

Research Article

# Glucose signalling pathway controls the programmed ribosomal frameshift efficiency in retroviral-like element Ty3 in *Saccharomyces cerevisiae*

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## Abstract

Ty3 elements of *S. cerevisiae* contain two overlapping coding regions, *GAG3* and *POL3*, which are functional homologues of retroviral *gag* and *pol* genes, respectively. Pol3 is translated as a Gag3-Pol3 fusion protein dependent on a +1 programmed frameshift at a site with the overlap between the two genes. We show that the Ty3 frameshift frequency varies up to 10-fold in *S. cerevisiae* cells depending on carbon source. Frameshift efficiency is significantly lower in cells growing on glucose as carbon source than in cells growing on poor alternative carbon sources (glycerol/lactate or galactose). Our results indicate that Ty3 programmed ribosomal frameshift efficiency in response to glucose signalling requires two protein kinases: Snf1p and cAMP-dependent protein kinase A (PKA). Increased frameshifting on alternative carbon sources also appears to require cytoplasmic localization of Snf1p, mediated by the Sip2p protein. In addition to the two required protein kinases, our results implicate that Stm1p, a ribosome-associated protein involved in nutrient sensing, is essential for the carbon source-dependent regulation of Ty3 frameshifting. These data indicate that Ty3 programmed ribosomal frameshift is not a constitutive process but that it is regulated in response to the glucose-signalling pathway. Copyright © 2011 John Wiley & Sons, Ltd.

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## Introduction

Programmed ribosomal frameshift (PRF) is one of the major control mechanisms of gene expression in various metazoan viruses including retroviruses and also occurs more rarely in cellular genes of eukaryotes and prokaryotes (Jacks and Varmus, 1985; Farabaugh, 1996; Namy *et al.*, 2004; Jacobs *et al.*, 2007). During translation elongation, ribosomes change reading frame in a +1 or –1 direction relative to the start codon, resulting in the translation of a protein encoded in two reading frames in the mRNA. As the result of PRF, gag and pol proteins are synthesized at specific ratios in retroviruses and retroviral-like elements (Jacks *et al.*,

1988; Farabaugh, 1996; Brierley and Dos Ramos, 2006). Even small changes in the PRF frequency results in the unbalanced production of gag and pol polypeptides, leading to the formation of non-infective retroviral particles (Shehu-Xhilaga *et al.*, 2001). Hence strategies dealing with mutations that alter PRF rates in retroviruses have important clinical applications in the prevention of retroviral propagation (Dinman *et al.*, 1997, 1998; Gareiss and Miller, 2009; Park *et al.*, 2009).

Molecular mechanisms of PRF have been analysed in detail in various viruses. In viruses such as the retrovirus human immunodeficiency virus 1 (HIV-1) and the coronavirus infectious bronchitis virus (IBV), PRF occurs within the *gag-pol*

junction with a shift in the  $-1$  direction on the mRNA. Efficient  $-1$  PRF requires a heptameric slippery sequence and is stimulated by a downstream pseudoknot structure that causes pausing of the ribosome at the slippery heptamer (Brierley and Dos Ramos, 2006; Giedroc and Cornish, 2009; Plant and Dinman, 2008).  $+1$  frameshifting also occurs at a heptameric frameshift site and its efficiency depends on the structure and abundance of tRNAs that decode the codons at the frameshift site (Farabaugh, 1996).

Ty3 is a retrovirus-like element in the yeast *Saccharomyces cerevisiae* (Clark et al., 1988; Hansen et al., 1988). It was classified in the Metaviridae group of the Retrovirales order due to structural and genetic similarities to animal retroviruses (Capy, 2005). In addition to structural similarities, Irwin et al. (2005) showed that Ty3 and animal retroviruses require similar sets of host genes for their cellular propagation. The Ty3 genome encodes two overlapping peptides, Gag3 and Pol3, which are functionally similar to the retroviral gag and pol polypeptides, respectively (Farabaugh, 1995). Peptides derived from Gag3, after proteolytic cleavage, function as viral nucleocapsid proteins for the formation of Ty3 virus-like particles in the yeast cells. Proteolytic processing of Pol3 yields three enzymatic activities required for Ty3 transposition: protease, integrase and reverse transcriptase/RNaseH (Farabaugh, 1995; Kim et al., 1998). Translation of the Pol3 polypeptide requires a programmed ribosomal frameshift in the  $+1$  direction by out-of-frame binding of a valyl-tRNA at a GUU codon of the frameshift site (Farabaugh et al., 1993; Vimaladithan and Farabaugh, 1994).

