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The repressor Rgt1 and the cAMP-dependent protein kinases control the expression of the *SUC2* gene in *Saccharomyces cerevisiae*

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ABSTRACT

Background: A low level of glucose is required for maximal transcription of the *SUC2* gene in *Saccharomyces cerevisiae*. Although the repressor Rgt1 binds the *SUC2* promoter in gel-shift assays, it has been reported that Rgt1 has only minimal effects on *SUC2* expression. Rgt1 acts together with Mth1 to repress the *HXT* genes encoding glucose transporters, and the release of Rgt1 from some *HXT* promoters requires cAMP-dependent protein kinase (PKA) activity.
Methods: The genes *RGT1* and *MTH1* have been disrupted and the *SUC2* promoter modified in several *S. cerevisiae* backgrounds. Yeast cells were grown in different carbon sources in the presence or absence of 0.1 or 2% glucose, and invertase was assayed in whole cells.
Results: Galactose, glycerol or ethanol hindered invertase induction by low glucose, but lactate did not. During growth in lactate, deletion of *RGT1* or *MTH1* caused a marked increase in invertase levels, and elimination of the Rgt1-binding site in the *SUC2* promoter caused also invertase induction. PKA activity decreased invertase levels in cells growing in lactate, and increased them during growth in lactate + 0.1% glucose.
Conclusions: The low level of expression of *SUC2* in the absence of glucose is mainly due to repression by the Rgt1-Mth1 complex. Repression is dependent on PKA activity, but not on any specific Tpk isoenzyme.
General significance: The results show that previously overlooked regulatory elements, such as Rgt1 and Tpks, participate in the control of *SUC2* expression in *S.cerevisiae*.
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1. Introduction

The transcriptional profile of the yeast *Saccharomyces cerevisiae* is strongly affected by glucose, the expression levels of about 40% of its genes being up- or down-regulated when glucose becomes available to yeast cells growing on non-fermentable carbon sources [1,2]. Among the genes regulated by glucose *SUC2*, encoding invertase, has been considered a model to study the mechanisms underlying the repression caused by glucose [3,4]. The elements responsible for the repressing effect of high glucose (2%) on *SUC2* have been thoroughly investigated and the role of many regulatory proteins clearly established [5–9]. Although *SUC2* is repressed by 2% glucose, it requires a low concentration of glucose (below 0.2%) to be induced [10]. A similar pattern of expression had been described for genes such as *HXT2* or *HXT4*, which encode glucose transporters [11], and it has been shown that in the presence of low glucose the DNA-binding protein Rgt1 is removed from the promoters of the *HXT* genes, relieving its repressing effect [12].

Although Rgt1 can bind to the *SUC2* promoter at a consensus TNNTCCG site [13], it has been reported that in a *rgt1* mutant *SUC2* expression remains low in the absence of glucose [10]. Moreover, *SUC2* was not identified among the significant physiological targets of Rgt1 [14]. These results suggested that the activation of the transcription of *SUC2* and of the *HXT* genes by a low concentration of glucose is mediated by different mechanisms. We noticed, however, that in previous studies expression of *SUC2* in the absence of glucose was measured in cells grown on glycerol or galactose [10] and this could have influenced the results, since glycerol and galactose are able to repress *SUC2* (J.M. Gancedo preliminary experiments). We have, therefore, reexamined a possible role for Rgt1 in the control of *SUC2* expression, using a carbon source that does not repress this gene. Expression of *SUC2* has been assessed by measuring secreted invertase. To investigate whether Rgt1 has a direct effect on the *SUC2* promoter, or may also act indirectly, we compared invertase levels in mutants lacking either Rgt1 or the Rgt1-binding site in the *SUC2* promoter. We evaluated also the role of Mth1 on invertase expression, since the interaction of Mth1 with Rgt1 allows its binding to DNA [15].

As it has been shown that the cAMP-dependent protein kinases are required to relieve glucose repression of genes *HXT1* and *HXT3* by Rgt1 [12], we examined also whether these kinases are needed for the induction of *SUC2* by low glucose.

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Table 1
Strains used in this work.

