura4* reporter strains are viable on AA plates containing 5-FOA up to 1000U/ml P-factor

JY1192 (Rgs1$^{His171\rightarrow Arg}$) and JY1191 (Rgs1$^{Val305\rightarrow Ile}$) were cultured overnight in AA media lacking uracil and $5x10^4$ cells plated onto AA plates containing uracil and 5-FOA and a range of P-factor concentrations. Plates were incubated for 3d at 29°C.

From Figure 64 it can be seen that both the presence of the His171→Arg (JY1192) and the Val305→Ile (JY1191) szRgs1p mutations in the *Sz. pombe sxa2::ura4* reporter strain enabled the strains to grow on 5-FOA plates containing uracil in the presence of up to 1000U/ml P-factor. The *rgs1*$^-$ *sxa2::ura4* reporter strain was incapable of growth on AA plates containing 5-FOA and uracil regardless of the presence or absence of P-factor pheromone. This was due to increased pheromone-independent and pheromone-dependent signalling through the pheromone communication pathway compared to the *rgs1*$^+$ *sxa2::ura4* reporter strain, which was only capable of growth on AA plates containing 5-FOA and uracil up to 1U/ml P-factor.

Despite the fact that in the *sxa2::lacZ* reporter strain the two *Sz. pombe* Rgs1p mutants Rgs1$^{His171\rightarrow Arg}$ and Rgs1$^{Val305\rightarrow Ile}$ did not display phenotypes consistent with the mutations conferring increased RGS activity compared to the wild-type Rgs1 protein, these mutations did appear to enable the *sxa2::ura4* reporter strain to grow in the presence of 5-FOA at P-factor concentrations greater than that enabled by the wild-type szRgs1p (1000U/ml P-factor compared to 1U/ml P-factor). One reason for

this anomaly could be the timescales involved in the two different assays. In the β-galactosidase assay, LacZ activity was determined after 16h of pheromone stimulation. In the 5-FOA viability assay, strain viability was assessed after 3d of pheromone stimulation. The longer timescale of the viability assay potentially introduces greater physiological complexity, as a result of an increased number of cell doublings.

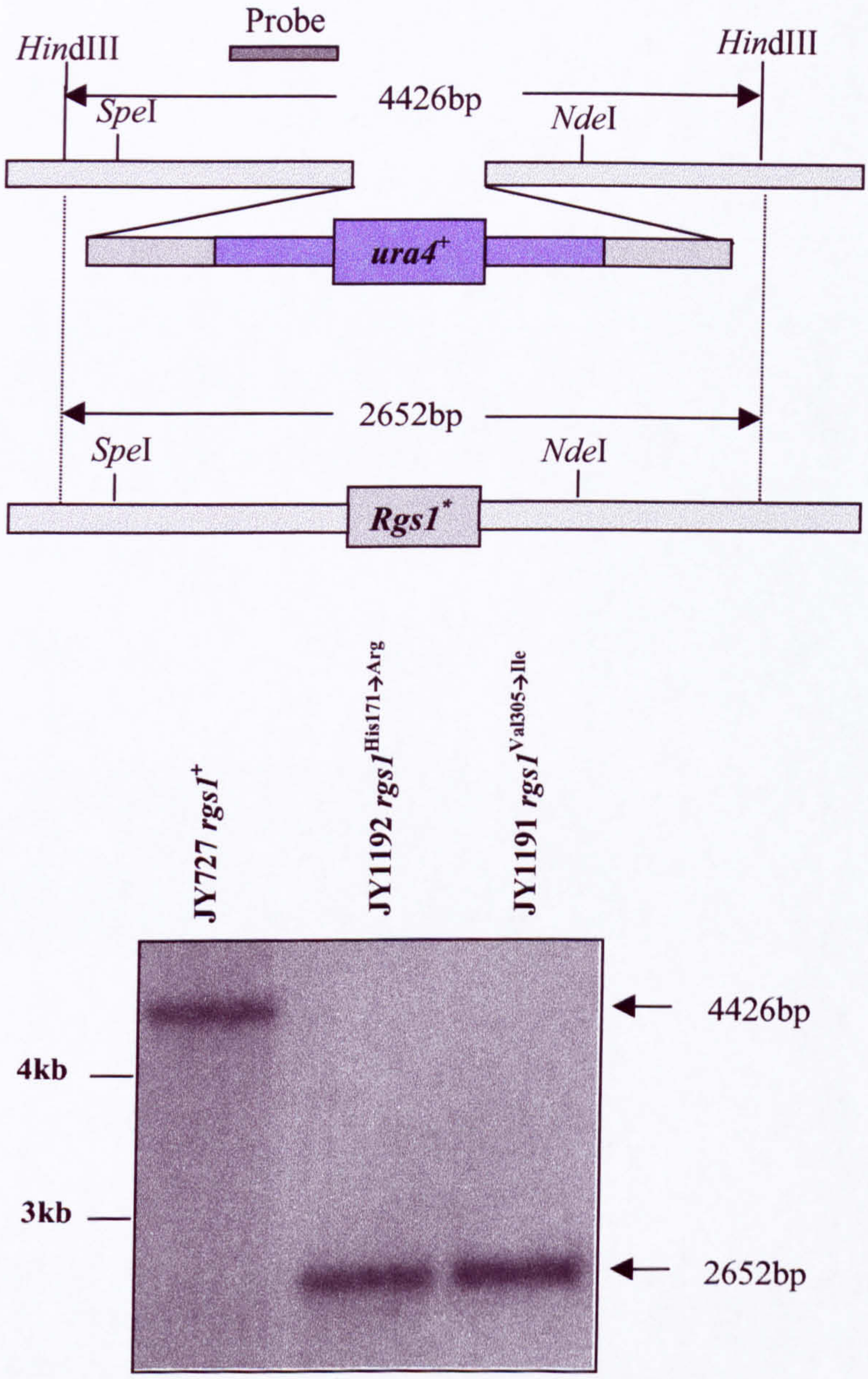


Figure 63. Southern blot analysis confirming integration of *rgs1*$^{His171\rightarrow Arg}$ and *rgs1*$^{Val305\rightarrow Ile}$ ORFs at the *rgs1* locus in the *rgs1*$^{-}$ *sxa2*::*ura4* reporter strain

Genomic DNA was prepared for JY727, JY1192 and JY1191, and the DNA *Hin*dIII-restricted and subjected to Southern blot analysis. A band of 2652bp indicated the correct integration of the mutant *rgs1* ORFs at the *rgs1* locus. Non-integration of the mutant *rgs1* ORF was indicated by a band of 4462bp.

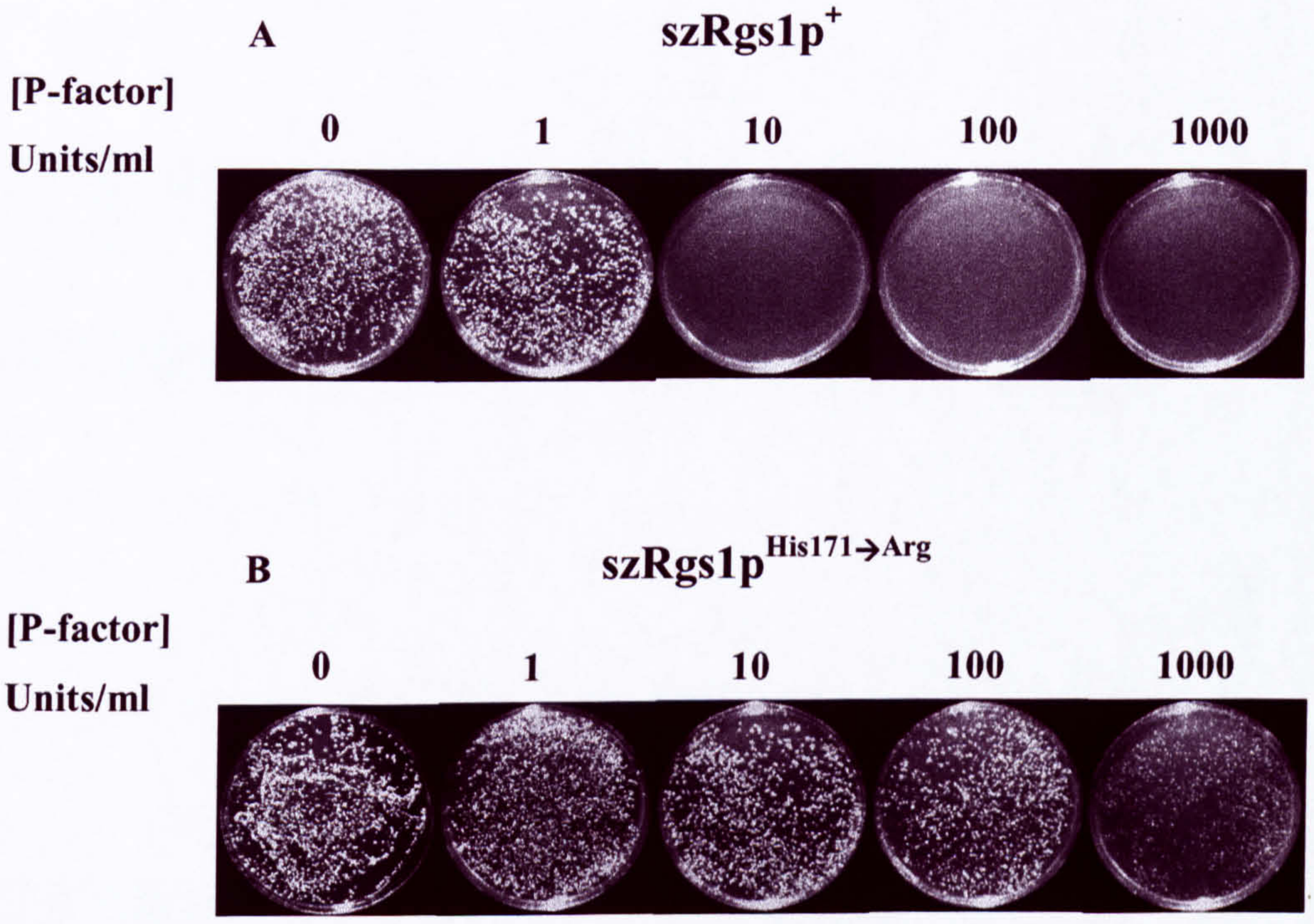


Figure 64. Rgs1$^{His171\rightarrow Arg}$ and Rgs1$^{Val305\rightarrow Ile}$ *sxa2*::*ura4* reporter strains are capable of growth on AA plates containing 5-FOA, uracil and 1000U/ml P-factor

JY603 (*rgs1*$^{+}$ [A]), JY1192 (Rgs1$^{His171\rightarrow Arg}$ [B]) and JY1191 (Rgs1$^{Val305\rightarrow Ile}$ [C]) were cultured overnight in AA media lacking uracil and $5x10^{4}$ cells plated onto AA plates containing uracil and 5-FOA and a range of P-factor concentrations. Plates were incubated for 3d at 29°C. JY603 formed colonies up to 1U P-factor/ml. JY1192 and JY1191 were capable of forming colonies in the presence of up to 1000U/ml P-factor.