Some cellular factors required for propagation of Ty3 element may regulate the efficiency of PRF. Because PRF occurs during the elongation stage of translation, it may be regulated by signalling pathways that target translation elongation factors (EF) or by ribosomal proteins. EF-kinases, which are activated by a glucose pulse, are known to control the translation elongation process by acting on EF2 in human cells (Horman et al., 2002; Yan et al., 2003). Mutations in cellular genes such as ribosomal proteins and tRNAs, can perturb the PRF frequency in both bacteria and yeast (Atkins and Björk, 2009). The function of these frameshift regulating factors may be controlled physiologically. For example, Stahl et al. (2004) also reported that the PRF frequency changes in Ty1 in a growth

stage-dependent manner in yeast; the growth stage effect may also function through frameshift-regulating signalling pathways. In addition, we reported that the Ty1 type  $+1$  PRF frequency decreases several fold in glycerol–lactate-grown *S. cerevisiae* cells (Türkel et al., 2009); this carbon source regulation may function through one or more signalling pathways.

Glucose is the preferred carbon and energy source for *S. cerevisiae*, and its availability regulates cellular processes such as growth, cell cycle, ribosome biogenesis and regulation of gene expression (Santangelo, 2006; Zaman et al., 2008; Busti et al., 2010). High levels of glucose in the growth medium repress the genes required for the utilization of alternative carbon sources like glycerol, lactate, sucrose and galactose (Gancedo, 1998). Addition of glucose to yeast cultures growing in non-fermentable carbon sources changes the expression patterns of approximately 30% of the *S. cerevisiae* genes (DeRisi et al., 1997; Wang et al., 2004). The cell membrane of *S. cerevisiae* contains low-affinity glucose sensors Gpr1p and Rgt2p and the high-affinity sensor Snf3p (Schneper et al., 2004; Santangelo, 2006). Activation of these glucose sensors leads to the transduction of the glucose signal to various cytoplasmic protein kinases, principally Snf1p, cAMP-dependent protein kinase-A (PKA), and TOR kinases in *S. cerevisiae* (Busti et al., 2010). Downstream targets of these protein kinases include transcriptional regulators, metabolic enzymes and structural proteins (Santangelo, 2006; Zaman et al., 2008; Busti et al., 2010). Snf1p and cAMP-dependent PKA are especially important for the glucose response and are conserved throughout eukaryotes (Hong and Carlson, 2007; Smets et al., 2010).

Snf1p is the yeast homologue of mammalian AMP-activated protein kinase (Mitchell et al., 1994). Snf1p becomes activated after autophosphorylation of its T210 residue in response to glucose limitation and certain stress conditions (Hong and Carlson, 2007; Hedbacker and Carlson 2008). However, the specific metabolites or signals that trigger the Snf1p activation are still unknown (Hedbacker and Carlson, 2008; Busti et al., 2010). The Snf1p complex can be found in various subcellular compartments depending on growth conditions. Snf1p localization depends on its association with three alternative  $\beta$ -subunits: Sip1p, Sip2p and Gal83p (Hedbacker et al., 2004; Vincent et al.,

2001). The Snf1p-Sip2p complex remains in the cytoplasm when cells are grown either on glucose or alternative carbon sources. Growing yeast cells in alternative carbon sources causes Gal83 to displace Sip2p and the resulting Snf1p-Gal83p kinase complex translocates from the cytoplasm to the nucleus. Moreover, in ethanol-grown yeast cells Sip1p replaces Sip2p and the Snf1p-Sip1p complex translocates from the cytoplasm to the vacuole. The nucleocytoplasmic distributions of active Snf1 complexes provide versatility to the physiological response and adaptation to alternative carbon sources.

The Snf1p, cAMP-dependent PKA and TOR kinase pathways regulate translation and various other cellular processes in an interdependent manner (Zurita-Martinez and Cardenas, 2005; Slattery *et al.*, 2008; Busti *et al.*, 2010). In addition to protein kinases, ribosome-associated proteins like Stm1p regulate translation in response to nutrient deprivation. Stm1p is a highly abundant and multifunctional protein that is present in both subunits of elongating ribosomes (Van Dyke *et al.*, 2006, 2009).

In this study we investigated the effects of glucose signalling on the PRF frequency in Ty3. We provide genetic evidence indicating that the PRF frequency can change up to 10-fold in Ty3 depending on the nature of the glucose signalling pathway. Our results indicated that glucose signalling regulates Ty3 type +1 PRF through the protein kinase Snf1p and the cAMP-dependent protein kinase-A (PKA) in *S. cerevisiae*.

