New mutations of *Saccharomyces cerevisiae* that partially relieve both glucose and galactose repression activate the protein kinase Snf1

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Abstract

We isolated from *Saccharomyces cerevisiae* two mutants, *esc1-1* and *ESC3-1*, in which genes *FBP1*, *ICL1* or *GDH2* were partially derepressed during growth in glucose or galactose. The isolation was done starting with a triple mutant *pyc1 pyc2 mth1* unable to grow in glucose-ammonium medium and selecting for mutants able to grow in the non-permissive medium. HXT1 and HXT2 which encode glucose transporters were expressed at high glucose concentrations in both *esc1-1* and *ESC3-1* mutants, while derepression of invertase at low glucose concentrations was impaired. REG1, cloned as a suppressor of *ESC3-1*, was not allelic to *ESC3-1*. Two-hybrid analysis showed an increased interaction of the protein kinase Snf1 with Snf4 in the *ESC3-1* mutant; this was not due to mutations in *SNF1* or *SNF4*. *ESC3-1* did not bypass the requirement of Snf1 for derepression. We hypothesize that *ESC3-1* either facilitates activation of Snf1 or interferes with its glucose-dependent inactivation.

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Keywords: Glucose signalling; Catabolite repression; Snf1; Protein kinase; Pyruvate carboxylase; *MTH1*

1. Introduction

*Saccharomyces cerevisiae* is found in wine cellars and breweries. When fermentations start the yeast finds media rich in sugars that in time become depleted of them and enriched in ethanol. The presence of glucose and related hexoses has an important influence on the expression of many yeast genes and consequently on the metabolism of the organism. In the presence of these sugars, the expression of a large number of genes, mainly those encoding enzymes required to use alternative carbon sources, is repressed. This phenomenon, known as catabolite repression, has been studied in deep and a broad picture of it is emerging [1–3]. Central actors in the repression process are the Mig1 repressor and the Snf1 kinase complex that counteracts it and whose activity is necessary for derepression of glucose-repressed genes [2,4]. Glucose availability in the medium is assessed by the sensor proteins Snf3 and Rgt2 [5–7] that transmit information about the external situation to the internal cellular machinery. These sensors interact with the proteins Std1 and Mth1, relieving their repressive effect on the expression of the genes encoding glucose transporters [8,9]. In the search for new elements involved in glucose signalling in yeast, one strategy used has been the isolation of suppressors of the toxic effects of glucose on different glycolytic mutants [10–12]. A common result of these approaches has been the isolation of mutated forms of *MTH1* less sensitive to the presence of glucose in the medium [9,13]. A possible way to avoid the isolation of mutants affected in *MTH1* is to start the search of new mutants in a strain lacking *MTH1*. This is in principle feasible since deletion of *MTH1* does not produce an appreciable effect on growth or gene expression in different media [14]. We have constructed a triple mutant strain with disruptions in the genes *PYC1 PYC2* that en-
code isoenzymes of pyruvate carboxylase [15,16] and in the gene \textit{MTH1}, and used it to isolate new genes implicated in catabolite repression or glucose signalling. The rationale of our approach is based on the finding that \textit{pyc1 pyc2} mutants do not grow in a minimal medium with glucose as carbon source and ammonium as nitrogen source [15]. This behavior is due to the fact that in these conditions pyruvate carboxylase is the only enzyme able to produce oxaloacetate to replenish the tricarboxylic acid cycle, since glucose represses the alternative anaplerotic enzymes isocitrate lyase and malate synthase [17] (Fig. 1). Mutations that abolish catabolite repression would allow growth of \textit{pyc1 pyc2} mutants in glucose–ammonium medium because the genes encoding the enzymes of the glyoxylate cycle would be expressed. We report here the isolation and analysis of mutants that partially alleviate the repressive effect not only of glucose but also of galactose.