5.3. Mutagenesis of human RGS4

Both huRGS1p and huRGS4p have been shown to be capable of negatively regulating the pheromone communication pathway in *Sz. pombe* when expressed from multicopy expression vectors (Chapter 4). The extent of huRGS1p and huRGS4p RGS activity upon this G protein signalling pathway depends upon the level of expression (and thus RGS activity) within the cell. When expressed from an expression vector, huRGS1p and huRGS4p appeared to modulate the pheromone signalling pathway to a comparable degree when compared to expression of *Sz. pombe* Rgs1p from the same vector. However, when expressed from single copy in a *rgs1*⁻ LacZ reporter strain, huRGS1p appeared capable of reducing signalling only at low P-factor concentrations (up to 1U/ml P-factor), and huRGS4p only appeared to lower the level of signalling through the pheromone communication pathway to a small degree compared to the *rgs1*⁻ LacZ reporter strain. The decreased ability of huRGS1p and huRGS4p to inhibit signalling through the pheromone communication pathway compared to that of szRgs1p was likely due to their lower affinities for the *Sz. pombe* G_α subunit, Gpa1.

At present no gain-of-function mutations have been identified for human RGS proteins. It was decided to initially concentrate on human RGS4 for mutagenesis, as structural analyses of RGS proteins have centred on this RGS protein. The *rgs1*⁻ *sxa2*::*ura4* reporter strain was chosen as a host strain to screen for RGS4 gain-of-function mutants, as in conjunction with the viability-based assay using 5-FOA, thousands of isolates could be screened.

5.3.1. Mutagenesis of the *rgs1*::*Rgs4* integration construct

The *rgs1*::*Rgs4*⁺ integration construct, comprised of the human *Rgs4* ORF flanked with *Sz. pombe rgs1* 5' and 3' non-coding regions (Section 4.4.4), was treated with hydroxylamine (Section 2.2.11) and the DNA purified. DH5 *E. coli* were then transformed with the purified hydroxylamine-treated *rgs1*::*Rgs4** construct. Approximately 45,000 bacterial colonies were combined to create a library of hydroxylamine-treated *rgs1*::*Rgs4** constructs. The *rgs1*::*Rgs4** integration fragment was liberated from the hydroxylamine-treated integration construct via *Nde*I/*Spe*I restriction, and the *rgs1*⁻ *sxa2*::*ura4* reporter strain (JY727) transformed

with the *rgs1*::*Rgs4** integration construct (Figure 65). Cells were grown to stationary phase in YEALU medium (approximately 48h), plated onto AA plates containing uracil, 5-FOA and 100U/ml P-factor, and grown for 5d at 29°C. The reasoning behind the use of 100U/ml P-factor in selective plates was to identify transformants in which there was a significant inhibition of the pheromone signal transduction pathway compared to the *rgs1*$^{+}$ and *rgs1*$^{-}$ reporter strains. One way this could be achieved is though increased RGS activity, as a result of gain-of-function mutations in the human *Rgs4* ORF. Transformants were patched onto AA plates containing uracil, 5-FOA and 100U/ml P-factor and the morphology of the cells observed microscopically.

The *rgs1*$^{-}$ *sxa2*::*ura4* reporter strain was incapable of growth on AA media containing 5-FOA due to its increased level of pheromone-independent signalling through the pheromone signal transduction pathway relative to the *rgs1*$^{+}$ *sxa2*::*ura4* reporter strain. As it had been shown that integration of the human *Rgs4* ORF at the *rgs1* locus in the *rgs1*$^{-}$ LacZ reporter strain did not appear to reduce the high level of pheromone-independent *sxa2* promoter activity seen for the *rgs1*$^{-}$ LacZ reporter strain, it was envisaged that strains capable of growth on 5-FOA plates containing P-factor may have the potential for expressing a mutated, hyper-activated form of huRGS4p.

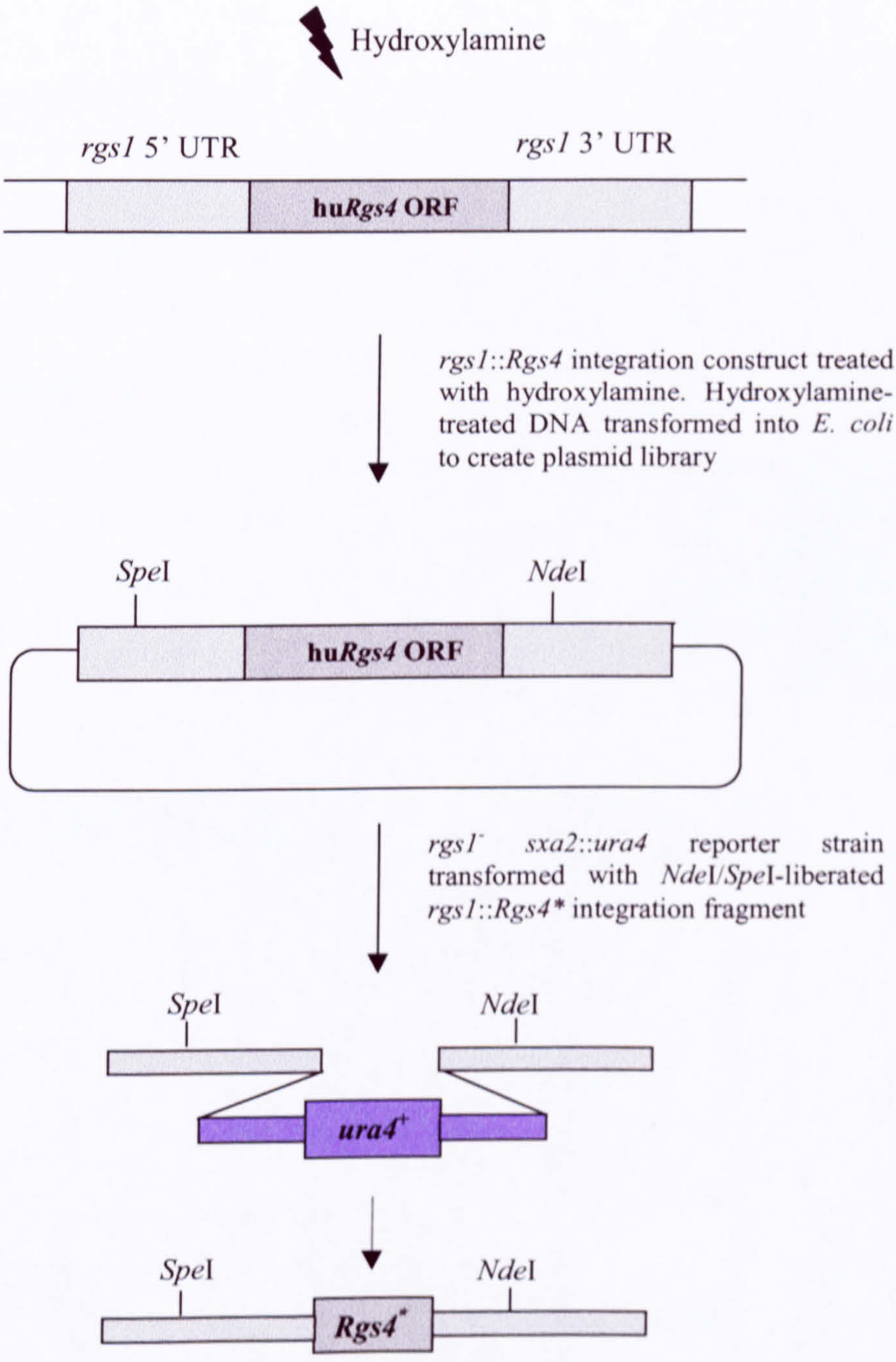


Figure 65. Hydroxylamine treatment of human *Rgs4* ORF and its subsequent integration at the *Sz. pombe rgs1* locus in the *rgs1⁻ sxa2::ura4* reporter strain

The *rgs1::Rgs4* integration construct was treated with hydroxylamine, and a bacterial library of the hydroxylamine-treated *rgs1::Rgs4* integration construct created. The *rgs1::Rgs4* integration construct was liberated from the hydroxylamine-treated integration construct via *Nde*I/*Spe*I restriction, and the *rgs1⁻ sxa2::ura4* reporter strain transformed with the *rgs1::Rgs4* integration fragment. Cells were grown to log phase in YEALU medium, plated onto AA plates containing uracil, 5-FOA and 100U/ml P factor.

5.3.2. Screening for *rgs1*::*Rgs4* sxa2*::*ura4*$^+$ LacZ reporter strains

As I was hoping to isolate huRGS4p gain-of-function mutants, it was anticipated that for those transformants in which the pheromone signalling pathway had been inhibited (as a result of increased RGS activity) there would be reduced sensitivity to pheromone stimulation. One morphological response to pheromone stimulation is the formation of shmoos (cells appear elongated as a result of unidirectional growth toward extracellular pheromone). Initially I microscopically studied transformants to determine which did not appear to shmoo when grown on 5-FOA plates in the presence of P-factor. 42 out of the 85 transformants (~50%) that were able to grow on 5-FOA plates containing 100U/ml P-factor did not appear to shmoo in response to P-factor pheromone stimulation. Those isolates that did not shmoo often had a more 'bulbous' morphology compared to the *Sz. pombe* wild-type morphology in the absence of pheromone.