## Materials and methods

### Yeast strains and plasmids

*Saccharomyces cerevisiae* BY4741-based strains (MATa, *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) were purchased from EUROSCARF (University of Frankfurt, Germany). Their accession numbers and relevant genotypes are: Y00000 (wild type), Y14311 (*snf1Δ*), Y04574 (*sip2Δ*), Y04615 (*pde1Δ*), Y01657 (*pde2Δ*), Y03967 (*reg1Δ*), and Y04107 (*stm1Δ*). The yeast strains Σ1278b (MATa, *ura3-52*) and Szy9a (MATa *ura3-52*; *yak1::HYG*; *tpk1::G418*; *tpk2::NAT*; *tpk3::G418*) are also congenic except for the *tpk* and *yak1* mutations (Zurita-Martinez and Cardenas, 2005).

Ty3 frameshift (Ty3-FS) and Ty3 frame fusion (Ty3-FF) reporter plasmids, used to determine

PRF rates in various growth conditions in *S. cerevisiae*, are derivatives of the 2 μm-*URA3*-based shuttle vector pMB38 (Belcourt and Farabaugh, 1990). In the Ty3-FS vector, the Ty3 frameshift site (GCG-AGT-T) is fused to the *E. coli lacZ* gene in the +1 reading frame. Therefore, the amount of translation of the *lacZ* fusion protein in this expression vector depends on the efficiency of the +1 frameshift event in the +1 direction at the Ty3 frameshift site. In the Ty3-FF plasmid, the central nucleotide A of the frameshift site has been removed, putting the *lacZ* reporter gene into the zero reading frame. In this reporter vector, translation of the *lacZ* fusion protein does not require a frameshift (Farabaugh *et al.*, 1993). Plasmid pLG-Δ312 is also a 2 μm-*URA3*-based shuttle vector, which contains a *CYCI-lacZ* gene fusion (Guarente, 1983). It was used as the control gene fusion because the transcription of *CYCI* is regulated by glucose signalling (Zitomer *et al.*, 1979). Plasmids were transformed into competent yeast cells as described previously using the lithium acetate-PEG method (Gietz and Schiestl, 1995). The 2 μm *URA3*-based plasmids can be stably maintained with no drastic change in copy number in different yeast transformants under selective growth conditions (Farabaugh *et al.*, 1989).

### Growth conditions and β-galactosidase assays

*Saccharomyces cerevisiae* strains were cultivated in standard YPD (1% yeast extract, 2% Bacto peptone, 2% glucose) medium for transformation. The yeast transformants were selected on synthetic complete glucose medium lacking uracil (Sc-Ura, + 2% glucose) (Rose *et al.*, 1990).

Transformants of *S. cerevisiae* strains were grown to logarithmic stage in Sc-Ura medium, supplemented with various carbon sources (2% w/v) as indicated in relevant tables, and then harvested for β-galactosidase assays. For nutritional downshift experiments, yeast transformants were first grown to logarithmic phase in liquid Sc-Ura media supplemented with 2% glucose and then harvested by centrifugation and washed twice with 5 ml of sterile distilled water. The cultures were then resuspended in 5 ml of Sc-Ura medium supplemented with various carbon sources and further grown for 8 h prior to harvest for β-galactosidase assays. For glucose upshift experiments, yeast transformants were first grown to logarithmic stage in Sc-Ura medium

containing ethanol or glycerol and lactate as sole carbon sources, then glucose was added to these cultures at repressing (2%) concentrations and further incubated for 5 h.

Yeast cells were harvested by centrifugation at the end of the growth periods, washed with 1 ml of sterile distilled water, and then re-suspended in 200  $\mu$ l of yeast cell breaking buffer and permeabilized by addition of 20  $\mu$ l of 0.1% SDS and 20  $\mu$ l of chloroform (Guarente, 1983).  $\beta$ -Galactosidase assays were done in triplicate; units are given in nmol of ONPG (2-nitrophenyl  $\beta$ -D-galactopyranoside) cleaved per minute per mg of protein. Protein concentrations were determined as described (Lowry et al., 1951).

Frameshift rates were calculated as the percentage of the ratio of  $\beta$ -galactosidase activities expressed from the Ty3-FS plasmid to the  $\beta$ -galactosidase activities expressed from the Ty3-FF plasmid in the yeast transformants. Yeast cultures were grown in triplicate. All experiments were repeated at least once under the same growth conditions. Standard deviations in these assays were less than 15%.