2. Materials and methods

2.1. Yeast strains, culture conditions and genetic methods

The \textit{S. cerevisiae} strains used in this work are shown in Table 1. Gene disruptions were done according to Rothstein [18]. To construct strain YCR006 an \textit{EcoRI–EcoRI} fragment from plasmid pMTH1::TRP1 [9] was introduced into strain YCR005, and to construct strains YCR012 and YCR013 a \textit{BamHI–BamHI} fragment from pUCl\textit{snf1D::KanMX4} (see below) was introduced into strains W303-1A and YCR011, respectively. Yeasts were grown at 30°C either in a rich medium (YP, 1% yeast extract, 2% peptone) or in a minimal medium (YNB, Difco) with 40 mM ammonium sulfate or aspartate and the adequate requirements. The indicated carbon sources were used unless indicated otherwise at 2%. Mutagenesis of strain YCR003 was done by ethyl methane sulfonate as in Lawrence [19]. After mutagenesis the cells were spread directly on minimal glucose–ammonium plates. Colonies appearing after 3–4 days were picked and purified on plates of the same composition. Crosses between strains, pre-sporulation and sporulation media and micromanipulation of spores were done by standard procedures. Transformation was done by the lithium acetate method [20].

2.2. Plasmids and library

Plasmid pUCl\textit{snf1D::KanMX4} contains an inner fragment of 537 bp of the \textit{SNF1} gene replaced by the \textit{KanMX4} selection marker [21]. Other plasmids used in this study were pSH18-18 (6\textit{lexAoplacZ}) and pLR1D1 (0\textit{lexAoplacZ}) [22], LexA-Snf1, LexA-Snf1K84R and Snf4-GAD [23], pACTII (GAD) [24], pEG202 (LexA) [25] and FBPI-lacZ [26].

The yeast genomic library GRF88, based in YCP50, was obtained from the ATCC collection (Rockville, MD, USA). PCR products were cloned in plasmid pGEM-T (Promega, Madison, WI, USA).

2.3. RNA extraction and Northern analysis

Total RNA was extracted from 50 mg cells (wet weight) with the Gibco BRL Trizol reagent (Invitrogen, Carlsbad,

<table>
<thead>
<tr>
<th>Strain</th>
<th>Name in text</th>
<th>Genotype</th>
<th>Origin or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1A</td>
<td>Wild-type</td>
<td>Mata ade2-1 his3-11,15 leu2-3,112 ura3-52 trpl-1</td>
<td>[42]</td>
</tr>
<tr>
<td>22BHL</td>
<td>\textit{pyc1 pyc2}</td>
<td>Mata ade2-1 his3-11,15 leu2-3,112 ura3-52 trpl-1 \textit{pycl::LEU2 pyc2::HIS3}</td>
<td>[12]</td>
</tr>
<tr>
<td>MJ002</td>
<td>\textit{pyc1 pyc2 mth1}</td>
<td>Mata ade2-1 his3-11,15 leu2-3,112 ura3-52 trpl-1 \textit{can1-100 mth1::URA3}</td>
<td>Opposite sex to MJL5 [9]</td>
</tr>
<tr>
<td>YCR003</td>
<td>\textit{pyc1 pyc2 mth1}</td>
<td>Mata ade2-1 his3-11,15 leu2-3,112 ura3-52 trpl-1 \textit{pycl::LEU2 pyc2::HIS3 mth1::URA3}</td>
<td>This work. From a cross 22BHL×MJ002</td>
</tr>
<tr>
<td>YCR005</td>
<td>\textit{pyc1 pyc2 mth1}</td>
<td>Mata ade2-1 his3-11,15 leu2-3,112 ura3-52 trpl-1 \textit{pycl::LEU2 pyc2::HIS3}</td>
<td>This work. From a cross 22BHL×MJ002</td>
</tr>
<tr>
<td>YCR006</td>
<td>\textit{pyc1 pyc2 mth1}</td>
<td>Mata ade2-1 his3-11,15 leu2-3,112 ura3-52 trpl-1 \textit{pycl::LEU2 pyc2::HIS3 mth1::URA3}</td>
<td>This work. Disruption of \textit{MTH1} with \textit{pMTH1::TRP1} in YCR005</td>
</tr>
<tr>
<td>YCR008</td>
<td>\textit{escl-1}</td>
<td>Mata ade2-1 his3-11,15 leu2-3,112 ura3-52 trpl-1 \textit{pycl::LEU2 pyc2::HIS3 mth1::URA3}</td>
<td>This work</td>
</tr>
<tr>
<td>YCR009</td>
<td>\textit{escl-1}</td>
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<td>This work</td>
</tr>
<tr>
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<td>This work</td>
</tr>
<tr>
<td>YCR011</td>
<td>\textit{ESC3-1}</td>
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<td>This work</td>
</tr>
<tr>
<td>YCR012</td>
<td>\textit{snfl}</td>
<td>Mata ade2-1 his3-11,15 leu2-3,112 ura3-52 trpl-1 \textit{snfl::KanMX4}</td>
<td>This work. Disruption of \textit{SNF1} in W303-1A with \textit{KanMX4}</td>
</tr>
<tr>
<td>YCR013</td>
<td>\textit{ESC3-1 snf1}</td>
<td>Mata ade2-1 his3-11,15 leu2-3,112 ura3-52 trpl-1 \textit{ESC3-1 snfl::KanMX4}</td>
<td>This work. Disruption of \textit{SNF1} in YCR011 with \textit{KanMX4}</td>
</tr>
</tbody>
</table>
CA, USA). The RNAs were separated in 1.5% formaldehyde agarose gels and transferred to Hybond N+ filters (Amersham, Little Chalfont, UK). Probes were labelled with 32P using conventional techniques [27]. The probes used were those described by Diderich et al. [28], kindly supplied by A.L. Kruckeberg (Amsterdam, The Netherlands).