Strain	Relevant genotype	Source/reference
CJM534	*MAT***a** ade2-*1 his3-11,15 trp1 ura3-52*	This laboratory
CJM561	*MAT***a** *ade2-1*	This laboratory
CJM922	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1 ura3-52 rgt1::LEU2*	This study
CJM926	*MAT***a** *SUC2-33 ade2-1 his3-11,15 trp1 ura3-52*	This study
CLF61	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1 ura3-52 mth1::TRP1*	[21]
MB23	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1 ura3-52 tpk2::HIS3 tpk3::URA3*	[22]
MB13	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1 ura3-52 tpk1::LEU2 tpk3::URA3*	[22]
MB12	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1 ura3-52 tpk1::LEU2 tpk2::HIS3*	[22]
CJM553	*MAT***a** *ade2-1 his3-11,15 trp1 msn2::HIS3 msn4::TRP1*	This laboratory
CJM549	*MAT***a** *ade2-1 his3-11,15 trp1 ura3-52 msn2::HIS3 msn4::TRP1 tpk1::URA3 tpk2::HIS3 tpk3::TRP1*	This laboratory
CJM564	*MATα ade2-1 yak1::KanMX*	This laboratory
CJM1066	*MAT***a** *ade2-1 his3-11,15 trp1 ura3-52 msn2::HIS3 msn4::TRP1 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 rgt1::LEU2*	This laboratory
W *yak1 tpk1/2/3*	*MATα ade2-1 his3-11,15 leu2-3,112 trp1 ura3-52 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2*	[23]
CJM1083	*MATα ade2-1 his3-11,15 leu2-3,112 trp1 ura3-52 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2 rgt1::ADE2*	This laboratory
CJM319	*MATα ade2-1 his3-11,15 trp1 ura3-52 hxk2::LEU2*	This laboratory
DBY1315	*MATα leu2-3,112 lys2-801 ura3-52*	[24]
CJM866	*MATα leu2-3,112 lys2-801 ura3-52 rgt1::LEU2*	This study
CJM991	*MATα SUC2-33 leu2-3,112 lys2-801 ura3-52*	This study
CJM957	*MATα leu2-3,112 lys2-801 ura3-52 mth1::URA3*	This study
MSY230	*MAT***a** *his4-912δ lys2-128δ leu2Δ1 ura3-52*	[19]
CJM868	*MAT***a** *his4-912δ lys2-128δ leu2Δ1 ura3-52 rgt1::LEU2*	This study
MSY233	*MAT***a** *SUC2-33 his4-912δ lys2-128δ leu2Δ1 ura3-52*	[19]
CJM956	*MAT***a** *his4-912δ lys2-128δ leu2Δ1 ura3-52 mth1::URA3*	This study

2. Materials and Methods

2.1. Yeast strains

Yeast strains used in this study are listed in Table 1. Strains CJM534 and CJM561 were derived from strain W303-1A [16], strains CJM553 and CJM549 from strains W*msn2msn4* [17] and W303 *tpk1/2/3 msn2msn4* [18], respectively, and strain CJM564 from *yak1::KanMX* (gift from C. Brocard) by substituting in the original strains the mutated genes (*leu2, his3, trp1, ura3*) by their wild-type alleles, as needed. In strains CJM922, CJM866, CJM868 and CJM1066, *RGT1* was interrupted by substituting the *Eco*RV$_{1026}$-*Eco*RV$_{2766}$ sequence of the ORF by the *LEU2* gene. In strain CJM1083, *RGT1* was interrupted by substituting the same sequence by the *ADE2* gene. In strains CJM957 and CJM956, *MTH1* was interrupted by substituting the *Afl*II$_{-131}$-*Nco*1$_{1376}$ sequence of the ORF by the *URA3* gene. To replace the wild-type *SUC2* gene by the *SUC2-33* allele, with modifications in the -442 to -431 sequence of the promoter [19], we proceeded as follows. The *SUC2* gene from strains CJM534 and DBY1315 was interrupted from *Nco*1$_{-384}$ to *Nco*1$_{962}$ with the *URA3* gene. The *SUC2::URA3* strains were transformed with the fragment -534 to 1107 from *SUC2-33* obtained by PCR from strain MSY233 and transformants were selected in plates with 5-fluorouracil [20]. The correct integration was checked by PCR. In strain CJM319 the complete reading frame of *HXK2* and its flanking sequences were replaced by the *LEU2* gene in the W303 background.