## Results

### The glucose signalling pathway affects the Ty3 programmed ribosomal frameshifting

In order to investigate whether glucose signalling affects the PRF frequency in Ty3, haploid wild-type yeast transformants that contained a Ty3 frameshift (Ty3-FS), a Ty3 frame fusion (Ty3-FF) or a control gene fusion (*CYC1-lacZ*) were grown in

Sc-Uracil medium supplemented with 2% glucose or alternative carbon sources such as galactose, sucrose, ethanol or glycerol and lactate. The PRF frequency for Ty3 in yeast cells grown in glucose medium was 4.4%, similar to values reported previously for these standard conditions (Farabaugh et al., 1993). However, our results indicated that, when the yeast cells were grown in alternative carbon sources, the frameshift frequency changed depending on the carbon source. Glycerol–lactate-grown yeast cells showed the highest increase (2.8-fold) in the PRF frequency relative to glucose-grown yeast cells (Table 1). The increase in the PRF frequency was less in galactose or sucrose-grown yeast cells, increasing 1.8-fold in galactose-grown yeast cells and 1.5-fold in sucrose-grown yeast cells (Table 1). Unexpectedly, when the yeast cells were grown in ethanol containing growth medium, PRF frequency decreased to 1.4%. The difference in the PRF frequency between glycerol–lactate and ethanol grown yeast cell is approximately 10-fold (Table 1).

Moreover, in a carbon source downshift experiment in which glucose-grown yeast transformants were transferred to the glycerol/lactate medium, the PRF frequency increased 1.8-fold. When glucose was added to the glycerol–lactate-grown yeast cells, PRF frequency decreased 3-fold (to 4.2%) (Table 2). However, when glucose was added to ethanol-grown yeast transformants, PRF frequency increased approximately 3-fold and rose to standard wild-type level (Table 2). To ensure that the cells were responding as expected to changes in carbon source, we measured the transcription of the control gene fusion *CYC1-lacZ* and found

**Table 1.** Carbon sources affect the PRF frequency in Ty3 in *S. cerevisiae* strain

Gene expression	Carbon sources				
	Glucose	Glycerol/lactate	Galactose	Sucrose	Ethanol
% FS	4.4 $\pm$ 0.3	12.3 $\pm$ 2.0	8.6 $\pm$ 1.0	6.6 $\pm$ 0.8	1.4 $\pm$ 0.1
<i>CYC1-lacZ</i>	200 $\pm$ 5	11200 $\pm$ 340	1025 $\pm$ 43	520 $\pm$ 14	4960 $\pm$ 85

**Table 2.** Glucose shifts affects the PRF frequency in Ty3

Gene expression	Carbon sources shifts		
	Glucose to glycerol/lactate	Glycerol/gactate to glucose	Ethanol to glucose
% FS	8.0 $\pm$ 0.3	4.2 $\pm$ 0.4	4.0 $\pm$ 0.5
<i>CYC1-lacZ</i>	687 $\pm$ 23	2200 $\pm$ 74	1235 $\pm$ 62

expected increases of up to 56-fold in alternative carbon sources relative to growth in glucose (Table 1). In addition, addition of high levels of glucose (2%) to the yeast cells growing on alternative carbon sources resulted in rapid repression of *CYC1* transcription as expected (Table 2). Nonetheless, expression level of the *CYC1-lacZ* gene fusion in glycerol–lactate-grown yeast cells is much higher than for yeast cultures shifted to glycerol–lactate from the glucose medium (Tables 1 and 2). This difference in the *CYC1-lacZ* transcription may result from the differential localization of Snf1p in steady state growth of yeast cells in glycerol–lactate medium as explained by Vincent *et al.* (2001).

These data show that the efficiency of PRF is sensitive to the carbon source in which the cells are grown, suggesting a possible link between glucose signalling and frameshifting.

### Snf1p and PKA regulate Ty3 programmed ribosomal frameshifting

These initial results indicate that glucose signalling has a significant effect on the regulation of PRF in Ty3. The protein kinases Snf1p and cAMP-PKA are known to be the major factors regulating diverse metabolic events by carbon sources in *S. cerevisiae*. Having shown that glucose signalling regulates the PRF frequency in Ty3, we tested whether Snf1p and PKA were involved in the regulation of the Ty3 frameshift frequency. First, we used mutant strains in which PKA or Snf1p is constitutively active. Reg1p is the negative regulator of Snf1p kinase responsible for reduced activity in glucose-grown cells. In a *reg1Δ* mutant, Snf1p activity is constitutively high (Ludin *et al.*, 1998) and we found that Ty3 PRF in a *reg1Δ* mutant was nearly 2-fold higher than in wild-type yeast grown in glucose medium (Table 3). The high-affinity cAMP phosphodiesterase

Pde2p is a negative regulator of PKA activity; the increased levels of cytoplasmic cAMP in a *pde2Δ* mutant cause elevated PKA activity (Park *et al.*, 2005) and we found that Ty3 PRF in a *pde2Δ* mutant was nearly 2-fold higher than wild type (Table 3). By contrast, a *pde1Δ* mutant lacking low-affinity cAMP phosphodiesterase Pde1p has no effect on the cytoplasmic cAMP levels (Park *et al.*, 2005) and we found that Ty3 PRF is unchanged from wild type (Table 3). As expected, expression of the control *CYC1-lacZ* gene fusion was increased relative to wild type more than 3-fold in the *reg1Δ* mutant strain grown in glucose, demonstrating that Snf1p is constitutively active in this mutant yeast strain. In addition, *pde1Δ* and *pde2Δ* caused no significant change in expression of the *CYC1-lacZ* reporter.