2.4. Preparation of extracts and enzymatic assays

Cell extracts were prepared by vortexing at 4°C four times for 1 min, with 1-min intervals in ice about 100 mg wet weight yeast cells with 1 g glass beads (0.4–0.5 mm diameter) and 0.5 ml of 20 mM imidazole, pH 7. After centrifugation in the cold at 8000×g for 15 min, the supernatants were used for enzyme assays and determination of protein concentration. Fructose-1,6-bisphosphatase was assayed as in Gancedo and Gancedo [29], glutamate dehydrogenase as in Witt et al. [17] and isocitrate lyase as in Dixon and Kornberg [30]. Invertase activity was assayed in whole cells as described by Jiang and Carlson [23]. L-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units [31]. Protein was assayed using the commercial Pierce (Rockford, IL, USA) assay.

2.5. Measurement of glucose consumption, fermentation and respiration

Glucose consumption was determined by following glucose disappearance in yeast suspensions (20 mg ml−1) in 50 mM phosphate buffer, pH 6, at 30°C. Fermentation and respiration were measured at 30°C in a conventional Warburg respirometer in the same conditions.

3. Results

3.1. Isolation and genetic characterization of mutations that suppress the pyc1 pyc2 phenotype

To isolate new genes of *S. cerevisiae* involved in catabolite repression or glucose signalling and to avoid the isolation of mutations in the gene *MTH1* (see Section 1) we started our search with a triple pyc1 pyc2 mth1 mutant strain (YCR003, see Table 1). *S. cerevisiae* pyc1 pyc2 mth1 mutants do not grow in glucose-ammonium but do so in glucose-aspartate (Fig. 1). After conventional mutagenesis we isolated several independent mutants able to grow in glucose-ammonium. Each mutant was backcrossed to a pyc1 pyc2 mth1 isogenic strain (YCR006, see Table 1) of opposite mating type; the diploids were isolated and tested for growth in glucose-ammonium. Ten diploids derived from mutants with a clear phenotype were sporulated and 12–15 complete tetrads of each cross were scored for growth in glucose-ammonium medium. All mutants that produced a progeny indicative of a monogenic mutation were rechecked. Finally we retained one mutant carrying a recessive mutation and another one carrying a semidominant one; we named the observed phenotype esc (escape of sugars control), the genes responsible for it *ESC1* and *ESC3*, respectively, and the corresponding alleles esc1-1 and *ESC3-1*. During the analysis of tetrads derived from diploids heterozygous for the *ESC3-1* mutation we observed a 2+:2− segregation of small-size colonies; all the segregants forming small colonies carried the *ESC3-1* allele.

Minimal galactose-ammonium medium is also a non-permissive medium for *pyc1 pyc2 mth1* mutants due to the repressive effect of galactose on the genes encoding the enzymes of the glyoxylate cycle [32]. Therefore *pyc1 pyc2* mutants do not grow in minimal glucose-ammonium medium; they may grow, however, on glucose-aspartate. During growth on non-repressing carbon sources the joint action of isocitrate lyase and malate synthase replenishes the tricarboxylic acid cycle.