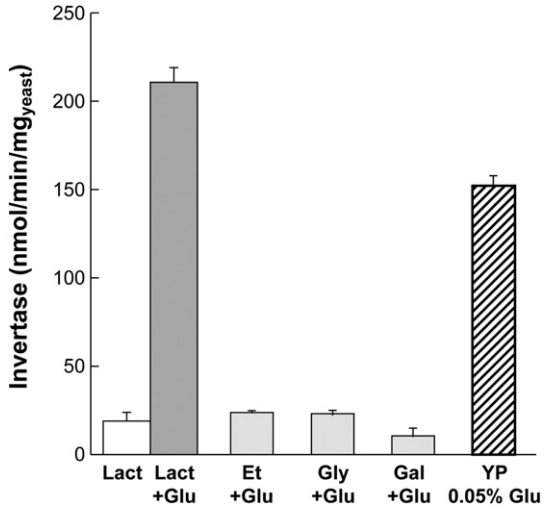


Fig. 1. Invertase activity from yeast cells grown on different carbon sources. *S. cerevisiae* strain CJM561 was grown in SC medium with the indicated carbon sources (Lact, 2% lactate; Glu, 0.1% glucose; Et, 2% ethanol; Gly, 3% glycerol; Gal, 2% galactose). The yeast was also grown in YPD and derepressed for 3 h in YP0.05% glucose. Invertase was measured as described in Materials and Methods, values are averages (± SD of the sample) of three independent cultures.

2.2. Culture conditions and enzymatic measurements

The yeasts were grown in a synthetic medium (SC dropout) [25] with the necessary supplements at a final concentration of 20 mg/l and with different carbon sources (2% lactate, 2% ethanol, 3% glycerol or 2% galactose) in the absence or presence of glucose (0.1% or 2%) and collected at the mid-log phase of growth. Invertase was tested as [26] using whole cells and measuring the glucose formed in the reaction with hexokinase and glucose-6P dehydrogenase; activity is expressed as nanomol/min/mg yeast (wet weight).

3. Results

3.1. Induction of invertase by low glucose in yeast growing in a synthetic medium

Two different transcripts originate from the *SUC2* gene, one of them encoding a constitutive cytoplasmic invertase, and the second one encoding a periplasmic invertase, whose levels are regulated by glucose [3]. We have tested invertase in whole cells, measuring therefore only the periplasmic, regulated form. To examine factors which affect the induction of invertase by low glucose, a prerequisite is to identify a carbon source which does not cause repression of invertase. Therefore we compared invertase levels in yeasts grown on different carbon sources in the presence of 0.1% glucose. Since the yeast extract used to prepare rich media contains some glucose, we used a synthetic medium for these experiments. In addition, we measured also invertase in yeast cells grown on YPD and incubated for a few hours in YP0.05% glucose, as this has been the standard method to determine derepressed levels of invertase [27].

As shown in Fig. 1, a low concentration of glucose (0.1%) induced high levels of invertase when added to lactate medium, similar to those measured after incubation in YP0.05% glucose, while invertase

activity was low in cultures grown with ethanol, glycerol or galactose together with 0.1% glucose. Repression by galactose or glycerol was also observed in strains with different genetic backgrounds (results not shown). Lactate, therefore, is a carbon source adequate for investigating the regulation by glucose of *SUC2*. In the following experiments, invertase has been measured in cells grown in lactate, lactate + 0.1% glucose, or lactate + 2% glucose to compare, in the different strains, basal levels of invertase with those induced by low glucose and those repressed by high glucose.

3.2. The regulatory circuit that involves Rgt1controls invertase levels

To investigate the role of Rgt1 on *SUC2* expression we measured invertase in sets of strains with different genetic backgrounds. We used a diversity of genetic backgrounds because preliminary experiments showed that the quantitative effects on invertase levels of the mutations studied depended on the strain tested. Each set of strains included a wild-type reference strain, a deleted *rgt1* mutant, a mutant ($SUC2_{mut}$) where the Rgt1 binding site of the *SUC2* promoter had been modified [19], and a deleted *mth1* mutant strain.