### Activation of Ty3 programmed frameshifting requires Sip2-dependent Snf1 activity

Ty3 PRF frequency increased 2-fold in glucose-grown *reg1Δ* mutant yeast cells and 3-fold in glycerol–lactate-grown wild-type yeast cells: two conditions that result in elevated Snf1 kinase activity. Furthermore, in cells grown in ethanol, which causes sequestration of Snf1p in the vacuole, Ty3 PRF frequency decreased 3-fold. These results suggest that cytoplasmic Snf1 kinase may be necessary for stimulation of PRF in Ty3 in response to alternative carbon sources. To test this hypothesis, we measured the effects of *snf1Δ* and *sip2Δ* deletions on PRF efficiency. Sip2p, one of the β-subunits of Snf1p, promotes cytoplasmic localization and stimulates the kinase activity of Snf1p (Vincent *et al.*, 2001). *snf1Δ* and *sip2Δ* mutants are each viable; although *sip2Δ* cells grow in alternative carbon sources, *snf1Δ* cells do not. To test the effect of Snf1p on Ty3 PRF, *snf1Δ* or *sip2Δ* mutant yeast strain was grown in glucose medium to logarithmic stage and then shifted to glycerol–lactate medium for 8 h. In wild-type cells, this nutritional shift resulted in a 1.8-fold increase in PRF (Table 2), but in *snf1Δ* or *sip2Δ* mutant cells there was no increase in the frameshift frequency when these strains were grown in glycerol and lactate medium (Table 4). However, when the *sip2Δ* mutant yeast strain was grown in ethanol medium, frameshift frequency decreased to 1.8%, as in the wild-type strain. Ethanol-induced localization of the Snf1p kinase to the vacuole, which occurs both in the wild-type strain or a

**Table 3.** Protein Kinase A and Snf1 involves in the regulation of PRF in Ty3

Gene Expression	<i>S. cerevisiae</i> strains*			
	wild type	<i>Δpde1</i>	<i>Δpde2</i>	<i>Δreg1</i>
% FS	4.4 ± 0.3	4.6 ± 0.2	7.1 ± 0.5	8.4 ± 0.2
<i>CYC1-lacZ</i>	200 ± 5	260 ± 9	220 ± 14	790 ± 32

\*Yeast strains were grown in Sc-Uracil medium supplemented with 2% glucose.

**Table 4.** Snf1 and Sip2 affect carbon source dependent regulation of PRF in Ty3

Gene expression	$\Delta snf1$		$\Delta sip2$		
	Glucose	Gly/lact <sup>a</sup>	Glucose	Gly/lact	Ethanol
% FS	3.7 ± 0.6	4.2 ± 0.5	4.5 ± 0.4	4.1 ± 0.8	1.8 ± 0.2
<i>CYC1-lacZ</i>	168 ± 6	136 ± 14	265 ± 12	7400 ± 390	8500 ± 320

<sup>a</sup> $\Delta snf1$  mutants grown in glucose medium to log stage first, then shifted to glycerol and lactate medium for 8 h.

*sip2*Δ strain, should reduce cytoplasmic Snf1 activity and also reduces Ty3 PRF, consistent with our hypothesis (Table 4). The results obtained from *reg1*Δ, *snf1*Δ and *sip2*Δ mutants strongly suggest that the cytoplasmic Snf1p-Sip2p complex stimulates Ty3 PRF in response to alternative carbon sources (Table 4).

Expression of the control gene fusion *CYC1-lacZ* was not activated in  $\Delta snf1$  mutants when the yeast cells were shifted to glycerol–lactate medium. However, in  $\Delta sip2$  mutants, the expression was regulated as in the wild type. This result suggests that the nuclear functions of activated Snf1 kinase are not affected by  $\Delta sip2$  mutations (Table 4).