![Fig. 1. Situation and role of pyruvate carboxylase in yeast metabolism. During growth in media with ammonium as nitrogen source, inorganic nitrogen enters metabolism via NADP+—glutamate dehydrogenase (Gdh) that drains away K−ketoglutarate from the tricarboxylic acid cycle to form glutamate. If glucose or other repressing sugars are used as carbon sources, pyruvate carboxylase (Pyc) is the only enzyme able to replenish the cycle due to the repression of isocitrate lyase (Icl) and malate synthase (MLs). Therefore *pyc1 pyc2* mutants do not grow in minimal glucose-ammonium medium; they may grow, however, on glucose-aspartate. During growth on non-repressing carbon sources the joint action of isocitrate lyase and malate synthase replenishes the tricarboxylic acid cycle.](FEMSYR 1512 13-2-03)
are referred to in the text as esc1-1 and ESC3-1, respectively.

3.2. Physiological characteristics of the mutants

While the esc1-1 mutant grew as the wild-type in rich or minimal glucose or galactose media, the ESC3-1 mutant grew always slower in those media with an increase of about 1.7-fold in generation time (Table 2). The rate of glucose utilization was lower than that of the wild-type in both mutants and the glucose fermentation rate was strongly decreased (Table 2). Although the rate of glucose respiration increased (Table 2) the mutants were able to grow in the presence of antimycin A, at difference with other strains carrying suppressors that relieve the effect of glucose [11] or galactose [32].

The mutants were not thermosensitive and their microscopic morphology appeared normal in the conditions used.

3.3. The mutations esc1-1 and ESC3-1 decrease repression by glucose and galactose

Since pyruvate carboxylase is the only known anaplerotic enzyme that replenishes the tricarboxylic acid cycle during growth in repressing sugars, the fact that the suppressor mutations allowed growth in glucose or galactose-ammonium media implies that the enzymes of the glyoxylate cycle, the alternative anaplerotic pathway (Fig. 1), should be at least partially active. We tested the activity of isocitrate lyase as well as that of other enzymes encoded by catabolite-repressed genes during growth in glucose or galactose and found that all of them were partially derepressed. The degree of derepression was different for the different enzymes but the derepression was always higher in the ESC3-1 mutant (Table 3). It is noteworthy that in all cases the level of enzymes found in derepressing conditions was higher in the esc1-1 mutant than in the wild-type. Curiously, although no activity of fructose-1,6-bisphosphatase was found in the assay in the esc1-1 mutant grown in glucose, we found that in these conditions a \( FBPlacZ \) fusion was expressed at low level in the mutant but not in the wild-type (result not shown). This difference between the two situations may indicate that although some derepression of \( FBP \) occurs in the mutant, fructose-1,6-bisphosphatase activity is not detected due to the effect of catabolite inactivation that affects the yeast enzyme but not the \( \beta \)-galactosidase reporter enzyme [33].

High levels of glucose repress invertase but low levels of the sugar have an inducing effect [34]. As shown in Table 3, low levels of glucose were unable to produce induction of invertase in the mutants. Also in the ESC3-1 mutant at high glucose the level of activity remained similar to that found at 0.05% glucose, indicating a difficulty in receiving or processing adequately the glucose signal.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain</th>
<th>Wild-type</th>
<th>esc1-1</th>
<th>ESC3-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generation time (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YP glucose</td>
<td></td>
<td>90</td>
<td>90</td>
<td>150</td>
</tr>
<tr>
<td>YP galactose</td>
<td></td>
<td>120</td>
<td>130</td>
<td>200</td>
</tr>
<tr>
<td>YNB glucose</td>
<td></td>
<td>120</td>
<td>120</td>
<td>210</td>
</tr>
<tr>
<td>Glucose consumption (µmol g(^{-1}) min(^{-1}))</td>
<td></td>
<td>26</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Fermentation (µmol CO(_2) g(^{-1}) min(^{-1}))</td>
<td></td>
<td>26</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Respiration (µmol O(_2) g(^{-1}) min(^{-1}))</td>
<td></td>
<td>3</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

The yeasts were grown in the media indicated and the different parameters determined as described in Section 2. Values are the mean of three independent experiments. Variation between individual values was <15%.

