DDSE: downstream targets of the *SNF3* signal transduction pathway

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Abstract

Mutations in the yeast *SNF3* gene affect glucose sensing and *snf3* mutants show defective growth on glucose. DNA sequence dependent suppressing elements (DDSEs) are regions located in the promoters of yeast glucose transporter (*HXT*) genes that when present in high copy suppress the *snf3* growth defect. Here we provide evidence that the multicopy DDSE suppression is due to the titration of the Rgt1p transcriptional repressor. The DDSE region from *HXT4* was found to function as a UAS sequence rendering a UASgal-less LacZ gene fused to the *GAL1* promoter responsive to glucose induction. Expression mediated by the UASDDSE was dependent upon the presence of Snf3p. Expression was elevated to a high level in an *rgt1* mutant in the absence of Snf3p suggesting that this DDSE region contains binding sites for the Rgt1p transcriptional repressor/activator. The UASDDSE led to expression in a *grr1* mutant background, which confers a defect in inactivation of Rgt1p, as predicted from the model. The presence of tandem repeats of the putative Rgt1p binding site gave results similar to those of the DDSE, suggesting that loss of repression is due to the presence of Rgt1p footprint in the multicopy DDSE. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Glucose sensing; Glucose transporter; *SNF3*; *Saccharomyces*

1. Introduction

Glucose uptake in the yeast *Saccharomyces cerevisiae* is catalyzed by multiple transporters encoded by members of the *HXT* gene family [1-4]. These genes display different modes of regulation that serve to match the affinity of the transporter with the substrate concentration of the medium. Under low substrate conditions, the *SNF3* gene plays a key role in transporter regulation [5-11]. Snf3p is involved in both induction and repression of a subset of the *HXT* genes (*HXT2, 3, 4, 6 and 7*) [7-12]. The *snf3* mutants in the S288C genetic background display a phenotype consistent with defects in glucose transport of poor growth on low substrate concentrations [5,6,11]. However, it appears that the defect is due to an inability to rapidly adapt to low substrate concentrations as well as poor substrate utilization rates [11]. Several lines of evidence indicate that *SNF3* encodes a glucose sensor that regulates cellular activities in addition to the *HXT* genes ([3,13], J.A. Brown and L.F. Bisson, to be reported).

Numerous multicopy and genomic suppressors of the *snf3* growth defect on low glucose have been isolated [1,14,15]. Dominant genomic suppressors identified a high glucose sensor, *RGT2* [9,14]. The *RGT2* gene bears striking homology to *SNF3* [9,16,17]. The *RGT1* gene was discovered as a recessive genomic suppressor of *snf3* and subsequently shown to encode an Snf3p-responsive transcription factor [16]. Not surprisingly, many of the multicopy suppressors of *snf3* encoded *HXT* genes, the presumed downstream targets of Snf3p regulation [1,15]. The presence of these genes in multicopy was thought to increase the basal level of expression of the transporter, thus negating the need for Snf3p-mediated induction. Further analysis revealed, however, that some of the *HXT* genes identified as multicopy suppressors were not able to suppress the *snf3* mutation when expressed from a heterologous promoter [3]. It was found that these *HXT* genes contained a promoter region, termed ‘DDSE’ for DNA sequence dependent suppressing element responsible for the multicopy suppression phenotype [15]. The promoter regions of *HXT1, 2, 3, 4* [15] and of the *SKS1* gene [13] were capable of suppressing the growth defect...
resulting from loss of \( snf3 \) in the absence of their downstream coding sequence. This suppression was dependent on the presence of either the \( HXT2 \) or \( HXT3 \) gene, suggesting a model in which the multiple copies of the regulatory region lead to a titration of a repressing transcription factor [15]. The repressor encoded by the \( RGT1 \) gene is a likely candidate for titration by the DDSE regions. The goal of this study was to further characterize the DDSE regions and test the hypothesis that suppression is due to loss of Rgt1p-mediated repression.

2. Materials and methods

2.1. Yeast strains and media

The yeast strains used in this study are listed in Table 1. LBY500 is YPH500 carrying the integrated reporter gene construct. LBY970 was a segregant of a cross between strains MCY1516 [18] and LBY403/pGTDZ. Standard methods for yeast manipulation were used [19]. Yeasts were transformed using the lithium acetate method [20]. Suppression of \( snf3 \) mutations was tested by patching yeast strains onto YEP (yeast extract, 10 g l\(^{-1}\); peptone, 20 g l\(^{-1}\)) medium containing 0.05% glucose or 2% raffinose with Antimycin A (2 \( \mu \)g ml\(^{-1}\) added to inhibit respiration. For the \( \beta \)-galactosidase assays yeasts were grown in a synthetic complete medium, YNB (0.67% yeast nitrogen base without amino acids), supplemented with required amino acids and 40 mg l\(^{-1}\) each of adenine and uracil. All media were from Difco and all chemicals used were reagent grade unless otherwise specified.