Isolates that did not exhibit a morphological response to P-factor were then screened for the presence of the human *Rgs4* ORF at the *rgs1* locus using the oligonucleotides JO930 and JO931. The sense primer JO930 contains 18 bases complementary to the *rgs1* 5' non-coding region (initiating at nucleotide position –811 relative to the *rgs1* initiator codon). The antisense primer JO931 contains 18 bases complementary to the *rgs1* 3' non-coding region (initiating at nucleotide position +1888 relative to the *rgs1* initiator codon). JO930 and JO931 amplify the *rgs1* 5' and 3' non-coding regions, and any intervening sequence. Isolates in which the human *Rgs4* ORF had integrated at the *rgs1* locus gave a band of approximately 1.8kb, whereas isolates in which the human *Rgs4* ORF had failed to integrate at the *rgs1* locus gave a band of approximately 3kb, indicating the presence of the *ura4* cassette at the *rgs1* locus (Figure 66). 32 of the 42 isolates (~75%) that did not shmoo in response to P-factor stimulation appeared to contain the human *Rgs4* ORF at the *rgs1* locus, as determined by PCR amplification.

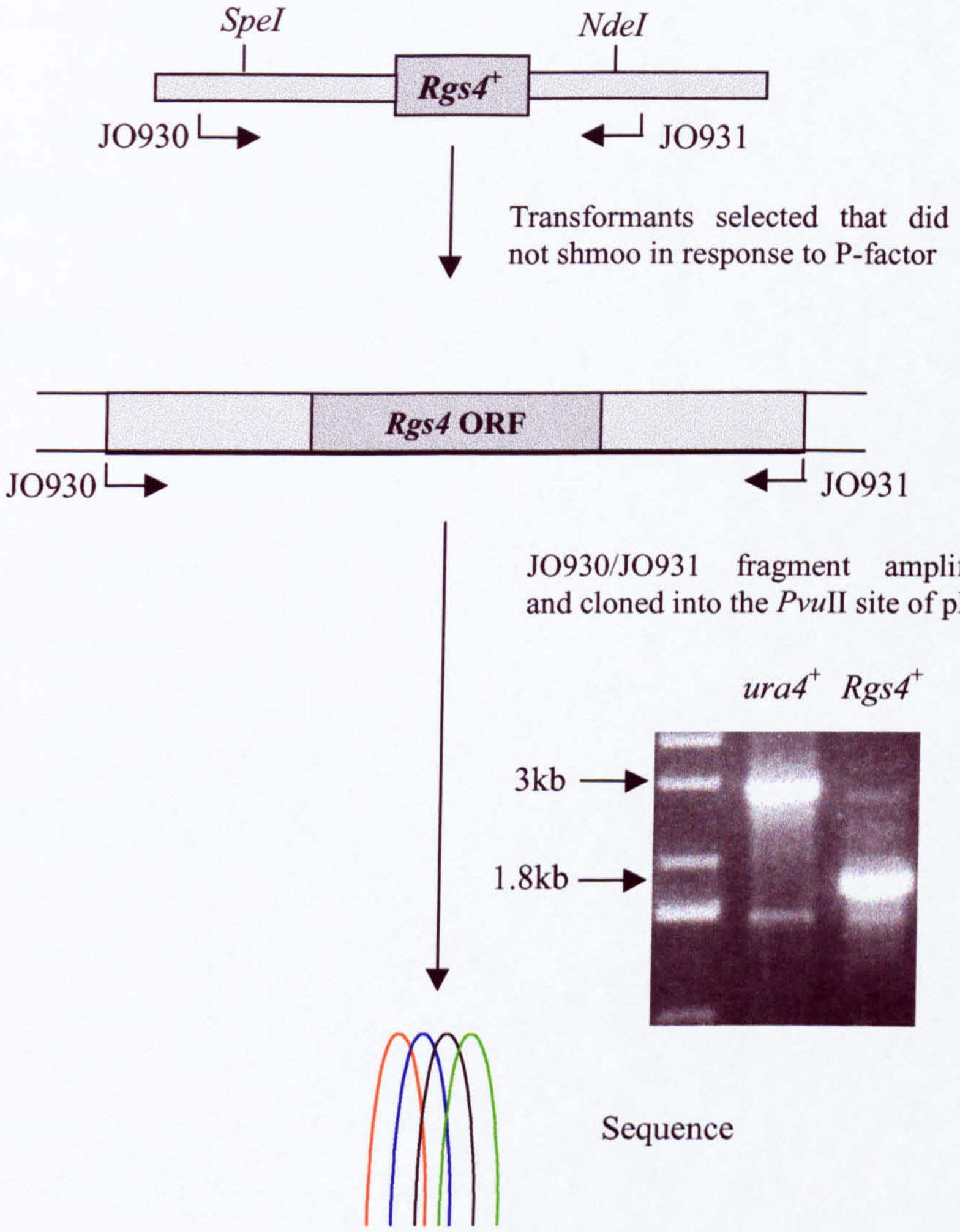


Figure 66. Screening for *rgs1*::*Rgs4*, *sxa2*::*ura4* transformants

Transformants were microscopically observed to determine which did not exhibit the typical morphological response to P-factor. These isolates were then screened for the presence of the human *Rgs4* ORF at the *rgs1* locus using the oligonucleotides JO930 and JO931. A band of 1.8kb indicated successful integration of the *rgs1*::*Rgs4* construct at the *rgs1* locus. Non-integration of the *rgs1*::*Rgs4* construct was indicated by a band of approximately 3kb. The JO930/JO931 fragments amplified from the *rgs1*::*Rgs4*$^{+}$ strains were cloned into the *Pvu*II site of pKS and sequenced.

5.3.3. Recovery of human *Rgs4* ORFs from *rgs1*::*Rgs4** LacZ reporter strains

Genomic DNA was prepared from strains containing the human *Rgs4* ORF at the *rgs1* locus identified from the screening procedure described in Section 5.3.2. The *Rgs4* ORF and flanking *rgs1* 5' and 3' non-coding regions were amplified using the oligonucleotides JO930 and JO931. The amplified *rgs1*::*Rgs4* fragments were cloned into the *Pvu*II site of pKS and sequenced either manually (via the dideoxynucleotide method) or via the departmental automated sequencing system.

5.3.4. Sequencing of human *Rgs4** ORFs

25 out of 32 (78%) separate human *Rgs4* ORFs were sequenced (time restraints prevented the sequencing of all isolates), and three separate mutations were identified.

Two silent mutations were identified in the human *Rgs4* ORF (C333→T and C504→T), with no resulting change in the huRGS4p primary sequence (Figure 67). The third mutation occurred at the nucleotide position 58 (A→G) in the human *Rgs4* ORF and results in a Lysine to Glutamate substitution at amino acid residue 20 in the primary sequence of huRGS4p (Figure 68).

The mutation identified at residue 20 in the RGS4 primary amino acid sequence is not in the conserved RGS domain of the protein, and no mutational analysis has as yet been carried out for this lysine residue. However, the N-terminal domain of RGS4 has been reported to confer receptor-selective inhibition of G protein signalling (Zeng *et al.*, 1998), and it is possible that the Lysine to Glutamate substitution at residue 20 could effect receptor selectivity. The N-terminus of RGS4 is essential for high potency inhibition of G protein-coupled signalling, and contributes significantly to RGS4 interaction with the receptor-G_{α} protein complex (Zeng *et al.*, 1998).

Silent mutations generated in the *Rgs4* ORF

		AGT	CCC	AAG	**GCC**	AAA	AAG	ATC
		TCA	GGG	TTC	**CGG**	TTT	TTC	TAG
Position within huRGS4p	**108**	Ser	Pro	Lys	Ala	Lys	Lys	Ile **114**
		AGT	CCC	AAG	**GCT**	AAA	AAG	ATC
		TCA	GGG	TTC	**CGA**	TTT	TTC	TAG
	108	Ser	Pro	Lys	Ala	Lys	Lys	Ile **114**

		TAC	CGC	CGC	**TTC**	CTC	AAG	TCT
		ATG	GCG	GCG	**AAG**	GAG	TTC	AGA
Position within huRGS4p	**165**	Tyr	Arg	Arg	Phe	Leu	Lys	Ser **171**
		TAC	CGC	CGC	**TTT**	CTC	AAG	TCT
		ATG	GCG	GCG	**AAA**	GAG	TTC	AGA
	165	Tyr	Arg	Arg	Phe	Leu	Lys	Ser **171**

Figure 67. Silent mutations identified in the *Rgs4* ORF resulting from hydroxylamine treatment of the *rgs1*::*Rgs4* integration construct.

The *rgs1*::*Rgs4** fragments amplified from the *rgs1*::*Rgs4** *sxa2*::*ura4* strains were cloned into the *Pvu*II site of pKS and sequenced. Two mutations identified in the human *Rgs4* ORF were silent (C333→T and C504→T), with no resulting change in protein primary sequence.