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>FbPase (mU (mg protein)(^{-1}))</th>
<th>GDH (mU (mg protein)(^{-1}))</th>
<th>ICL (mU (mg protein)(^{-1}))</th>
<th>Invertase (nmol glucose (mg yeast)(^{-1}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glu Gal Gly</td>
<td>Glu Gal Gly</td>
<td>Glu Gal Gly</td>
<td>2% Gluc 0.5% Gluc</td>
</tr>
<tr>
<td>Wild-type</td>
<td>&lt;1 1 10 40</td>
<td>9 40 30 60</td>
<td>&lt;1 1.5 38 40</td>
<td>3 230</td>
</tr>
<tr>
<td>esc1-1</td>
<td>&lt;1 10 40</td>
<td>9 30 60 80</td>
<td>4 10 80 205</td>
<td>3 17</td>
</tr>
<tr>
<td>ESC3-1</td>
<td>8 10 40</td>
<td>68 80 205</td>
<td>9 10 76 12</td>
<td>12 20</td>
</tr>
</tbody>
</table>

For the assay of fructose-1,6-bisphosphatase (FbPase), NAD-glutamate dehydrogenase (GDH) and isocitrate lyase (ICL), yeasts were grown in rich medium with 2% glycerol (Gly), glucose (Glu) or galactose (Gal) as carbon source as described in Section 2, and harvested during the exponential phase of growth. For the invertase assay yeasts were grown on YPD, harvested during the exponential phase of growth and resuspended in YP 2% glucose and YP 0.05% glucose, respectively, for 5 h. Assays for the activities reported were as described in Section 2. Values are the mean of three independent cultures done on different days. Variation between individual values was <15%.
3.4. Expression of the genes encoding sugar transporters in the esc-1 and ESC3-1 mutants

*S. cerevisiae* has several glucose transporters that are differentially expressed as a function of the glucose concentration in the medium [35]. We studied in the *esc* mutants the expression of *HXT1* and *HXT2* as representatives of a low- and a high-affinity transporter. As seen in Fig. 2, expression of *HXT1* that is induced at high glucose had a pattern of expression similar in the wild-type and in the mutants; however, expression of *HXT2* that is induced by low glucose and repressed by high glucose [36] was no longer repressible in the mutants. Neither *HXT1* nor *HXT2* was expressed during growth on galactose (Fig. 2), giving evidence that in the mutants the induction pathway controlled by glucose was intact.

3.5. Cloning of REG1 as a suppressor of the phenotype of the ESC3-1 mutant

We attempted to clone the genes responsible for the suppressor phenotype found. The high rate of spontaneous reversion of the *esc-1*-1 phenotype precluded a direct approach to the cloning of the *ESC1* gene; an indirect approach using the derepression of a *FBP1-lacZ* fusion in the mutant also failed.

In the case of the *ESC3* gene we tried to take advantage of our finding that glucose did not repress enzymes needed for galactose utilization in the *ESC3-1* mutant (e.g. specific activity of galactokinase in a glucose+galactose culture was 740 mU (mg protein)$^{-1}$ in the mutant while it was below detection level in the wild-type). Our rationale was the following: a wild-type yeast pregrown in a medium with galactose will grow when transferred to a medium with fructose and the toxic analogue 2-deoxygalactose because the genes of the galactose pathway are repressed by fructose; however, a strain with the *ESC3-1* mutation will not grow in these conditions since the *GAL* genes are not repressed and metabolism of the toxic analogue will be possible. Therefore overexpression of genes that complement the *ESC3-1* mutation would allow growth of a transformed-complemented mutant in fructose+2-deoxygalactose because the *GAL* genes will be repressed and 2-deoxygalactose will not be metabolized. We transformed an *ESC3-1* mutant with a yeast genomic library selecting for the *URA3* marker of the library plasmid on galactose plates and we then replica-plated about 15000 transformants to plates with 25 mM fructose+1 mM 2-deoxygalactose. On these plates 10 colonies grew; plasmids were isolated from them and analyzed. Two different restriction patterns with an overlapping region were found; this region contained the sequence of *REG1*, a gene encoding a regulatory subunit of the phosphatase Glc7 [37]. Overexpression of *REG1* restored a normal colony size to the *ESC3-1* mutant during growth on glucose and allowed growth of the mutant on fructose in the presence of 2-deoxygalactose.

It was important to verify if *ESC3-1* was an allelic form of *REG1* or if *REG1* was an extragenic suppressor. To discriminate between these possibilities we used two approaches. In one of them we made use of the fact that *REG1* is linked to *TRP1*, therefore if *ESC3-1* is an allelic form of *REG1*, in a cross *ESC3-1 trp1×ESC3 TRP1* most of the tetrads should be of parental di-type and the majority of the *ESC3-1* mutant sensitive to 2-deoxygalactose should require tryptophan due to the *trp1* mutation. We analyzed 13 complete tetrads and found a segregation of the markers indicative of non-linked genes, demonstrating that although overexpression of *REG1* suppressed the phenotype studied, *REG1* was not the gene affected in the mutant.