2.2. Plasmid manipulations

Standard methods for DNA manipulation were used throughout [21,22]. pGT4 was constructed by cloning the \( PstI \) fragment containing the DDSE region of the \( HXT4 \) promoter into the 2-micron plasmid pRS424 [23], and pGT3 contains the same DDSE region in plasmid pRS423. Plasmid pRS424 contains the \( TRP1 \) gene and pRS423 \( HIS3 \) as selectable markers. Plasmids pGT201, pGT203 and pGT295 were constructed using PCR primers (\( 5^\prime\)-TATCCGAGTCACTGGAATCCGGG-3' and \( 5^\prime\)-CTGCAGGAAATATCTTCTCTTCTCC-3') to amplify a 200-bp internal fragment (−724 to −519 from the \( HXT4 \) start site). The fragment was cloned into the \( Smal \) site of pRS424. pGT201, pGT203 and pGT205 contain one, three and five tandem copies of the fragment, respectively. Plasmid pSOT4 contains three tandem repeats of the Rgt1p binding site of the \( HXT2 \) promoter and was a gift from Dr. Sabire Ozcan [10]. To obtain plasmids with increasing numbers of this binding site, an EcoRI-XbaI fragment containing the three tandem copies was excised and the ends were filled using Klenow. The blunt-end fragment was cloned into the \( Smal \) site of pRS424. Plasmids pGTR3, pGTR6, and pGTR12 contain three, six and 12 copies of the Rgt1p footprint. In all cases the number and orientation of inserts were confirmed by DNA sequencing conducted by the DNA Sequencing Facility of the Division of Biological Sciences, University of California, Davis, CA, USA.

2.3. Construction of integrated reporter gene

Plasmid pGTDZ was generated using PCR primers (\( 5^\prime\)-CCAAGAAACGCTTGGATCCTTAAAGTC-3' and \( 5^\prime\)-GCAGGCCGGGAATATGGGGATCCCCA-3') containing BamHI restriction sites, to amplify a 349-bp fragment corresponding to the previously identified DDSE region of the \( HXT4 \) gene. The fragment was digested with BamHI and cloned into the \( BgII \) site of the one hybrid construct pJL638 [24]. pGTDZ contains the region of the \( HXT4 \) promoter from −770 to −460 cloned into the one hybrid vector in the same orientation with respect to the start codon as in the \( HXT4 \) promoter. This construct replaces the UAS\(_{gal}\) regulatory region of the \( GAL1 \) gene with the DDSE from \( HXT4 \). pGTDZ was digested with \( Smal \), the fragment containing the reporter construct was gel-purified and integrated into the \( ura3 \) locus. Southern blot analysis was used to confirm single copy integration at \( URA3 \).

2.4. Assay of \( \beta \)-galactosidase activity

Cells for \( \beta \)-galactosidase assays were pre-grown in YNB containing casamino acids (2 g l\(^{-1}\)), with 2% glucose or 2% galactose as carbon and energy source. Cells were inoculated into the same medium with the appropriate carbon source (0.5%, 2% glucose, 2% galactose or 2% galactose with 0.05% glucose) as indicated, at an initial OD\(_{580}\) of 0.05. Cells were grown to late exponential phase and 20 OD units were harvested (enough volume to equal a total of 20 OD\(_{580}\)). Cell extracts were made using the glass bead method as previously described [19]. Samples were taken in triplicate for protein determination using the Bradford reagent (Bio-Rad, Hercules, CA, USA) according to manufacturer’s instructions. Samples were also taken in triplicate for assay of \( \beta \)-galactosidase activity [25]. \( \beta \)-Galactosidase values reported are the average of triplicate determinations of replicate (2–8) experiments.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Yeast strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Genotype</td>
</tr>
<tr>
<td>LBY500</td>
<td>MATa URA3::DDSE-LacZ</td>
</tr>
<tr>
<td>LBY403</td>
<td>MATa snf3::HIS3 URA3::DDSE-LacZ</td>
</tr>
<tr>
<td>LBY311</td>
<td>MATa snf3::TRP1 URA3::DDSE-LacZ</td>
</tr>
<tr>
<td>LBY921</td>
<td>MATa grr1::LEU2 URA3::DDSE-LacZ</td>
</tr>
<tr>
<td>LBY970</td>
<td>MATa snf3-M::HIS3 rgt1::URA3::DDSE-LacZ</td>
</tr>
</tbody>
</table>

All strains also carry: ura3-52 lys2-801 ade2-100 trp1-363 his3-D200 leu2-3A and were constructed in the YPH500 background.
lactosidase activity values (10–50 units) varied by a factor of 2–3 and high values (≥200 units) by 20–30%. Activity was expressed in nmol of \( \text{o-nitrophenol} \) released from \( \text{o-nitrophenyl-}\beta-\text{D-galactopyranoside} \) (ONPG) \( \text{min}^{-1} \text{mg}^{-1} \) total protein.