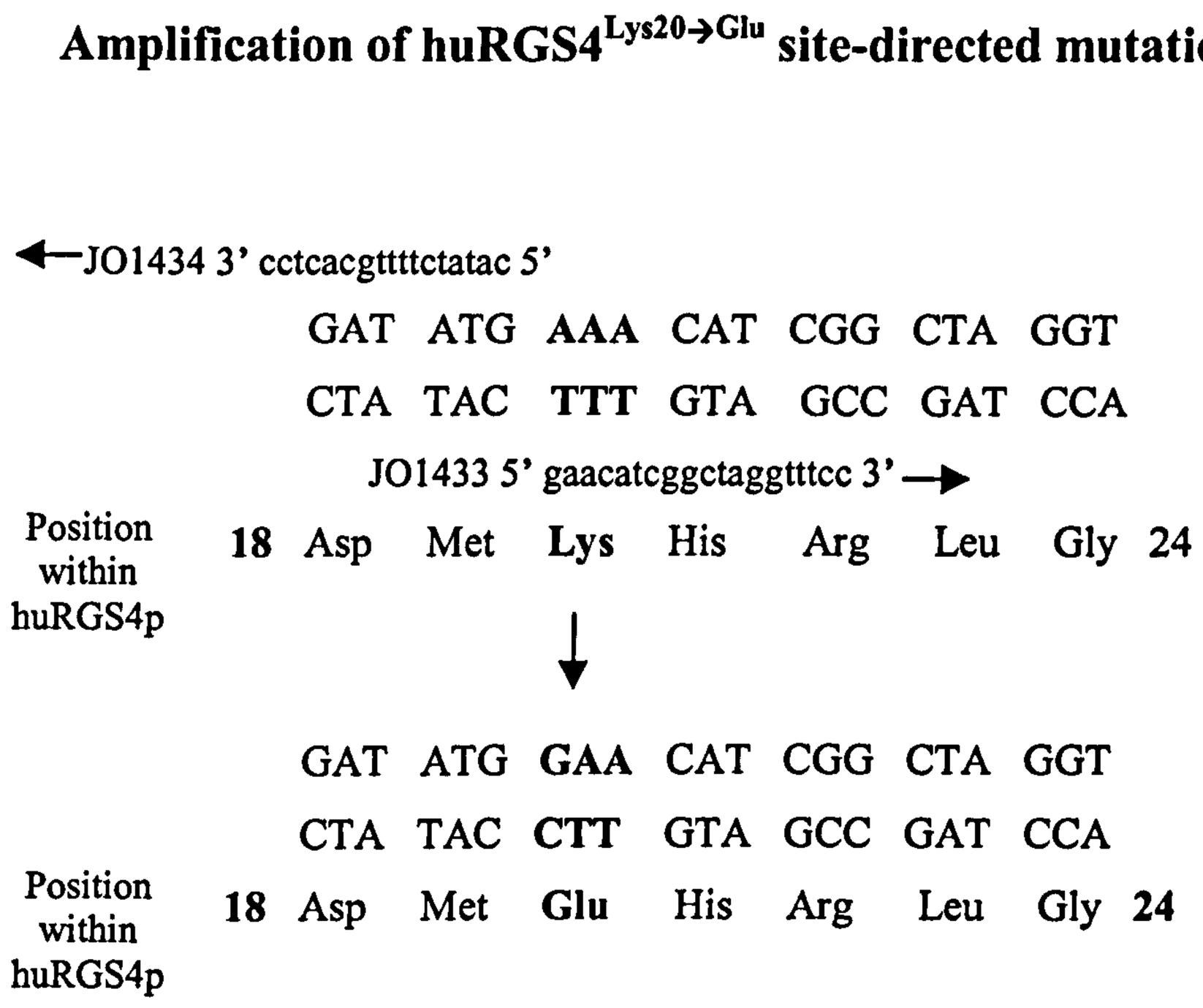


Figure 68. A58→G mutation identified in the *Rgs4* ORF resulting from hydroxylamine treatment of the *rgs1*::*Rgs4* integration construct.

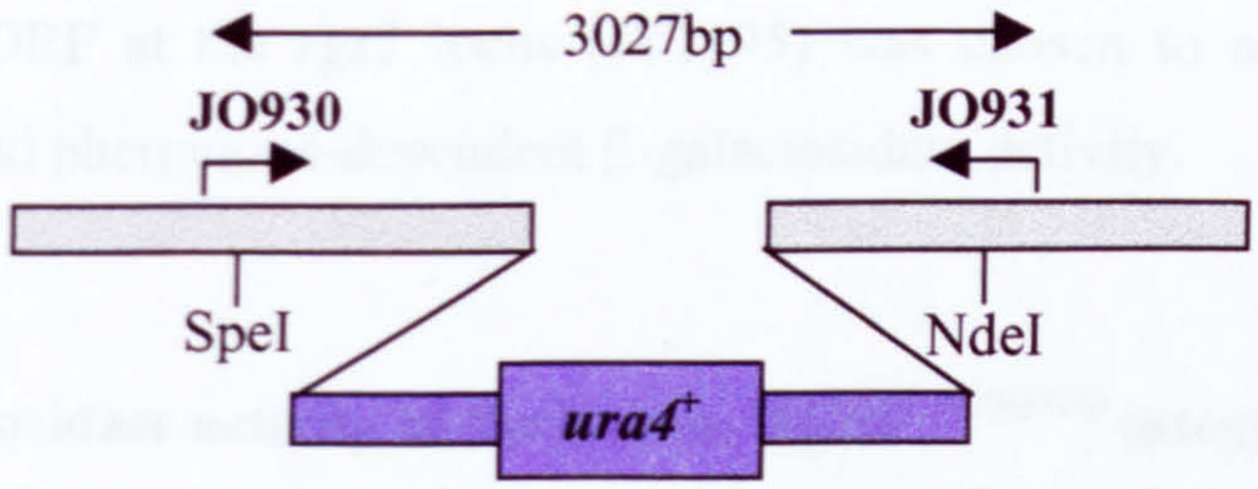
The *rgs1*::*Rgs4** fragments amplified from the *rgs1*::*Rgs4** *sxa2*::*ura4* strains were cloned into the *Pvu*II site of pKS and sequenced. A mutation at nucleotide position 58 in the *Rgs4* ORF replaces an adenine nucleotide with guanine. This results in a Lysine to Glutamate substitution at amino acid residue 20 in the human RGS4 primary sequence. Site-directed amplification using the primers JO1433 and JO1434 recreates the A58→G mutation in the *Rgs4* ORF.

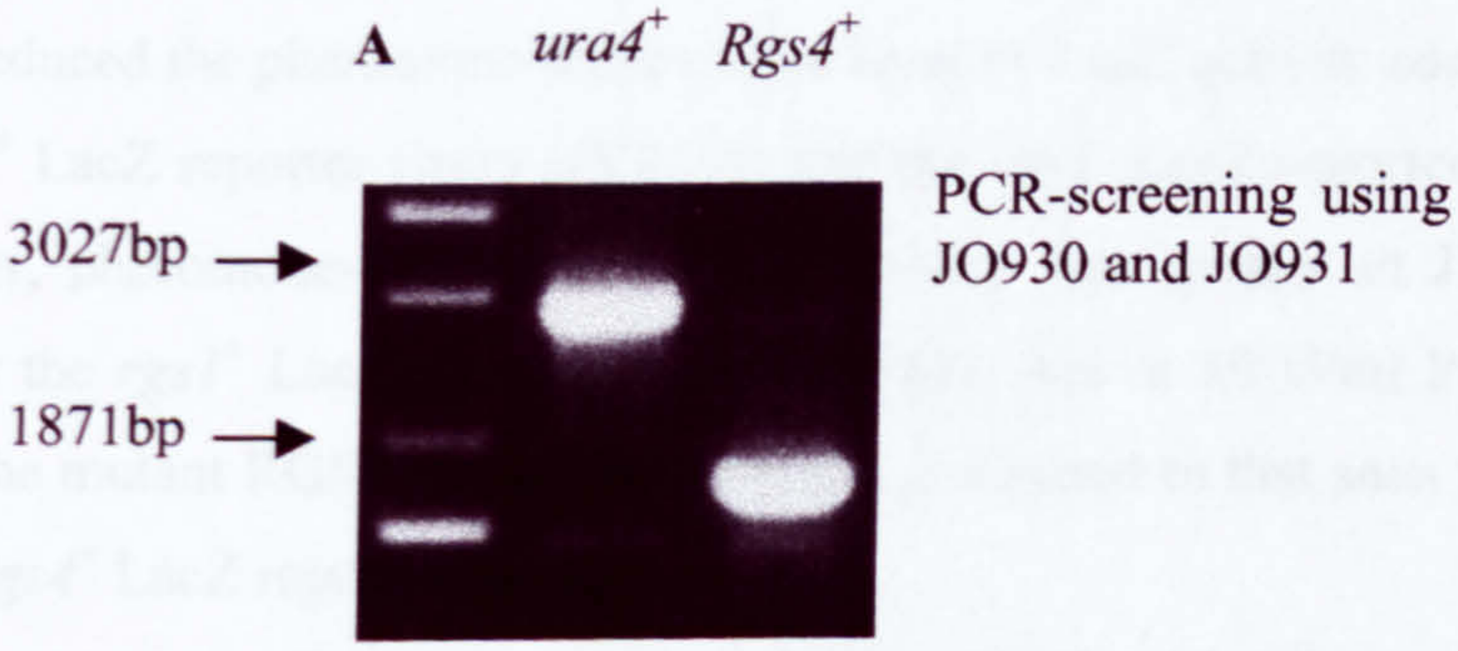
5.3.5. Recreation of human *Rgs4*$^{Lys20\rightarrow Glu}$ mutation

The Lys20→Glu mutation identified in Section 5.3.4 was re-created using the oligonucleotides JO1433 and JO1434 (Figure 68) in conjunction with the *rgs1*::*Rgs4* integration construct described in Chapter 4. The sense primer JO1433 contains 19 bases complementary to the human *Rgs4* ORF initiating at nucleotide position +58 relative to the *Rgs4* initiator codon, and introduces a guanine nucleotide at nucleotide position 58 in the resulting *Rgs4* ORF (replacing adenine in the wild-type *Rgs4* ORF). The antisense primer JO1434 contains 18 bases complementary to the human *Rgs4* ORF initiating at nucleotide position +57 relative to the *Rgs4* initiator codon. These primers re-create the A→G mutation at nucleotide position 58 in the *Rgs4* ORF that results in the Lysine to Glutamate substitution at amino acid residue 20 in the huRGS4p primary sequence. Subsequent ligation circularised the amplified fragment, creating the G→A mutation at nucleotide +58 in the *Rgs4* ORF. The substitution was confirmed by sequencing.

5.3.6. Integration of *Rgs4*$^{Lys20\rightarrow Glu}$ at the *rgs1* locus in the *rgs1*$^{-}$ LacZ reporter strain

Site-directed PCR amplification of the mutant *Rgs4* ORF created a construct in which the *Rgs4*$^{Lys20\rightarrow Glu}$ ORF is flanked with *Sz. pombe rgs1* 5' and 3' non-coding regions. The *rgs1*$^{-}$ *sxa2*::*lacZ* reporter strain JY629 was transformed with the *rgs1*::*Rgs4*$^{Lys20\rightarrow Glu}$ integration fragment liberated via *Spe*I/*Nde*I restriction of the *rgs1*::*Rgs4*$^{Lys20\rightarrow Glu}$ integration construct. Cells were cultured to stationary phase in YEALU medium (approximately 48h), plated onto AA plates containing 5-FOA and uracil and incubated for 5d at 29°C. Colonies were patched onto AA plates containing 5-FOA and uracil, and screened via PCR amplification using oligonucleotides complementary to the *rgs1* 5' non-coding region, the *Rgs4* ORF and the *rgs1* 3' non-coding region for the presence of the *Rgs4*$^{Lys20\rightarrow Glu}$ ORF at the *rgs1* locus (Figure 69). One *sxa2*::*lacZ* strain identified as possessing an integrated