#### Regulation of Ty3 programmed ribosomal frameshifting requires protein kinase A

A significant increase in the Ty3 frameshift frequency in  $\Delta pde2$  mutant yeast cells indicated that PKA may be involved in the activation of Ty3 frameshifting. In addition, increase in the Ty3 PRF frequency upon addition of glucose to ethanol-grown yeast cells further supports the idea that activation of PKA and inactivation of Snf1p complex upon glucose addition regulates the PRF frequency in Ty3. Alternatively, it was possible that increased cAMP might regulate Ty3 PRF by a pathway not involving PKA. To test the role of cAMP-dependent PKA in the activation of Ty3 frameshift, we quantified Ty3 PRF in a strain lacking the three PKA structural genes, *tpk1*Δ, *tpk2*Δ, and *tpk3*Δ. Because PKA is essential, the triple deletion mutant is inviable; viability can be restored by deletion of the *YAK1* gene, a non-essential gene encoding another kinase involved in the glucose response. This mutant was derived from the *S. cerevisiae* strain  $\Sigma 1278b$  background, which has an intrinsically high level of cAMP and hence very high levels of PKA activity (Stanhill et al., 1999). This higher level may explain why the PRF frequency (14%) for the  $\Sigma 1278b$  strain grown in glucose is 3.1-fold higher

than in the S288c-based BY4741 series of wild-type yeast strains used in other experiments (Table 5). In the absence of PKA activity in the *tpk1*Δ *tpk2*Δ *tpk3*Δ *yak1*Δ mutant strain, PRF was reduced 4.7-fold (to 2.9%), indicating that PKA is involved in activating Ty3 frameshifting (Table 5).

Expression of control gene fusion *CYC1-lacZ* was similar in the  $\Sigma 1278b$  wild type and the *tpk1*Δ *tpk2*Δ *tpk3*Δ *yak1*Δ deletion mutant. However, the expression level of *CYC1-lacZ* gene fusion in  $\Sigma 1278b$  strain is much higher than the glucose-grown BY series of yeast strains. Transcription of *CYC1* is regulated by three distinct mechanisms. These are glucose repression, respiration status of the yeast cells and haem metabolism (Hörtner et al., 1982). The major transcriptional activators of *CYC1* are the Hap activator complex (Hap2/3/4/5) and Hap1p. Genome-wide analysis of  $\Sigma 1278b$  strain showed that many transcription factors (such as Hap4p, Snf11p, Spt6p) and *CYC1* itself are overexpressed in this strain (Jin et al., 2008). Hence we think that the high-level expression of *CYC1-lacZ* gene fusion in  $\Sigma 1278b$  strain is one of the intrinsic features of this strain, in addition to its well-known hyperactive Ras2/cAMP pathway and increased pseudohyphal growth.

#### Regulation of Ty3 programmed ribosomal frameshifting requires the ribosome-associated protein Stm1p

Stm1p is a highly abundant ribosome-associated protein that may regulate translation in response

**Table 5.** Protein kinase A activates the PRF in Ty3

Gene expression	<i>S. cerevisiae</i> strains	
	$\Sigma 1278b$	$\Delta tpk$ , $\Delta yak1$
% FS	14.0 ± 2.0	2.9 ± 0.5
<i>CYC1-lacZ</i>	7100 ± 300	5300 ± 340

Yeast strains were grown in Sc-Uracil medium supplemented with 2% glucose.

to nutrient deprivation, perhaps at the level of translation elongation (Van Dyke *et al.*, 2006, 2009). Because Snf1p and Tpk1p phosphorylate Stm1p (Ptacek *et al.*, 2005), we wanted to analyse the effects of Stm1p on the regulation of PRF in Ty3.

When *stm1Δ* mutant yeast transformants were grown in glucose medium, Ty3 PRF was slightly higher than the normal wild-type yeast strain (Table 6). However, contrary to the wild-type yeast strain, Ty3 PRF in Ty3 did not change significantly when the *stm1Δ* mutant yeast cells were grown in glycerol and lactate medium (Table 6). However, when the *stm1Δ* mutant yeast cells were grown in ethanol medium, PRF frequency decreased to 1.2% as in the wild-type yeast strain. This result indicated that while Stm1p is essential for the Snf1p kinase dependent activation of PRF frequency in Ty3, it is not required for vacuolar localization of Snf1p in ethanol-grown yeast cells (Table 6). By contrast, expression of the *CYCI-lacZ* reporter was regulated in a carbon source-dependent manner in *stm1Δ* mutant yeast strain. This result indicates that Stm1p is not involved in the Snf1p-dependent regulation or derepression of glucose-repressed genes such as *CYCI*.