As shown in Fig. 2 the levels of invertase in cells grown in lactate in the absence of glucose increased markedly in all the *rgt1* mutant strains with respect to the corresponding wild-type yeasts. There are, however, quantitative differences between strains; the invertase activity may become as high as in the wild-type strain grown in the presence of low glucose (Fig. 2B) or remain significantly lower (Fig. 2A, C). In the *rgt1* mutants, the addition of low glucose to the lactate medium may have a slight effect on invertase activity or increase it 2–3fold depending on the genetic background.

In strains $SUC2_{mut}$, lacking the Rgt1-binding site in the *SUC2* promoter, invertase activity in the absence of glucose increases; in some cases the increase is close to that found in the *rgt1* strain (Fig. 2A, C), while in another genetic background (Fig. 2B) the increase is much lower. In all cases activity is further induced in the presence of low glucose and becomes similar to that of the corresponding wild-type strains.

Deletion of *MTH1* increased also the basal invertase levels in all backgrounds. Induction by low glucose was still observed in two strains (Fig. 2B, C), while in the commonly used W303 background, invertase activity was not affected by the presence of low glucose in the culture medium (Fig. 2A).

These results show that both Rgt1 and Mth1 play an important role in blocking transcription in the absence of glucose.

3.3. Influence of cAMP-dependent protein kinases on invertase levels

A possible role for cAMP signalling in the control of invertase levels has been investigated by different groups, using different mutants. In *cyr1* mutants, defective in adenylate cyclase, and therefore impaired in cAMP production, levels of invertase in cells grown in induction conditions were very low [28,29]. This suggests that activation of PKA is required for *SUC2* expression. In a $tpk1^{w}$ *bcy1Δ* mutant, in which the single cAMP protein kinase has a low activity unregulated by cAMP, due to the absence of the regulatory subunit Bcy1, induced and repressed levels of invertase are similar to those of a wild-type strain [30]. This indicates that regulated expression of *SUC2* does not depend on a cAMP responsive PKA. Since these results seem to some extent contradictory, we tried to approach the problem in a different way, using strains completely lacking PKA activity.

In *S. cerevisiae* there are three protein kinases, Tpk1, Tpk2 and Tpk3 able to interact with Bcy1, and activated when Bcy1 binds cAMP [31]. While a *tpk1 tpk2 tpk3* strain is not viable, different suppressor mutations have been identified that allow yeast growth in the absence of PKA activity. One of them, *msn2 msn4* [32], lacks the homologous transcription factors Msn2 and Msn4 activated in stress conditions [17,33]. Another suppressor mutation is the deletion of *YAK1*, encoding a protein kinase [34] involved in growth control [35]. To test the effects of the total absence of PKA activity on *SUC2* expression, we compared invertase levels in mutants *tpk1 tpk2 tpk3 yak1* and *tpk1 tpk2 tpk3 msn2 msn4* with those of a wild-type strain and of isogenic *yak1* and *msn2 msn4* strains. To investigate possible specific effects of the different Tpk isoenzymes, we measured also invertase in strains with a single Tpk each.

The yeast strains expressing only one Tpk isoenzyme showed a behaviour similar to that of the wild-type (Fig. 3A), although quantitative differences among the strains were observed. The strains with only Tpk1 or Tpk2 reached only about half of the induced levels of the wild-type, while in the strain with only Tpk3 the non-induced level was slightly higher than in the wild-type. In the absence of all three Tpks (Fig. 3B, C) the levels of invertase in yeast grown in lactate were high, pointing to the possibility that Tpks repress invertase in the absence of glucose. After growth in lactate with 0.1% glucose, invertase activity was reduced but the nature of the suppressing mutations affected the extent of the decrease. The decrease was 2-fold in the *msn2 msn4* background, while activity was completely lost in the *yak1* background. The control strain *msn2 msn4* behaved as the wild-type reference strain; in contrast, the *yak1* strain showed in the absence of glucose a higher