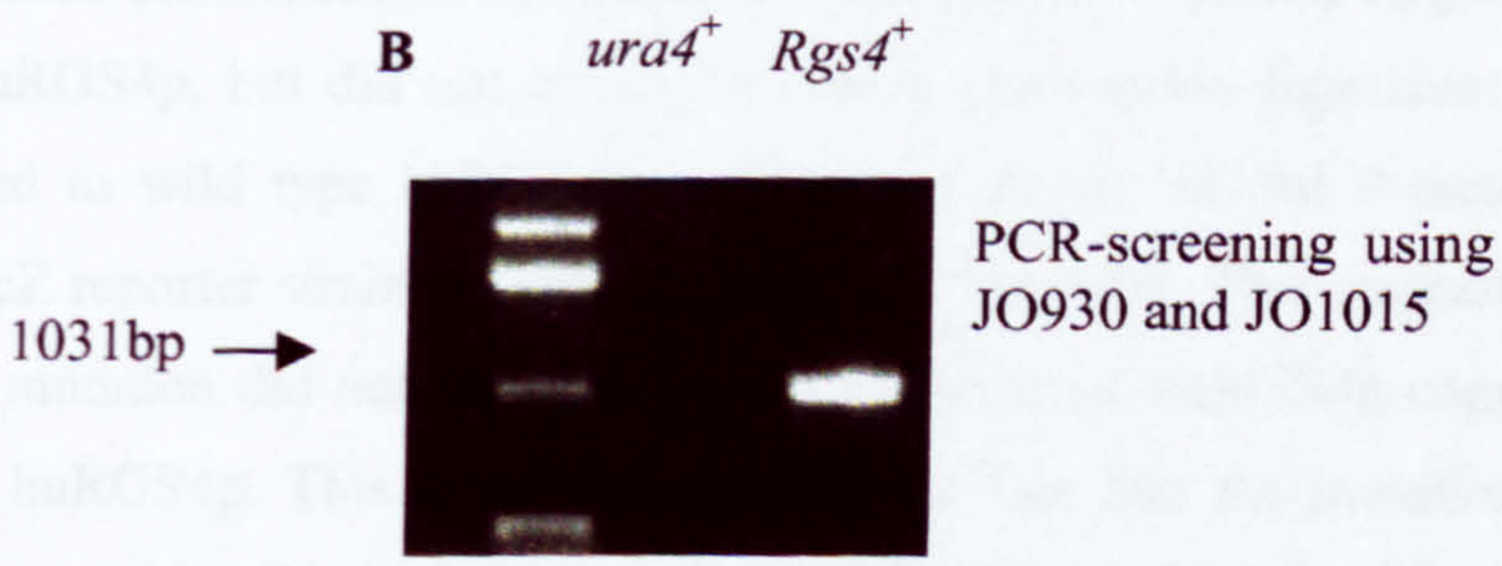


Figure 69. PCR amplification confirming the integration of *Rgs4*$^{Lys20\rightarrow Glu}$ ORF at the *rgs1* locus in the *rgs1*$^{-}$ LacZ reporter strain

The *rgs1*$^{-}$, *sxa2*::*lacZ* reporter strain JY629 was transformed with the *rgs1*::*Rgs4*$^{Lys20\rightarrow Glu}$ integration fragment liberated via *Spe*I/*Nde*I restriction from the *rgs1*:: *Rgs4*$^{Lys20\rightarrow Glu}$ integration construct. Transformants were screened via PCR amplification for the presence of the *Rgs4*$^{Lys20\rightarrow Glu}$ ORF at the *rgs1* locus using oligonucleotides complementary to the *rgs1* 5' non-coding region (JO930), the *Rgs4* ORF (JO1015) and the *rgs1* 3' non-coding region (JO931).

Rgs4$^{Lys20\rightarrow Glu}$ ORF at the *rgs1* locus (JY1195) was chosen to assess pheromone-independent and pheromone-dependent β-galactosidase activity.

5.3.7. β-galactosidase activity of the human RGS4$^{Lys20\rightarrow Glu}$ integrant

The β-galactosidase activity of JY1195 (*rgs1*::*Rgs4* $^{Lys20\rightarrow Glu}$, *sxa2*::*lacZ*) was determined following stimulation with a range of P-factor concentrations. The *rgs1*$^{+}$, *rgs1*$^{-}$ and *rgs1*::*Rgs4*$^{+}$ LacZ reporter strains were included in the assay to determine whether the RGS4$^{Lys20\rightarrow Glu}$ mutant displayed a phenotype consistent with the Lys20→Arg mutation conferring a huRGS4p gain-of-function phenotype (Figure 70).

Integration of the human *Rgs4*$^{Lys20\rightarrow Glu}$ mutant ORF in the *rgs1*$^{-}$ LacZ reporter strain (JY1195) reduced the pheromone-independent level of LacZ activity compared to the *rgs1*::*Rgs4*$^{+}$ LacZ reporter strain (JY1153) and the *rgs1*$^{-}$ LacZ reporter strain (JY629). However, pheromone-independent LacZ activity was greater in JY1195 than that seen for the *rgs1*$^{+}$ LacZ reporter strain (JY544). Above 10 U/ml P-factor LacZ activity of the mutant RGS4 strain was increased compared to that seen for the *rgs1*$^{+}$, *rgs1*$^{-}$ and *Rgs4*$^{+}$ LacZ reporter strains.

The data presented in Figure 70. suggest that that the Lys20→Glu mutation in huRGS4p reduced pheromone-independent LacZ activity to a greater degree than the wild type huRGS4p, but did not appear to reduce pheromone-dependent LacZ activity compared to wild type huRGS4p, and indeed above 10U/ml P-factor the *Rgs4*$^{Lys20\rightarrow Glu}$ LacZ reporter strain exhibited greater LacZ activity. This suggests that the Lys20→Glu mutation did not increase the RGS activity of huRGS4p compared to the wild-type huRGS4p. This is anomalous with the fact that the mutation was present in a strain capable of growth in the presence of 5-FOA when stimulated with 100U/ml P-factor. As with the szRgs1p mutants described in Section 5.2.4., these results could be explained by the timescales of the assays. In the β-galactosidase assay, LacZ activity was determined after 16h of pheromone stimulation. In the 5-FOA viability assay, cells were stimulated for 3d. As well as pheromone communication, other physiological systems, such as cell growth and differentiation, may complicate the conclusions that can be reached.

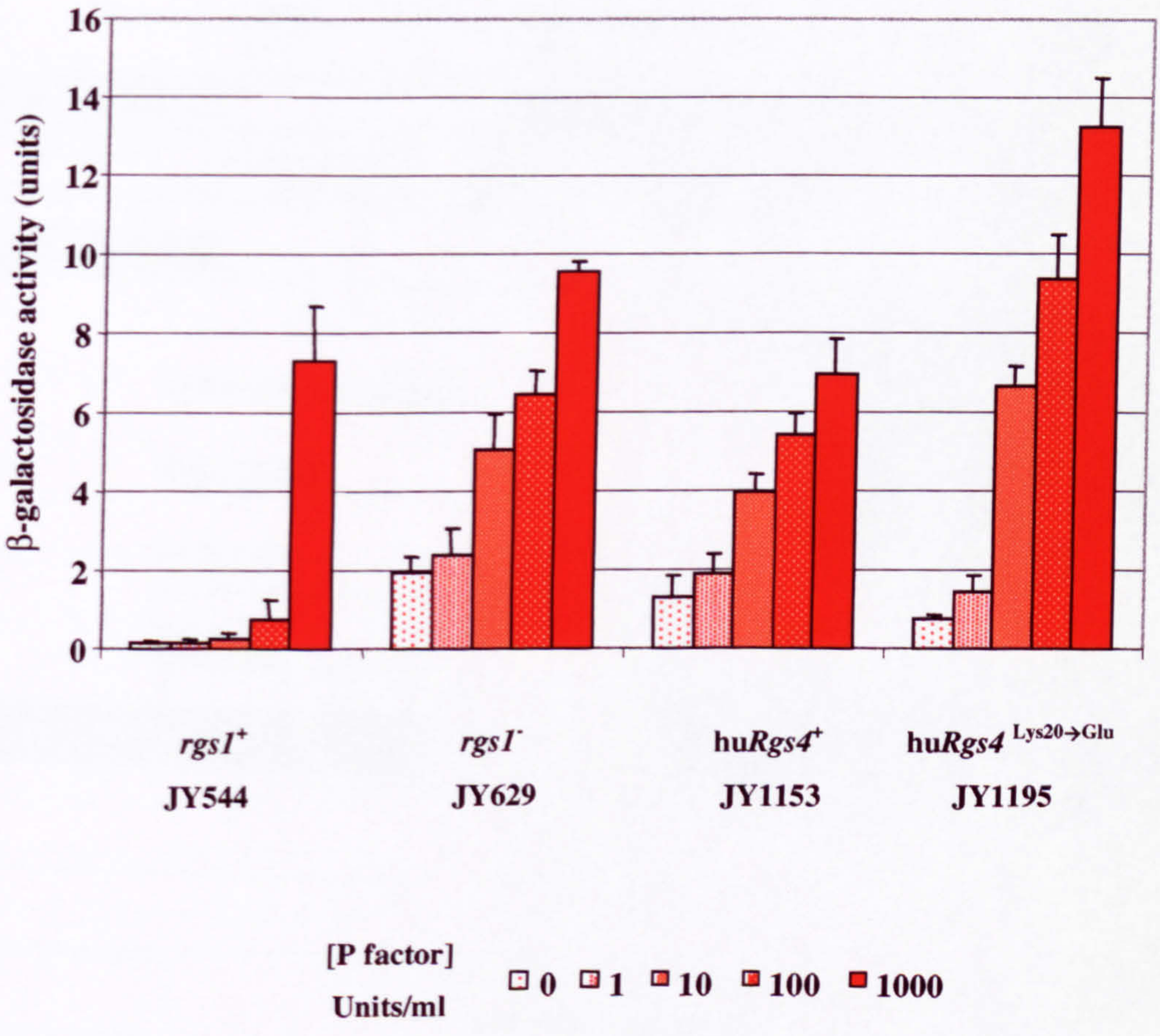


Figure 70. The *Rgs4*$^{Lys20\rightarrow Arg}$ LacZ reporter strain exhibits reduced pheromone-independent β-galactosidase activity compared to the *Rgs4*$^{+}$ LacZ reporter strain but increased pheromone-dependent β-galactosidase activity above 10U/ml P-factor

The *rgs1*$^{+}$ (JY544), *rgs1*$^{-}$ (JY629), *Rgs4+* (JY1153) and *Rgs4*$^{Lys20\rightarrow Arg}$ (JY1196) LacZ reporter strains were assayed for β-galactosidase activity following exposure to a range of P-factor pheromone concentrations. Reporter strains were cultured to a density of $5x10^5$ cells/ml in DMM medium prior to exposure to pheromone for 16h. LacZ activity was calculated as the ratio of *o*-nitrophenol product formed (OD_{420}) to cell number. Data shown is the mean of three assays; y-axis error bars represent standard deviation.