## Discussion

The molecular mechanisms of +1 or -1 PRF have been well-characterized in many viral and cellular genes (Farabaugh, 1996; Namy *et al.*, 2004; Brierley and Dos Ramos, 2006). However, the effects of different signal transduction pathways on the PRF frequency have not been elucidated yet. In this study we have shown that glucose-signalling regulates the PRF frequency in Ty3 in *S. cerevisiae*. Growth on several alternative carbon sources resulted in significant changes in PRF, consistent with the concept that PRF is regulated by one or more glucose-dependent signalling systems. We

provide genetic evidence indicating that activation of the protein kinases Snf1p and PKA controls the PRF frequency in Ty3. We also demonstrated that the ribosome-associated Stm1 protein is necessary for stimulation of Ty3 PRF by alternative carbon sources. Since Stm1p is a phosphorylation target of both Snf1p and the Tpk1p isozyme of PKA in yeast, we suggest that the phosphorylation of Stm1p may be the mechanism by which these kinases stimulate increased Ty3 PRF.

When released from glucose repression by growth on alternative carbon sources, Snf1p kinase regulates many metabolic pathways by phosphorylating various target proteins. We believe that the Snf1p kinase directly acts on the translation elongation complex in the cytoplasm. Our results clearly show that stimulation of PRF by Snf1p in alternative carbon sources requires the Sip2 protein. Snf1p is the alpha subunit of heterotrimeric G proteins that forms complexes with a single gamma subunit, Snf4p, and three alternative beta subunits, Sip1p, Sip2p and Gal83p. All three Snf1p complexes localize to the cytoplasm during growth on glucose as carbon source. During growth on alternative carbon sources two of the complexes shift to new cellular locations. Gal83p-associated Snf1p shifts to the nucleus in alternative carbon sources and mediates its transcriptional effect (Vincent *et al.*, 2001). Although overexpression of Sip1p can complement the transcriptional defect of a *gal83Δ* mutant (Mylin *et al.*, 1994), Sip1p-associated Snf1p is not normally nuclear but instead after a shift to ethanol medium it localizes to the vacuole (Vincent *et al.*, 2001); the purpose of this localization is unclear. A significant decrease in the PRF frequency in ethanol-grown yeast cells further supports the involvement of cytoplasmic Snf1p in the regulation of PRF in Ty3. The Snf1p-Sip2p complex remains cytoplasmically localized in all carbon sources. We have demonstrated that, on alternative carbon sources, transcription of *CYCI* is derepressed to nearly

**Table 6.** Ty3 frameshift efficiency in the *Δstm1* mutant *S. cerevisiae* strain grown in different carbon sources

Gene expression	Carbon sources				
	Glucose	Gly/lact	Galactose	Sucrose	Ethanol
% FS <sup>a</sup>	6.0 ± 0.1	5.0 ± 1.0	4.5 ± 0.5	5.4 ± 0.3	1.2 ± 0.1
<i>CYCI-lacZ</i>	260 ± 7	10940 ± 840	830 ± 25	500 ± 33	3000 ± 137

<sup>a</sup>Frameshift frequency in the glucose grown wild type yeast strain is 4.4 ± 0.3 %.

wild-type levels in  $\Delta sip2$  mutant, indicating that Sip2p-associated Snf1p is not required for the nuclear functions of Snf1p. Hence Sip2 may be essential for the Snf1p-dependent increase in Ty3 PRF, which occurs during cytoplasmic translation.

Adding glucose to the yeast cultures growing in non-fermentable carbon sources causes a rapid but transient increase in the cytoplasmic level of cAMP, which activates PKA. It is known that the PKA acts on Snf1p complex and regulates its activities (Hedbacker *et al.*, 2004). Hence it appears that the Snf1p and PKA control the PRF in an interdependent manner. PKA may exert its effect on the PRF, either directly by acting on elongation complex or by acting on Snf1p or both.

The stimulation of Ty3 PRF by Sip2p-associated Snf1p requires the well-characterized Reg1p-Glc7p mechanism. Reg1p is the regulatory subunit of the Glc7p type 1 serine/threonine protein phosphatase. In the presence of glucose, Reg1p directs Glc7p to Snf1p where it deactivates it by dephosphorylating threonine 210 (Ludin *et al.*, 1998). In a *reg1* $\Delta$  mutant strain, Snf1p is constitutively active. We find that a *reg1* $\Delta$  mutant has constitutively elevated levels of Ty3 PRF suggesting that stimulation of Ty3 PRF requires Snf1p to phosphorylate one or more protein targets.