In the other approach we studied the effect of the expression of the *REG1* gene isolated from the *ESC3-1* mu-

<table>
<thead>
<tr>
<th>Strain and LexA fusions</th>
<th>GAD fusions</th>
<th>β-Galactosidase (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (6lexAoplacZ)</td>
<td>LexA-Snf1</td>
<td>Snf4-GAD 8</td>
</tr>
<tr>
<td></td>
<td>LexA-Snf1</td>
<td>GAD &lt; 1</td>
</tr>
<tr>
<td><em>ESC3-1</em> (6lexAoplacZ)</td>
<td>LexA-Snf1</td>
<td>Snf4-GAD 68</td>
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<tr>
<td></td>
<td>LexA-Snf1</td>
<td>GAD 1050</td>
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<td></td>
<td>LexA-Snf1</td>
<td>GAD 4</td>
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<td>GAD 2</td>
</tr>
<tr>
<td></td>
<td>LexA-Snf1</td>
<td>GAD &lt; 1</td>
</tr>
</tbody>
</table>

A wild-type and an *ESC3-1* mutant were transformed with plasmids expressing the indicated fusions and grown on 4% glucose. They were harvested during the exponential phase of growth and β-galactosidase was assayed as described in Section 2; values are average activities of four to six transformants, with deviation among values lower than 15% in all cases.
tant by PCR on the behavior of a pyc1 pyc2 mutant. When a pyc1 pyc2 strain was transformed with a plasmid bearing the REG1 gene, none of the transformants grew in glucose–ammonium medium and all of them were resistant to 2-deoxygalactose, indicating that REG1 is different from ESC3. This result makes clear that REG1 was not mutated in the ESC3-1 mutant, and supports the conclusion reached with the classical genetic approach.

3.6. The ESC3-1 mutation acts upstream of the Snf1 complex

Derepression of glucose-repressed genes requires an active Snf1 complex [2]. Since in the esc mutants glucose repression was decreased, we studied in the ESC3-1 mutant, that presents the strongest phenotype, the interaction of Snf1 with Snf4, the activating subunit of the Snf1 kinase complex [23]. As shown in Table 4, expression of a LexA-dependent reporter gene during growth on high glucose was negligible in a wild-type strain expressing LexA-Snf1 and Snf4-GAD fusion proteins, and high in the ESC3-1 mutant expressing the same proteins. We observed also that in the ESC3-1 background a LexA-Snf1 fusion was by itself a potent transcriptional activator of the reporter gene (Table 4). This activation was dependent on Snf1 activity, as it was not observed when a fusion with a mutated, inactive Snf1 (SNF1K84R) was used (Table 4). Kuchin et al. [22] have reported that a hyperactive Snf1 bound as a LexA fusion to a promoter activates transcription in a glucose-regulated way. Our results suggest that the mutation ESC3-1 results in a permanently active Snf1. As proposed by Kuchin et al. [22], this activated Snf1 may interact directly with the RNA polymerase holoenzyme and thereby activate transcription. Although Snf4 has been shown to be required for the direct activation by a LexA-Snf1 fusion [22], our results indicate that in the ESC3-1 mutant, the simultaneous expression of Snf4-GAD interferes with the activation by LexA-Snf1. This is a surprising result for which we do not have a straightforward explanation. We have checked that the characteristics of the ESC3-1 mutant are not due to mutations in the SNF1 or the SNF4 genes. For this we obtained by PCR the SNF1 and SNF4 genes from the ESC3-1 mutant and transformed either a snf1 or a snf4 mutant with independent PCR products. Expression of invertase and of FBP1-lacZ in the transformants was regulated by glucose as in the wild-type, indicating that the behavior observed in the ESC3-1 mutant was not due to mutations in SNF1 or SNF4 (results not shown).

We also verified that expression of glucose-repressed genes did not occur in a double ESC3-1 snf1 mutant (Table 5). Therefore we conclude that the ESC3-1 mutation does not bypass the requirement for Snf1. The mutation likely acts upstream of the Snf1 complex, either facilitating the activation of Snf1 or interfering with its glucose-dependent inactivation. As described in the Section 3.5, an excess of the regulatory protein Reg1 counteracts this action of the ESC3-1 mutation.