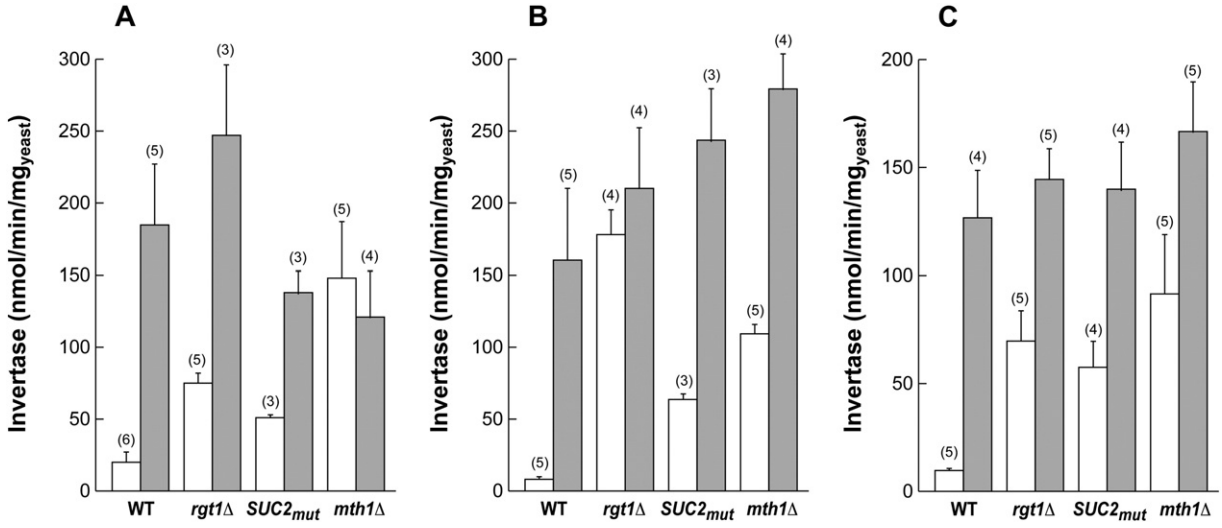
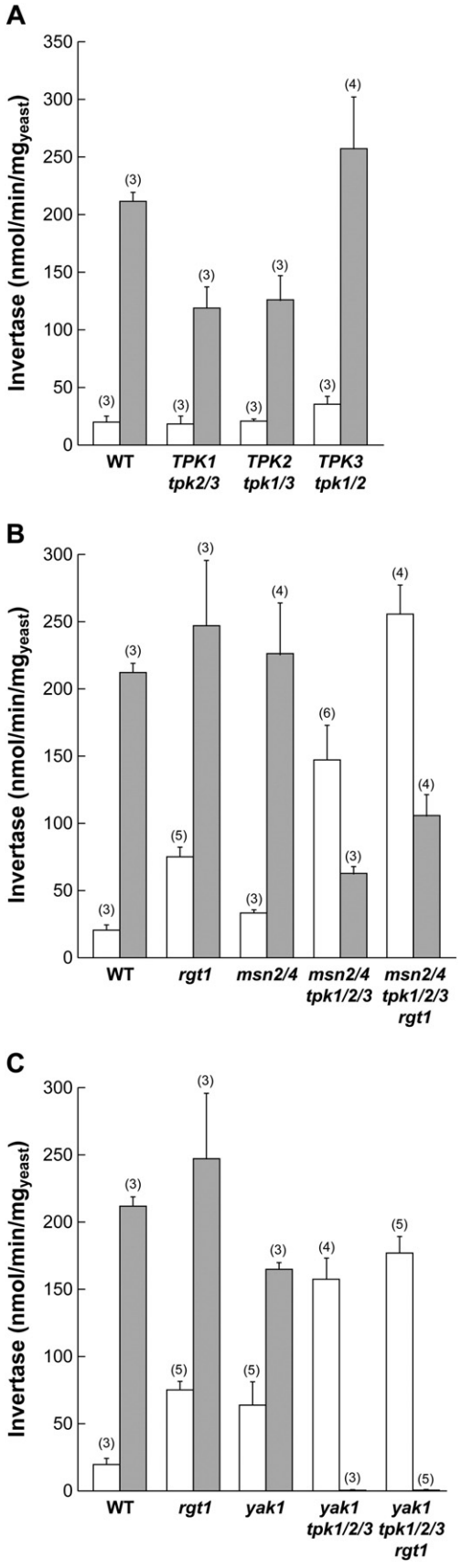


Fig. 2. Effect of mutations that affect the regulatory circuit involving Rgt1 on invertase levels. The different yeast strains were grown in lactate (▭) or lactate + 0.1% glucose (▬). Invertase was measured as described in Materials and Methods, activities are averages (± SD) of several independent cultures (number in brackets). A) W303 background (WT CJM534) B) DBY1315 background C) FY637 background (WT MSY230).

invertase activity than the wild-type, and while this activity increased in the presence of low glucose it did not reach that of the wild-type in these conditions. These results show that Tpks contribute to invertase induction in the presence of a low concentration of glucose. However, the effect of the absence of Tpks in *SUC2* expression in these conditions is much stronger in a *yak1* strain than in a *msn2 msn4* strain.