We have also found that Ty3 PRF is stimulated by at least one of the three isozymes of cAMP-dependent protein kinase A (PKA), Tpk1p, Tpk2p or Tpk3p. Elimination of the high-affinity cAMP phosphodiesterase Pde2p results in a greater than 2-fold increase in cAMP level (Park *et al.*, 2005); in a *pde2* $\Delta$  mutant Ty3 PRF is elevated 1.6-fold in glucose medium. Moreover, in a *tpk1* $\Delta$  *tpk2* $\Delta$  *tpk3* $\Delta$  strain, which is devoid of any PKA activity, PRF is decreased 4.7-fold relative to a congenic wild-type strain. Therefore, the efficiency of Ty3 PRF is sensitive to the level of active PKA, suggesting that PKA may also stimulate PRF by phosphorylating some protein target.

The Snf1p and PKA kinases have many cellular targets. We have investigated the role of one such target, the Stm1p protein, which is phosphorylated by both Snf1p and the Tpk1p isozyme of PKA (Ptacek *et al.*, 2005). Stm1p is an abundant non-essential ribosome-associated protein required for response to nutrient deprivation that appears to function at the level of translation elongation (Van Dyke *et al.*, 2006). Stm1p was an obvious candidate for the protein responsible for mediating

the effect of the two kinases on Ty3 PRF and we found that a strain lacking Stm1p showed no increase in Ty3 PRF on any of the alternative carbon sources tested. This suggests that phosphorylation of Stm1p by Snf1p or PKA may be required for the increase in PRF seen in alternative carbon sources.

It is already known that Stm1p is a phosphoprotein and required for the TOR pathway (Ptacek *et al.*, 2005; Van Dyke *et al.*, 2006). Stm1p is involved in the regulation of eEF3, a yeast-specific elongation factor that facilitates eEF1 $\alpha$ -dependent binding of aminoacyl-tRNA to the ribosomal A-site in *S. cerevisiae* (Van Dyke *et al.*, 2009). Therefore, it appears that Snf1p and PKA may act on translation elongation by modulating the activities of the ribosome-associated protein Stm1p. In addition to Snf1p, PKA and Stm1p, we have shown that Asc1p is also involved in the regulation of the PRF frequency in Ty3 (data not given). Asc1p is one of the subunits of the glucose sensor Gpr1p. Moreover, Asc1 is also associated with ribosomes (Gerbasí *et al.*, 2004; Zeller *et al.*, 2007).

Stimulation of Ty3 PRF frequency during shifts from glucose to alternative carbon sources occurs concomitantly with strong translational repression induced by glucose depletion. Translation is reduced up to 95% in as little as 5 min following removal of glucose (Ashe *et al.*, 2000) and is associated with mRNAs accumulating in P bodies (Ashe *et al.*, 2000; Teixeira *et al.*, 2005). This repression does not occur after withdrawal of alternative carbon sources like galactose or sucrose. Moreover, repression and P body association of mRNAs during glucose withdrawal do not occur in cells lacking Reg1p, PKA or Stm1p protein (Balagopal and Parker, 2009). Balagopal and Parker (2009) argue that the role of Stm1p in this response is, as a ribosome-bound protein, to promote ribosomal stalling by blocking translation elongation at some specific step of the elongation cycle. Recently, they showed that Stm1p inhibits *in vitro* translation by blocking elongation by 80S ribosomes (Balagopal and Parker, 2011). However, glucose removal induces transient repression, lasting about 2 h after cells are transferred from glucose to an alternative carbon source (Ashe *et al.*, 2000) whereas the Ty3 PRF effect is a long-term response to glucose withdrawal after the cells have adapted to growth on the alternative

carbon source. It is conceivable that Stm1 has different functional roles in the transient and long-term response to the carbon source shift.

It is known that the frameshift efficiency and the synthesis of polypeptides are directly proportional to the formation of viral particles in retroviruses and retroviral-like elements (Menees and Sandmeyer, 1996; Shehu-Xhilaga *et al.*, 2001; Gareiss and Miller, 2009). Menees and Sandmeyer (1996) also found that Ty3 transposition decreases dramatically in certain growth conditions such as high temperature and growth in ethanol and that, although the transcription of Ty3 mRNA is not affected, the synthesis of Pol3 polypeptides (integrase and reverse transcriptase) is undetectable in these conditions. Our demonstration that Ty3 PRF is drastically reduced when cells are transferred to ethanol suggests that this effect may be the cause of the decline in Pol3 expression and in Ty3 transposition. More generally, the response of PRF to changes in cellular physiology suggests that programmed frameshifting may be used as a sensor of the state of the host cell by retrotransposons in yeast and by extension by retroviruses and other metazoan viruses. Hence manipulation of signal transduction pathways that influence PRF could cause imbalances in Gag/Pol ratio and therefore interfere with viral propagation. Such signal transduction systems may provide targets for development of alternative strategies to interfere with propagation of retroviruses and retroviral-like elements.

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