4. Discussion

We have isolated mutants from S. cerevisiae that relieve, partially and simultaneously, the repression produced by glucose and by galactose. Although this is not generally realized, galactose is able to produce in yeast many of the effects caused by glucose, although with less intensity [32,38]. The isolation of the mutants reported here suggests that both sugars share some common step(s) in their respective repression pathways. Mutants with the characteristics described here have not been reported previously, in spite of various searches for mutants that resist the glucose or the galactose effects [10,11,32]. In the case of glucose, mutant alleles of MTH1 have appeared in different screens, while in those used to search for galactose resistance, mutations affecting either GAL2 or GAL4 have been found [32]. These results indicate that an interference with the glucose signal, which involves the glucose sensors Snf3 and Rgt2, as well as Mth1 [8,9], produces the strongest mutants, and that only a deletion of the MTH1 gene allows recovery of mutations in other genes. It is noteworthy, however, that the mutations described in this work acted even in a MTH1 wild-type background.

A marked decrease in glucose transport can release glucose repression in yeast [11]. However, this mechanism is unlikely to account for the phenotypes observed here, since an important although reduced glucose consumption was measured in the mutants. Also the release of galactose repression argues against such a mechanism. Moreover, HXT1 expression was similar in the mutants and in the wild-type, and HXT2 had lost its repression by glucose. The characteristics of the mutants make it also unlikely that they are alleles of recently isolated mutations conferring resistance to catabolite repression such as gsf2 or GSF4 [39,40]. The recessive gsf2 mutation causes a syn-

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Invertase</th>
<th>FBP1-lacZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>snf1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>ESC3-1</td>
<td>5</td>
<td>255</td>
</tr>
<tr>
<td>ESC3-1 snf1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

The yeasts were grown in minimal medium with 4% glucose and harvested in the exponential phase of growth. Enzymatic assays were as described in Section 2. For the β-galactosidase assays, values are the mean of four to six independent transformants, with differences among values lower than 10% in all cases.
thetic lethal phenotype with snf1, which was not the case with the ESC3-I mutant as shown in Table 5. The GSF4 mutants exhibit a 20-fold higher invertase than the wild-type when grown at high glucose levels [40], making them also different from the mutants reported here.

According to present ideas, two conditions shall be met for derepression of genes repressed by both glucose and galactose. First, the protein Snf1 shall be in its active, phosphorylated state and second, Snf1 shall be localized in the nucleus [41]. This localization occurs only when the protein Gal83, a component of the Snf1 complex, is in a certain conformation. The mutant ESC3-I behaved as having a permanently active Snf1 as shown by the derepression of galactokinase in the presence of glucose. Moreover, the significant derepression of genes encoding gluconeogenic enzymes observed in the mutants indicates that at least part of the Snf1 protein is localized in the nucleus both in glucose and in galactose cultures. A possible explanation of the results is that the mutation influences some signal that affects the phosphorylation of Snf1 and the conversion of Gal83 to its active conformation.

It is worth remarking that the phenotype of the ESC3-I mutant is completely dependent on the presence of the protein kinase Snf1: double mutants ESC3-I snf1 are permanently repressed. Moreover, the ESC3-I mutation is not localized in SNF1 or in SNF4; it appears therefore that the mutation acts upstream of Snf1 and that ESC3-I could encode a protein implicated in the function of the Snf1 complex.

The isolation of REG1 as a suppressor of the ESC3-I phenotype is also consistent with an upregulated activity of Snf1 due to a mutated Esc3 protein. Reg1 is a regulatory subunit of the Gcl7 phosphatase complex that targets it to specific substrates. When REG1 is overexpressed, the complex Gcl7/Reg1 is able to convert the active form of Snf1 to the inactive one, thus causing the reappearance of a normal phenotype in the mutant.

An additional, unexpected feature found in the mutant ESC3-I was that in this background, a LexA-Snf1 fusion was by itself a potent transcriptional activator of a LexA fusion gene reporter in glucose-growing cells. Although it has been described that a hyperactive Snf1 kinase, Snf1G53R, bound to a LexA fusion was also a potent transcriptional activator, its activity was otherwise regulated as in a wild-type [22], in contrast with the behavior observed in the ESC3-I mutant. Curiously, expression of Snf4-GAD interfered with the activation by LexA-Snf1, a result for which we presently do not have an adequate explanation.

The results presented indicate that there are still unknown elements in the signal transduction pathways for sugars and give evidence that there is a common link in the pathways that transduce the glucose and galactose signals.

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