5.4. Summary

Chapter 5 describes the creation of two szRgs1p mutants, and the isolation of a huRGS4p mutant, that enabled the Ura4 reporter strain to grow at P-factor concentrations above that enabled by the wild-type szRgs1p.

The use of a *sxa2*::*ura4* reporter strain to screen for proteins that inhibit the pheromone communication pathway has been described, and represents a simple tool to screen thousands of colonies. This system could also be used to isolate dominant-negative proteins, which interfere with the GAP activity of wild-type RGS proteins.

Chapter 6. Discussion

6.1. Overview

Within this thesis I have described the generation of a temperature-sensitive *Sz. pombe* Ura4p marker (Chapter 3), which expands the currently limited number of markers available for the genetic manipulation of *Sz. pombe*. The *ura4*^{ts} cassette described can be used to carry out multiple gene disruptions, and presents greater scope when sequential gene disruptions are desired in conjunction with the selection of plasmid-borne markers.

Within Chapter 4, I describe the use of *Sz. pombe* LacZ reporter strains to assess the ability of mammalian RGS proteins to rescue the hypersensitive phenotype of the *rgs1*$^{-}$ LacZ reporter strain. The ability of the C-terminal RGS domain of *Sz. pombe* was also investigated for its ability to negatively regulate the pheromone signalling pathway. The human *Rgs1* and human *Rgs4* ORFs were integrated at the *rgs1* locus in the *rgs1*$^{-}$ LacZ and Ura4 reporter strains to determine whether they could rescue the hypersensitive phenotype when expressed from single copy.

The generation of RGS mutants is described in Chapter 5. Two site-directed szRgs1p mutants were created following the isolation of potential gain-of-function szRgs1p mutants previously in the Davey laboratory. The mutagenesis of human RGS4 via hydroxylamine treatment to isolate a gain-of-function huRGS4p mutant is also described, which resulted in a single missense mutation being identified.

6.2. A temperature-sensitive *Sz. pombe* Ura4p marker has been created

The *Sz. pombe ura4* gene encodes a biosynthetic gene required for growth in the absence of extracellular uracil. Ura4p has number of advantages over other *Sz. pombe* markers, including the availability of compounds for negative selection, for example 5-FOA (Boeke *et al.*, 1984), a fluoropyrimidine analogue that is lethal to cells expressing a functional *ura4* gene.

The *Sz. pombe ura4* cassette was integrated downstream of a cloned copy of the *Sz. pombe krp1* ORF. This construct was then used as a template for mutagenic PCR using *Taq* DNA polymerase and a modified PCR buffer. Integration of the amplified *krp1>ura4* fragment into a *ura4-D18* strain resulted in some of the

transformants exhibiting a temperature-sensitive Ura4p phenotype. The *ura4* ORF in one of these strains was found to contain a T-to-C mutation at nucleotide position +782 (relative to the *ura4* initiator codon). This mutation results in a leucine to proline substitution at residue 261 in the primary sequence of Ura4p. Recreation of the T-to-C mutation at position +782 in the *ura4* ORF confirmed that this mutation was responsible for the temperature-sensitive phenotype of Ura4p.

6.2.1. The *ura4*^{ts} cassette aids sequential gene disruptions

To demonstrate the use of the *ura4*^{ts} cassette in gene disruption strategies, the *irp1* gene of *Sz. pombe* was disrupted with an *irp1*::*ura4*^{ts} disruption construct, and transformants screened on medium lacking uracil at 23°C. Homologous integrants were unable to grow on plates lacking uracil at 37°C. The *prk1* gene of the *irp1*::*ura4*^{ts} strain was subsequently disrupted with the wild-type *ura4* cassette, and transformants isolated on medium lacking uracil at 37°C.

One drawback of *Sz. pombe* as an experimental organism is the limited availability of selectable markers, and while some markers used for the selection of genetic manipulations in *S. cerevisiae* are capable of rescuing auxotrophic *Sz. pombe* strains when carried on multicopy plasmids, they are often incapable of complementing an auxotroph when in single copy (the URA3 gene of *S. cerevisiae* fails to rescue a *Sz. pombe ura4* auxotroph). The restricted availability of *Sz. pombe* markers limits the number of sequential disruptions that can be carried out within a strain. Some markers have been developed that enable a gene to be disrupted by a marker, with the ensuing excision of the marker leaving behind a "scar" within the chromosome (Waddell and Jenkins, 1995; Alani *et al.*, 1987). It was envisaged that the temperature-sensitive *ura4* cassette would aid sequential integration events in a single strain, limiting the number of intermediate steps.

The *ura4*^{ts} cassette also aids discrimination between homologous integration events and the presence of the *ura4*^{ts} cassette on autonomously replicating plasmids, enabling efficient screening of transformants. The presence of multiple copies of the *ura4*^{ts} cassette enables growth at restrictive temperatures, while the integration of a single genomic copy only enables growth at the permissive temperature. Thus, replicating transformants to plates lacking uracil at 37°C can screen out

transformants in which the *ura4*^{ts} cassette is borne on autonomously replicating plasmids.

6.3. Expression of mammalian RGS proteins in *Sz. pombe*

It has been well established that mammalian RGS proteins can inhibit the pheromone response in *S. cerevisiae* (Druey *et al.*, 1996; Chen *et al.*, 1997), and it was thus of interest to determine whether mammalian RGS proteins would also be capable of negatively regulating the *Sz. pombe* pheromone communication pathway. In order to investigate the activity of mammalian RGS proteins upon the *Sz. pombe* pheromone communication pathway, mammalian RGS proteins were expressed in reporter strains in which the expression of reporter proteins (LacZ and Ura4p) were directed by the activity of the pheromone-regulated *sxa2* promoter.

6.3.1. *Sz. pombe* reporter systems have been created

Sz. pombe reporter strains were created by Kevin Davis and Mark Didmon in the laboratory to aid investigation of the pheromone signal transduction cascade. In these strains the open reading frames of the reporter proteins LacZ and Ura4 were integrated immediately downstream of the *sxa2* promoter. Expression of *sxa2* in M cells is tightly regulated by P-factor stimulation, with *sxa2* activity only detectable following pheromone stimulation. Expression of *sxa2* is dose-dependent, with greater P-factor concentrations resulting in greater *sxa2* expression. Thus, in the *Sz. pombe* reporter strains, pheromone stimulation results in *lacZ* or *ura4* expression, which can be qualitatively or quantitatively measured, and reflects the level of signalling through the pheromone communication pathway.

6.3.2. Disruption of *Sz. pombe* Rgs1p increased sensitivity and response in a LacZ reporter strains

The *Sz. pombe* RGS protein, szRgs1p, was first reported by Tesmer *et al.* (1997) as a hypothetical RGS ORF. This protein has been characterised by Watson *et al.* (1999) and Pereira and Jones (2001), and acts as a GTPase-activating protein for

the *Sz. pombe* G_α subunit, Gpa1p. Both groups reported that loss of szRgs1p increased signalling through the pheromone communication pathway, and resulted in the loss of mating ability.

Data presented in this thesis confirm that a LacZ reporter strain lacking the *rgs1* gene (*rgs1*$^-$) exhibited increased sensitivity and response to pheromone (Chapter 4). Expression of szRgs1p from a multicopy expression vector rescued the hypersensitive phenotype of the *rgs1*$^-$ LacZ reporter strain.

At high levels of P-factor (1000U/ml) the *rgs1*$^+$ LacZ reporter strain possessed greater lacZ activity compared to the *rgs1*$^-$ LacZ reporter strain (reflecting a greater level of signalling). This was in contrast to LacZ activities observed at lower P-factor concentrations, as the *rgs1*$^-$ LacZ reporter strain exhibited increased sensitivity and response to P-factor. This could reflect the ability of RGS proteins to increase the kinetics of the G protein activation and inactivation cycle, as observed by other groups (Doupnik *et al.*, 1997). This consequence of RGS proteins is thought to result from faster rates of deactivation, and the fact that receptor/G protein complexes are kept as functional units at the plasma membrane by RGS proteins. The absence of an RGS protein has the consequence that G proteins are inactivated slowly as a consequence of their intrinsic GTPase activity. The ability of RGS proteins to accelerate the rate of GTP hydrolysis may thus result in a greater concentration of inactive G proteins in G protein/receptor complexes at the plasma membrane that are available for re-activation.

6.3.3. Mammalian RGS proteins rescue the hypersensitive phenotype of the *rgs1*$^-$ LacZ reporter strain

It had been observed that expression of szRgs1p within an *rgs1*$^-$ LacZ reporter strain rescued the hypersensitive phenotype, and the ability of several mammalian RGS proteins to negatively regulate the pheromone signalling pathway of the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains was investigated.

Human RGS1 and human RGS4 displayed the greatest ability to inhibit the pheromone communication pathway in *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains, to similar degrees as szRgs1p. Human RGS2, human RGS3, human RGS9-2 and

murine RGS2 complemented the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains to lesser, varying degrees.

The most obvious reason for differential activity of mammalian RGS proteins upon the pheromone communication pathway is G_α subunit specificity. HuRGS4p has been reported to act as a GAP for numerous G_α subunits, both *in vitro* and *in vivo*, while human RGS2 displays a preference for $G_{\alpha q}$ over $G_{\alpha i}$ subunits (Heximer *et al.*, 1997). It is thus likely that the different abilities of the RGS proteins to negatively regulate the *Sz. pombe* pheromone signalling pathway reflects their affinity for the *Sz. pombe* G_α subunit, Gpa1p. The fact that heterogeneous RGS proteins can function as GAPs for the *Sz. pombe* G_α subunit reflects their promiscuity for G_α subunits. The ability of mammalian RGS proteins to act as GAPs in *Sz. pombe* also means that caution should be taken when extrapolating the results to eukaryotic systems.

To further characterize mammalian RGS protein activity in *Sz. pombe*, it would be interesting to investigate their localization, for example using proteins tagged with the green fluorescent protein (GFP). It is possible that some of the RGS proteins investigated did not display significant GAP activity, as a result of mis-localisation.