To check to what extent the lowered expression of invertase in the absence of Tpks during growth in low glucose is related to the operation of Rgt1, we have deleted the *RGT1* gene in strains *msn2 msn4 tpk1/2/3* and *yak1 tpk1/2/3*. As shown in Fig. 3B, C, the lack of Rgt1 caused an increase of about 70% in the activity of invertase both in the absence and presence of low glucose in the *msn2 msn4 tpk1/2/3* strain and had no effect in the *yak1 tpk1/2/3* strain. Therefore the decrease in invertase activity caused by low glucose in the strains without Tpks does not require Rgt1.



4. Discussion

It is well established that *SUC2* expression is high in media with low glucose and is strongly reduced in the presence of high glucose [4,10]. There is much less information on the effect of other carbon sources on invertase levels. Repression by xylose has been described [36], and we have shown in this work that invertase levels are repressed in media containing galactose, glycerol or ethanol together with a low level of glucose. In contrast, we have found that lactate is a carbon source which does not affect induction by low glucose (Fig. 1). It should be noted than in the lactate cultures the pH of the medium remains stable at about 5, while in the case of the other carbon sources the medium is poorly buffered and the pH drops at around 4. We have therefore measured invertase levels in cultures buffered at pH 5 with succinate. In this case the invertase activity in galactose, glycerol or ethanol together with 0.1% glucose was about 5-fold higher than in the unbuffered media, but did not reach the activity measured in the lactate medium. Repression by glycerol and galactose can explain an earlier report showing that invertase was only partially induced in a *rgt1* mutant grown on glycerol + galactose [10]. Similarly, the low level of invertase in a *mth1* mutant grown on ethanol [37] is consistent with the repression by ethanol. The use of lactate as a carbon source has allowed us to reexamine the induction of *SUC2* by low glucose and reach different conclusions.

The fact that mutations in the sequence of the single canonical Rgt1-binding site present in the *SUC2* promoter increased invertase levels during growth in the absence of glucose indicates that this site participates in the regulation of *SUC2* expression, likely by binding Rgt1. However, the lack of Rgt1 has a stronger effect on invertase levels in yeast grown in lactate than the elimination of the Rgt1-binding site in the *SUC2* promoter. We suggest, therefore, that in addition to its repressing role, depending on its binding to the *SUC2* promoter, Rgt1 may also play another, indirect, negative effect on *SUC2* transcription. The effect is most marked in the DBY1315 background, where invertase levels in the *rgt1Δ* mutant are similar in the presence or absence of low glucose (Fig. 2B). We may note that Rgt1 is required to fully induce the *HXT1* gene [38]; in this case it was proposed that Rgt1 acts as an activator of *HXT1* transcription by an unknown, indirect mechanism. Our observation that in two strains removal of Rgt1 is not sufficient to allow full expression of invertase in the absence of glucose (Fig. 2A, C) points to the existence of a further mechanism, independent of Rgt1, for regulating the expression of this enzyme in these conditions.

Repression of gene transcription through Rgt1 requires the presence of the paralogous proteins Mth1 or Std1 [39]. In the case of *SUC2*, as happens for some other genes [39], Std1 cannot fully substitute for Mth1, as shown by the fact that removal of Mth1 is sufficient to increase strongly *SUC2* expression in the absence of glucose (Fig. 2). Here, again,

Fig. 3. Invertase activity from yeast strains affected in the cAMP-dependent protein kinases. Mutants in the W303 background were grown in lactate (▭) or lactate + 0.1% glucose (▬), and invertase was measured as described in Materials and Methods; activities are averages (± SD) of several independent cultures (number in brackets). A) Effect of the expression of a single cAMP-dependent protein kinase. B) Effect of the lack of PKA activity in a *msn2msn4* background. C) Effect of the lack of PKA activity in a *yak1* background.

quantitative differences between strains were observed and, in a given genetic background, the lack of Rgt1 did not always cause the same increase in invertase activity as the lack of Mth1. In cases where the lack of Mth1 had a weaker effect on invertase levels than the lack of Rgt1, it is likely that Std1 is partially replacing Mth1; when invertase expression during growth in lactate is higher in the *mth1* mutant than in the corresponding *rgt1* strain it would appear that in this background Mth1 plays a repressing role independent of Rgt1.