3. Results and discussion

3.1. The \( \text{HXT4} \) DDSE defines a \( \text{UAS} \) responsive to glucose

The DDSE regions are located in the promoters of genes that are downstream targets of Snf3p activation. The model for DDSE-mediated suppression proposes that these regions bind a repressing element(s) leading to the expression of \( \text{HXT2} \) or \( \text{HXT3} \) in the genome [15]. In order to test this hypothesis, our first goal was to integrate a reporter gene into the genome containing a DDSE region that would therefore be responsive to the DDSE region in multicopy. The DDSE region of the \( \text{HXT4} \) gene was used to replace the UAS\( \text{gal} \) site of the \( \text{GAL10-GAL1} \) promoter region using the ’one hybrid’ system plasmid, which also carries a fusion of the LacZ gene to the \( \text{GAL1} \) promoter [24]. This reporter construct can be used to test the ability of a given sequence to function as a \( \text{UAS} \) and to analyze the mechanism of transcriptional activation. Analysis of transformants carrying the original vector without the DDSE sequence did not reveal any \( \beta \)-galactosidase activity in response to glucose shift (data not shown), nor was there any response to low glucose of the lactosidase activity in response to glucose shift (data not shown). Analysis of transformants carrying the original vector only (\( \text{pRS423} \) [23]) or with plasmid \( \text{pGT93} \) carrying the LacZ gene as mentioned by allowing expression of \( \text{HXT} \) genes via titration of a transcriptional repressor. If true, then the presence of the DDSE region in multicopy would be predicted to lead to expression of \( \beta \)-galactosidase from the integrated reporter construct as well. Wild-type, \( \text{LBY500} \), and \( \text{snf3} \) null strains, \( \text{LBY403} \) and \( \text{LBY311} \), containing the reporter gene construct as well. Wild-type, \( \text{LBY500} \), and \( \text{snf3} \) null strains, \( \text{LBY403} \) and \( \text{LBY311} \), containing the reporter construct integrated at the \( \text{URA3} \) locus.

Basal level of expression of \( \beta \)-galactosidase activity was low under repressing sugar concentrations (2%), but was highly induced (18-fold) by shift to low glucose conditions in the wild-type strain, \( \text{LBY500} \) (Table 2). The basal level of expression on galactose was somewhat elevated (3-fold) as compared to glucose growth, but was further induced by the addition of a low concentration of glucose. The induction of \( \beta \)-galactosidase did not occur in an \( \text{snf3} \) null background (\( \text{LBY403} \)) regardless of carbon source indicating that it is dependent on \( \text{SNF3} \) as is observed for \( \text{HXT2} \). Thus, gene expression from this construct is comparable to what has been reported for the \( \text{HXT2} \) gene [12] indicating that the DDSE region is sufficient to confer this pattern of Snf3p-mediated regulation.

3.2. Impact of multicopy DDSE on gene expression from the reporter construct

Previous work suggested that the DDSE regions functioned by allowing expression of \( \text{HXT} \) genes via titration of a transcriptional repressor. If true, then the presence of the DDSE region in multicopy would be predicted to lead to expression of \( \beta \)-galactosidase from the integrated reporter construct as well. Wild-type, \( \text{LBY500} \), and \( \text{snf3} \) null strains, \( \text{LBY403} \) and \( \text{LBY311} \), containing the reporter construct integrated at the \( \text{ura} \) locus were transformed with vector only (\( \text{pRS423} \) [23]) or with plasmid \( \text{pGT93} \) carrying the DDSE region. The DDSE region in multicopy did not lead to \( \beta \)-galactosidase expression in the presence of a repressing (2%) concentration of glucose (Table 3). Expression in both the \( \text{snf3} \) null and wild-type strain was elevated in the presence of multicopy DDSE under low glucose conditions (Table 3). Thus, the DDSE region in multicopy leads to the expression of genes located in the genome containing a UAS\( \text{DDSE} \) in the promoter. The presence of a repressing concentration of glucose overrides induction mediated by loss of the repressing factor. Presumably the Snf1p glucose repression pathway is the overriding mechanism, but this remains to be determined.