6.3.4. Human RGS2 and human RGS4 are expressed in the *Sz. pombe rgs1*$^-$ LacZ reporter strain and may be covalently modified

Expression of huRGS2p did not result in as great a reduction in LacZ activity in the LacZ reporter strains as that seen for huRGS4p, and it is possible that this could have been due to a lower expression level compared to that of huRGS4p. Western blot analysis was therefore carried out to determine whether huRGS2p and huRGS4p were expressed in the *rgs1*$^-$ LacZ reporter strain. For both RGS proteins a band approximately 4kD larger than the reported size was visualised. These bands were only seen for the *rgs1*$^-$ LacZ reporter strain transformed with the huRGS2p or huRGS4p expression constructs. These results indicate the huRGS2p and huRGS4p are expressed in *Sz. pombe* from the pREP3X expression vector, and that the proteins may be covalently modified in *Sz. pombe*. Palmitoylation of huRGS4p has been

reported (Srinivasa *et al.*, 1998a), and huRGS2p contains an N-terminal cysteine ring that may represent a target site for palmitoylation. Palmitoylation of G_α subunits has been reported to contribute to plasma membrane localisation (Degtyarev *et al.*, 1994), and this may hold true for RGS proteins. To determine whether huRGS2p and huRGS4p are palmitoylated in *Sz. pombe*, the covalent attachment of radiolabelled palmitate molecules to huRGS2p and huRGS4p could be monitored following isotopic labelling of the cellular palmitoyl-CoA pool. Palmitoyl-Co-A is the metabolic intermediate in palmitoylation reactions. Phosphorylation of RGS proteins may also play a role in regulating their localisation and/or activity, as potential phosphorylation sites have been identified in several RGS proteins, including GAIP (DeVries *et al.*, 1996; Fischer *et al.*, 2000). HuRGS2p has been reported to be phosphorylated by protein kinase C (Cunningham *et al.*, 2001).

The anti-RGS2p antibody also bound to a 35kD protein that was present in all strains analysed (*rgs1*$^-$, *rgs1*$^-$ pREP3X-szRgs1p, *rgs1*$^-$ pREP3X-huRGS2p). The identity of this band is at present unknown, and N-terminal sequencing of the protein could be carried out to ascertain its identity. The band is too small for it to be the *Sz. pombe* RGS protein, szRgs1p (55.4kD), and no other RGS proteins have been isolated in *Sz. pombe* at the time of writing.

6.3.5. The N-terminal domain of *Sz. pombe* Rgs1 is required for full RGS activity

It has been reported by a number of groups that the RGS domain of several RGS proteins are active *in vitro*, but do not function *in vivo* (for example RGS16, Chen and Lin, 1998). It was therefore decided to investigate whether the C-terminal RGS domain of szRgs1p would also prove to be inactive in *Sz. pombe* LacZ reporter strains. Expression of the RGS domain of szRgs1p reduced LacZ activity of the *rgs1*$^+$ and *rgs1*$^-$ LacZ reporter strains at low pheromone concentrations, but at high pheromone concentrations, the szRgs1p RGS domain was not as successful as the full-length szRgs1p in reducing LacZ activity. This indicates that N-terminus of szRgs1p is required for full GAP activity in szRgs1p.

The N-terminus of szRgs1p contains additional signalling motifs, including a DEP domain and a novel Fungal-DR domain (Pereira and Jones, 2001) that may be required for full szRgs1p activity.

6.4. Integration of human *Rgs1* and human *Rgs4* ORFs into *Sz. pombe* reporter strains

Out of the mammalian RGS proteins investigated, huRGS1p and huRGS4p exhibited the greatest ability to reduce LacZ activity in the LacZ reporter strains, and it was decided to integrate the human *Rgs1* and human *Rgs4* ORFs at the *rgs1* locus in the *rgs1*$^-$ LacZ and Ura4 reporter strains. This enabled the determination of the ability of the two human RGS proteins to negatively regulate the pheromone communication when expressed from a single genomic copy. This was important, as multi-copy expression vectors can give rise to indiscriminate expression levels.

The *Rgs1* and *Rgs4* ORFs were flanked with *Sz. pombe rgs1* 5' and 3' non-coding regions, and the *rgs1*$^-$ LacZ reporter strain transformed with the resulting integration constructs. The presence of *rgs1* 5' and 3' non-coding regions enabled homologous integration of the human RGS ORFs to be directed by the flanking *rgs1* sequences, resulting in the integration of the ORFs at the *rgs1* locus, exactly replacing the *rgs1* ORF. This resulted in the expression of the human *Rgs1* and human *Rgs4* ORFs being directed by the *Sz. pombe rgs1* promoter.

6.4.1. Human RGS1 reduced pheromone signalling at low pheromone concentrations when expressed from single copy

Expression of huRGS1p from single copy rescued the hypersensitive phenotype of the *rgs1*$^-$ LacZ reporter strain at low pheromone concentrations. The ability of huRGS1p to negatively regulate the pheromone communication pathway at low signalling levels was also observed when huRGS1p was expressed from single copy in the Ura4 reporter strain. This indicates that at low levels of signalling through the pheromone communication pathway huRGS1p had sufficient GAP activity towards the *Sz. pombe* G_α subunit to inhibit signalling, but at high

pheromone concentrations, the affinity of huRGS1p for the G_α subunit was insufficient to inhibit signalling to the level seen for szRgs1p.

6.4.2. Human RGS4 did not reduce pheromone signalling when expressed from single copy

Expression of huRGS4p from single copy did not rescue the hypersensitive phenotype of the *rgs1*⁻ LacZ reporter strain. I was unable to isolate *rgs1*::*Rgs4* integrants in the *rgs1*⁻ Ura4 reporter strain, and this was probably a consequence of high levels of pheromone-independent signalling through the pheromone signal transduction pathway. Thus, when expressed from a multicopy vector, RGS4 is capable of rescuing the *rgs1*⁻ hypersensitive phenotype, but is unable to when expressed from a single genomic copy. These results indicate that huRGS4p has lower affinity for the *Sz. pombe* G_α subunit than huRGS1p.

6.5 Mutagenesis of RGS proteins

Mutagenesis of proteins enables investigators to determine how amino acid residues contribute to protein activity. The mutagenesis of RGS proteins has resulted in the isolation of dominant-negative RGS proteins, RGS proteins with diminished or no GAP activity towards G_α subunits (Srinivasa *et al.*, 1998b; Druey and Kerhl, 1997). No gain-of-function mammalian RGS proteins have yet been identified. A gain-of-function SST2 protein (a *S. cerevisiae* RGS protein) has been isolated, but the mutation conferring the gain-of-function phenotype lies outside of the conserved RGS domain.

6.5.1. Creation of *Sz. pombe* Rgs1 gain-of-function mutants

Two potential szRgs1p gain-of-function mutants were isolated by Peter Watson in the Davey laboratory using a hydroxylamine mutagenesis approach. The *rgs1* ORF was mutagenised via hydroxylamine treatment, and the mutated *rgs1* ORF re-introduced into the *Sz. pombe rgs1*⁻ Ura4 reporter strain. Transformants were screened on selective plates that identified isolates with potentially reduced

signalling through the pheromone communication pathway. Sequencing of transformants revealed two mutations that potentially increased the GAP activity of the mutant szRgs1 proteins. The first mutation resulted in a histidine to arginine substitution at residue 171, and the second resulted in a valine to isoleucine substitution at residue 305. Both of these residues lie outside the C-terminal RGS domain in szRgs1p.

The two potential gain-of-function mutations were re-created via site-directed mutagenesis, and the mutant *rgs1* ORFs re-introduced into the *rgs1*⁻ LacZ and Ura4 reporter strains. The mutant proteins did not exhibit a gain-of-function phenotype in the LacZ reporter strain, but enabled the Ura4 reporter strain to grow on FOA plates in the presence of 1000-fold higher P-factor concentrations than the wild-type szRgs1p. This anomaly can be explained by the time-scales of the assays used to monitor signalling through the pheromone communication pathway. The longer timescale of the Ura4 viability assay (3d) compared to the LacZ assay (16h) means that results obtained with the Ura4 reporter strain may more truly reflect the activity of the szRgs1p mutants upon the pheromone signalling cascade.

6.5.2. Mutagenesis of human RGS4

The huRGS4p integration cassette contains the human *Rgs4* ORF flanked with *Sz. pombe rgs1* 5' and 3' non-coding regions. This construct was mutagenised via hydroxylamine treatment, and a plasmid library prepared. The *rgs1*::*Rgs4* integration fragment was liberated from this plasmid library and introduced into the *rgs1*⁻ Ura4 reporter strain. Transformants were screened on selective plates that prevented the growth of the *rgs1*⁻ Ura4 reporter strain, but would enable growth of strains in which the pheromone communication pathway had been inhibited to a sufficient degree to allow growth on 5-FOA-supplemented growth media containing 100U/ml P-factor. Transformants were screened for the presence of the human *Rgs4* ORF at the *rgs1* locus, and the *Rgs4* ORFs sequenced.

6.5.3. A single missense mutation in huRGS4p was identified

A single missense mutation was identified that resulted in a lysine to glutamate substitution at residue 20 in the primary sequence of huRGS4p. Integration of the mutant *Rgs4* ORF at the *rgs1* locus in the LacZ reporter strain did not reduce LacZ activity appreciably compared to the wild-type huRGS4p integrant, but considering the results obtained with the szRgs1p gain-of-function mutants, the possibility of the lysine to glutamate mutation conferring a gain-of-function phenotype should not be ruled out. The lysine residue does not lie within the conserved RGS domain of huRGS4p, but rather within the non-conserved N-terminus. The N-terminus of huRGS4p has been reported to be important for membrane targeting (Srinivasa *et al.*, 1998a; Bernstein *et al.*, 2000) and receptor selectivity (Zeng *et al.*, 1998), and it is possible that this mutation increases the affinity of huRGS4p for the *Sz. pombe* G_α subunit and/or P-factor receptor.

6.6. Summary

The ability of human RGS proteins to function on the *Sz. pombe* pheromone communication pathway means that this system can be used as a model to assess signalling proteins involved in G protein-coupled signal transduction pathways. This is not limited to RGS proteins, and could involve receptors, G proteins and down-stream effector proteins, enabling whole signalling pathways to be reconstituted in the *Sz. pombe* reporter strains. The RGS mutants described in this thesis are currently being investigated for their ability to negatively regulate G protein-coupled signalling in human tissue culture (Allen and Davey, personal communication).

Chapter 7. References

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Chapter 8. Appendices

8.1. Oligonucleotide primers

8.1.1. Oligonucleotide primers for PCR amplification

Upper case letters represent sequences homologous to genomic sequence.

Lower case letters represent non-homologous sequence.

Restriction sites are underlined.