We have found that the disruption of genes *RGT1* or *MTH1* in three different yeast backgrounds does not cause the same quantitative responses. The differences between the strains are not large, but they are reproducible. This is surprising as the three strains are closely related with the reference strain S288c [19,40,41]. It is interesting to note, however, that different *SUC* alleles (*SUC1-SUC5, SUC7*) are present in different *S. cerevisiae* strains [42]. Although only *SUC2* is active in the S288c strain, this strain carries suc^0 alleles at the other *SUC* loci. It could happen, therefore, that in derivatives from S288c one of these alleles could have been activated; in fact, in FL100 [43], a strain related with S288c [44], the only *SUC* gene active is *SUC7* [42]. Differences in the environment of the *SUC* gene are only one possible explanation for our results; while the strains diverged there could also have been some subtle changes in the corresponding regulatory circuits, as reported for those governing the adhesion phenotype in *S. cerevisiae* [45].

In this work we find that, in yeast strains lacking Tpks, invertase is highly expressed in the absence of glucose. There is not an obvious explanation for this observation, which contrasts with earlier reports on the requirement of Tpks for releasing Rgt1 from the promoters of the genes *HXK2* [46] or *HXT1* and *HXT3* [12]. A possibility would be that PKA activity is required for the formation of the Rgt1 repressing complex and/or its binding to the promoter of the gene. Since there is a single Rgt1 binding site in the *SUC2* promoter, it could be thought that for efficient binding Rgt1 requires the cooperation of another protein, phosphorylated by PKA. If such were the case, in the absence of PKA Rgt1 would not be able to repress *SUC2* transcription and invertase levels would be high. A candidate for a cooperating protein could be histone 3, as it has been reported to interact with Rgt1 [47] and could be a substrate for PKA. However the evidence is still weak, as the interaction was identified in a high throughput experiment and phosphorylation by PKA has not been demonstrated.

Another intriguing finding is the fact that, in mutants lacking Tpks, a low level of glucose causes a decrease in invertase levels in the *msn2 msn4* background, instead of the usual increase and a block of expression in the absence of Yak1. The effect of low glucose is still observed when the *RGT1* gene is interrupted, thus indicating that the repressing effect of glucose is not dependent on Rgt1. It is not clear, at present, how PKA may act to increase invertase levels in cells growing in the presence of low glucose.

Under repressing conditions, in yeasts grown in lactate + 2% glucose, invertase remains strongly repressed in all the mutants described above, activity being usually less than 1 nanomol/min/mg yeast and never higher than 3 nanomol/min/mg yeast (results not shown). These data are consistent with the fact that the repression circuit operating in high glucose does not require Rgt1, Mth1 or Tpks, but involves other elements, such as Mig1, Hxk2, Cyc8 or Tup1 [5–9].

Our results indicate that there are important similarities between the regulatory mechanisms which control the expression of *SUC2* and those described for the *HXT2* and *HXT4* genes [11], although in some cases there are small quantitative differences. While the expression of the glucose transport genes is strongly reduced in *hxk2* mutants, high levels of invertase have been reported in such mutants in the presence of glucose [5,37] and we have checked that in an *hxk2* mutant invertase activity is low during growth on lactate (27 nanomol/min/mg yeast) and increases 3- to 4-fold in the presence of low or high glucose, reaching 55–65% of the levels measured in the induced isogenic wild-type strain.

5. Conclusions

In the absence of glucose, expression of *SUC2* is repressed by Rgt1 and by Mth1. The main mechanism appears to be the binding of the Rgt1-Mth1 complex to a Rgt1-binding site in the *SUC2* promoter. In addition, depending on the genetic background, Rgt1 and Mth1 may also play indirect repressing roles. PKA activity decreases invertase levels in cells growing in the absence of glucose, and increases them during growth in the presence of low glucose.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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