The \( \text{grr1} \) mutation [18, 26] defines an F-box protein thought to function downstream of Snf3p in the inactivation of the Rgt1p protein [27]. If the DDSE region were also involved in loss of this repressor then multicopy DDSE should lead to expression of the reporter construct in a \( \text{grr1} \) mutant background. The DDSE reporter gene

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### Table 2

Expression of the reporter gene construct at varying glucose concentrations and in the presence and absence of the \( \text{SNF3} \) gene

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>% (g 100 ml(^{-1}))</th>
<th>β-Galactosidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{LBY500} )</td>
<td>glucose</td>
<td>2</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>652</td>
</tr>
<tr>
<td></td>
<td>galactose</td>
<td>2</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>galactose+glucose</td>
<td>2+0.05</td>
<td>328</td>
</tr>
<tr>
<td>( \text{LBY403} )</td>
<td>glucose</td>
<td>2</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>galactose</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>galactose+glucose</td>
<td>2+0.05</td>
<td>17</td>
</tr>
</tbody>
</table>

*Activity is expressed as nmol of ONPG hydrolyzed \( \text{min}^{-1} \text{mg}^{-1} \) total protein.

Data also include average values for \( \text{LBY311} \). There was no observable effect of \( \text{snf3} \) null background on activity values.

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### Table 3

Effect of multicopy DDSE region on expression from the reporter gene construct

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Glucose (%)</th>
<th>β-Galactosidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{LBY500} )</td>
<td>vector</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>586</td>
</tr>
<tr>
<td></td>
<td>pGT94 (DDSE)</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>983</td>
</tr>
<tr>
<td>( \text{LBY403} )</td>
<td>vector</td>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>pGT94 (DDSE)</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>965</td>
</tr>
<tr>
<td>( \text{LBY921} )</td>
<td>vector</td>
<td>0.05</td>
<td>259</td>
</tr>
<tr>
<td>( \text{LBY970} )</td>
<td>vector</td>
<td>0.05</td>
<td>1517</td>
</tr>
<tr>
<td></td>
<td>pGT94 (DDSE)</td>
<td>0.05</td>
<td>681</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>875</td>
</tr>
</tbody>
</table>

*See legend to Table 2.
construct was generated in a grr1 background via genetic cross, LBY921. The basal level of expression from the reporter construct under low glucose conditions was slightly less than that observed for the wild-type strain, but greater than that of the snf3 mutant (Table 3). Presence of the DDSE region in a grr1 background led to an even greater induction than observed with the other strains under low glucose conditions. This finding suggests that the DDSE region functions downstream of GRR1, consistent with a model in which the Rgt1p repressor (or a co-repressor) is being titrated by the DDSE region.

The effect of the DDSE region on expression of the reporter construct in an rgt1 snf3 null background, LBY970, was also evaluated (Table 3). Basal levels of β-galactosidase activity were high in this background, a consequence of loss of the Rgt1p repressor. The presence of DDSE in multicopy led to a very slight but reproducible further increase in expression in this strain. A dramatic increase, as would be predicted if the DDSE region were operating via an entirely different mechanism, was not observed. However, we cannot rule out the possibility that a co-repressor rather than Rgt1p itself is the target of DDSE titration.

### 3.3. Deletion analysis of the HXT4 DDSE region

The Rgt1p binding site of the HXT2 promoter has been identified [10]. The 394-bp region of the HXT4 DDSE contains seven sites with some homology to this footprint (Fig. 1). Key features of these binding sites are the CGG triplet flanked by a 3’ AT rich region. Interestingly, deletion analysis revealed that a 200-bp subclone of this region, DDSE200 (−724 to −519 which had lost the binding site matches at −746, −712 and −438), could not suppress snf3 if present in single copy in the plasmid (data not shown). This construct did not lead to a high level of expression of the reporter gene, but activity was elevated somewhat over the snf3 null strain carrying the vector only (Table 4). If multiple copies of the DDSE200 were inserted into the multicopy plasmid, suppression was restored. This suggests that if the Rgt1p binding site is required for suppression, multiple copies are required.

It is equally possible that the region responsible for titration is unrelated to the putative binding site of Rgt1p. To test this possibility directly, the Rgt1p binding site of HXT2 was cloned directly into a multicopy plasmid and used to transform cells carrying the reporter construct. The construct containing three tandem repeats of the binding site was not able to lead to expression of the reporter (Table 4). However, if more copies of this site were present in tandem, there was an increase in the level of expression of β-galactosidase. This observation suggests that the presence of a putative binding site for the Rgt1p repressor on a multicopy plasmid can lead to expression of genes in the genome under the regulation of this transcriptional factor. Insertion of random pieces of Lambda DNA into this region did not lead to suppression of the snf3 growth defect, suggesting that the results are indeed due to the presence of the specific sequence of the Rgt1p binding site.

The work presented here strongly supports the hypothesis that DDSE regions lead to suppression of the snf3 mutation due to relief of negative regulation of target genes. The observation that a similar suppression occurs if the putative Rgt1p binding site is present in multicopy suggests that removal of this transcriptional repressor is likely responsible for activation of the HXT2 and HXT3 genes. However, it is also possible that a co-repressor or other DNA binding proteins may also be involved since multiple Rgt1p binding sites were required to be present in tandem for suppression to occur.

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References