Primer (5' to 3')	Direction relative to ORF	Position relative to ORF
Generation of the *krp1>ura4* fragment		
JO65 TTGCCAGTGAACGACCG	Sense	1035 to1051
JO66 GTTTCGAGATGACTCCG	Antisense	2695 to 2679
Amplification of the *krp1>ura4* fragment		
JO61 GTTAATAAATCATTCGG	Sense	1484 to 1500
JO496 gggcagCTGTAAGTTCACAGCTTCT GCC	Antisense	2966 to 2945
Generation of the *irp1*::*ura4*^{ts} integration construct		
JO497 AAACGCGTATCCCAGCGC	Sense	-1228 to -1211
JO498 GGGGATCCTTTTTATTTATGAAA GGAA	Antisense	-1 to -27
JO499 GGGGATCCTTTTTTTTCCCTAATCCG	Sense	2911 to 2936
JO500 TCAATGATTCGTAAGTCC	Antisense	4821 to 4804

Generation of the *prk1*::*ura4*$^+$ integration construct

JO490	gggggatccGCTGAAATGAGACCC TACCC	Sense	1060 to 1080
JO491	gggggatccATCAACCTATTCATTA TCC	Antisense	-1 to -19

Amplification of the *ura4* ORF

JO1056	gggggatccaccATGGATGCTAGAGTA TTTC	Sense	1 to 19
JO1057	gggggatccGTCTTTTTTTAATGCTGAG	Antisense	803 to 784

Amplification of human *Rgs1* ORF

JO910	ggggatccaccATGCCAGGAATGTTCTTC TCTGC	Sense	1 to 23
JO911	ggggatccTCACTTTAGGCTATTAGC CTGC	Antisense	591 to 570

Amplification of human *Rgs2* ORF

JO912	ggggatccaccATGCAAAGTGCTATGTTC TTGGC	Sense	1 to 23
JO913	ggggatccTCATGTAGCATGAGGCTC TGTGG	Antisense	636 to 614

Amplification of murine *Rgs2* ORF

JO912	ggggatccaccATGCAAAGTGCTATGTT CTTGGC	Sense	1 to 23
JO913	ggggatccTCATGTAGCATGAGGCTCT GTGG	Antisense	636 to 614

Amplification of human *Rgs3* ORF

JO914 ggggatccaccATGTTTGAGACGGAGGCA GATG	Sense	1 to 22
JO915 ggggatccCTAAAGCGGGGGACTCATC TTC	Antisense	1560 to 1539

Amplification of human *Rgs4* ORF

JO917 ggggatccaccATGTGCAAAGGGCTTGC AGGTC	Sense	1 to 22
JO918 ggggatccTTAGGCACACTGAGGGAC CAG	Antisense	618 to 598

Amplification of human *Rgs9-2* ORF

JO1016 gggggatccaccATGACGATCCGACACC AAGGCCAGCAG	Sense	1 to 27
JO1017 gggggatccTGCTCAGCCCGCCTTCCCT TCCGC	Antisense	2031 to 2008

Amplification of *Sz. pombe rgs1* C-terminus

JO923 ggggatatcaccATGGAAACTGTTGCTA GCGATTTG	Sense	820 to 843
JO600 ggggatatCATTAAATACCGAGCCCCCC ATTC	Antisense	1448 to 1425

Amplification of human *Rgs1* ORF flanked with *rgs1* 5' and 3' non-coding sequences

JO1119 CACTTTATTATTTATGATAAAAGGC TACAAATTCGTAAAAGTAAGAATC AGCTTTGGatgccaggaatgttcttctctgc	Sense	-57 to -1 (*rgs1*) 1 to 12 (*Rgs1*)
JO1120 GTACAAAAAACAAGAGGAACTGCT AATGAATGATTTAAAACACGATT	Antisense	1504 to 1444 (*rgs1*)

	GTTTTCTATGCATTactttaggctattag cctgcag		588 to 568 (*Rgs1*)
Amplification of human *Rgs4* ORF flanked with *rgs1* 5' and 3' non-coding sequences			
JO1121	CACTTTATTATTTATGATAAAA GGCTACAAATTCGTAAAAGTA AGAATCAGCTTGatgtgcaaagggctt gcaggtctg	Sense	–57 to –1 (*rgs1*) 1 to 24 (*Rgs4*)
JO1122	GTACAAAAACAAGAGGAACT GCTAATGAATGATTTAAAACAC GATTGTTTTCTATGCATTAggcacact gagggaccaggg	Antisense	1504 to 1444 (*rgs1*) 618 to 596 (*Rgs4*)
Amplification of *Sz. pombe rgs1* 5' and 3' non-coding regions			
JO1267	ATGAACAAAAAGAATTGTG	Antisense	-58 to -75
JO1268	ATATTTCTTATTTGCATTATT	Sense	1505 to 1522
Amplification of *Sz. pombe rgs1* A512→G site-directed mutation			
JO1249	TAATTCTCAAGCCGAGCTTTAC	Sense	513 to 534
JO1250	CGTCCGTTATGTTGTAGAAAC	Antisense	512 to 492
Amplification of *Sz. pombe rgs1* G913→A site-directed mutation			
JO1247	TTTCCTATTTTTTGACTG	Sense	914 to 931
JO1248	TACCCTTTGCATAGCTAAAC	Antisense	913 to 894

Screening for *Rgs4* ORF at *rgs1* locus

JO930 GTCTCCAGGAGTGCTGGG	Sense	–811 to –794 (rgs1)
JO931 ATGAGGGTTAGTTCAGTG	Antisense	1888 to 1871 (rgs1)
JO1015 CCAGCCCACATTCATGAC	Antisense	220 to 203 (Rgs4)

Amplification of human *Rgs4* A58→G site-directed mutation

JO1433 GAACATCGGCTAGGTTTCC	Sense	58 to 76
JO1434 CATATCTTTTGCACTCC	Antisense	57 to 41

8.2. Oligonucleotide primers for sequencing

Sequencing human *Rgs1* ORF

JO910 ggggatccaccATGCCAGGAATGTTCTTC TCTGC	Sense	1 to 23
JO911 ggggatccTCACTTTAGGCTATTAGCCT GC	Antisense	591 to 570

Sequencing human *Rgs2* ORF

JO912 ggggatccaccATGCAAAGTGCTATGTTC TTGGC	Sense	1 to 23
JO913 ggggatccTCATGTAGCATGAGGCTCTG TGG	Antisense	636 to 614

Sequencing human *Rgs3* ORF

JO914 ggggatccaccATGTTTGAGACGGAGGCAGATG	Sense	1 to 22
JO915 ggggatccCTAAAGCGGGGGACTCATCTTC	Sense	1560 to 1539
JO1002 CTTCCACCCAACAAGGAC	Sense	127 to 144
JO1003 GCCTCCTAGCCAGGTCTC	Sense	414 to 431
JO1004 GCGCAGTGAGGCCAAGCG	Sense	690 to 707
JO1005 AGGACACAGGAAGATGAG	Sense	966 to 983
JO1006 AGTGAGGAGAATCTGGAG	Sense	1249 to 1266
JO1007 GTTGACCTCCTTGCATGC	Antisense	1380 to 1363
JO1008 TCCAGGGGACTCATTCCG	Antisense	1101 to 1084
JO1009 GGCTCCTGCAGCAGGCTG	Antisense	815 to 798
JO1010 CGGACCTCAGGGATCACG	Antisense	542 to 525
JO1011 GGTGGCACATCCTTGCTG	Antisense	260 to 243

Sequencing human *Rgs4* ORF

JO917 ggggatccaccATGTGCAAAGGGCTTGCAGGTC	Sense	1 to 22
JO918 ggggatccTTAGGCACACTGAGGGACCAG	Antisense	618 to 598
JO1012 TCTGATTCCTGTGAACAC	Sense	88 to 105
JO1013 TGGATTCTTGCACCAGGG	Sense	386 to 403
JO1014 TCGAGACTTGAGGAAGCG	Antisense	516 to 499
JO1015 CCAGCCCACATTCATGAC	Antisense	220 to 203

Sequencing human *Rgs9-2* ORF

JO1016	ggggatccaccATGACGATCCGACACC AAGGCCAGCAG	Sense	1 to 27
JO1017	ggggatccTGCTCAGCCCGCCTTCCCT TCCGC	Antisense	2031 to 2008
JO1018	TGGAGGCACAGAACCTGG	Sense	203 to 220
JO1019	GCGCTGGACTGCCAGGAG	Sense	535 to 552
JO1020	GATGCGAGTGGAGAGATG	Sense	870 to 887
JO1021	AAGGATTCTTACGCACGC	Sense	1192 to 1209
JO1022	AGGAAGCCCTTTGCTTCC	Sense	1501 to 1518
JO1023	ACACGGGAAGGTGCAGCC	Sense	1806 to 1823
JO1024	GAGTCCATCAGGCAGGTC	Antisense	1934 to 1917
JO1025	CTGTTCTCAGTGACTGAG	Antisense	1661 to 1644
JO1026	CTCTTCTTCTTCCAGCTG	Antisense	1359 to 1342
JO1027	CTGATCGCCGTACTTCAG	Antisense	1020 to 1003
JO1028	TTGGTAATACATGATCTC	Antisense	690 to 673
JO1029	TTCCGCTTCGCTAGATAG	Antisense	386 to 369
JO1030	AGCACGTCACCTCCTGTC	Antisense	167 to 150

Sequencing *Sz. pombe rgs1* ORF

JO603	GCAACGGAAATTCAATAG	Sense	62 to 79
JO604	TAAATGTTTATGTAATAC	Sense	363 to 380
JO605	AAACCCTCTTCTGGTACC	Sense	678 to 695
JO606	TGGACTAAAAATGTCTCC	Sense	955 to 972
JO607	TTCCCAGTGATCTTTATG	Sense	1262 to 1279

JO608 TACGGCTGTTTGCGCCTC		Antisense	1380 to 1363
JO609 ATAGGTTTGTAGATTCGG		Antisense	1071 to 1054
JO610 GCTATGTCAATGCCAAAC		Antisense	776 to 759
JO611 GTAACCTTTCCTTGTAAG		Antisense	477 to 460
JO612 ATCGTCGAGAAGAGCTCC		Antisense	176 to 159

Sequencing *Sz. pombe rgs1* 5' non-coding region

JO930 GTCTCCAGGAGTGCTGGG	Sense	-811 to -794
JO1267 ATGAACAAAAGAATTGTG	Antisense	-58 to -75

Sequencing *Sz. pombe rgs1* 3' non-coding region

JO1268 ATATTTCTTATTTGCATTATT	Sense	1505 to 1522
JO931 ATGAGGGTTAGTTCAGTG	Antisense	1888 to 1871