Amino acid residues in Std1 protein required for induction of SUC2 transcription are also required for suppression of TBPΔ57 growth defect in Saccharomyces cerevisiae

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Abstract

The STD1 gene of Saccharomyces cerevisiae was isolated independently as a high-copy suppressor of a dominant negative mutation in the TATA-binding protein and of a mutation in the Snf1/Snf4 kinase complex, suggesting that Std1 might couple the Snf1 kinase signaling pathway to the transcriptional machinery. In order to identify the protein domains that specify these activities of the Std1 protein, a plasmid library of randomly mutagenized STD1 genes was screened for loss of function alleles using complementation of the ratlinoise growth defect of a std-1, mth1- strain as an assay. One missense allele (P236S) with complete loss of function at 30°C and four missense alleles (L173F, E225K, S269L and E274K ) that conferred a temperature sensitive phenotype were identified. The C-terminal 20 residues of Std1 were essential for SUC2 derepression, whereas the deletion of the N-terminal 96 residues did not affect SUC2 gene induction. Std1 mutants that lost the ability to induce SUC2, were also unable to suppress the growth defect caused by the expression of the dominant negative TBPΔ57 protein, suggesting that these two genetic screens may be detecting the same biological activity. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The STD1 gene of Saccharomyces cerevisiae was independently identified as a high-copy suppressor of mutations in two seemingly unrelated genes, the TATA-binding protein gene (SPT15) and the SNF4 gene (Ganster et al., 1993; Hubbard et al., 1994). The STD1 gene was never isolated in loss of function screens presumably because the yeast genome encodes a STD1 homologue, MTH1, on chromosome IV. Std1 and Mth1 proteins are 61% identical and appear to be functional, as well as structural, homologues. Deletion of either gene fails to confer an obvious phenotype, but deletion of both STD1 and MTH1 results in cells that are defective in the induction of the SUC2 gene in response to glucose starvation (Hubbard et al., 1994). Consequently, std1−, mth1− cells display a growth defect on ratlinoise-antimycin media.

Biochemical and genetic characterization of the Std1 protein indicated that it was capable of binding to both the TATA-binding protein and the SNF1 protein kinase (Hubbard et al., 1994; Tillman et al., 1995). These findings lead to the hypothesis that the Std1 protein may be a physical link between the glucose signal transduction pathway and the transcriptional machinery. For instance, it is possible that the Std1 protein is in direct contact with the Snf1 kinase, as suggested by the GST-STD1 column binding experiments reported by Carlson and colleagues (Hubbard et al., 1994). However, Std1 protein did not affect Snf1 protein kinase activity in vitro or Snf1 protein accumulation in vivo (Hubbard et al., 1994). During times of glucose deprivation when the Snf1 kinase is activated, the Std1 protein may transmit the glucose starvation signal directly or indirectly to the TATA binding protein in the nucleus to alter patterns of gene expression. Two hybrid analysis and GST column binding assays support the idea that Std1 protein can bind directly to the TATA binding protein (Tillman et al., 1995). However, the Std1 protein does not activate reporter gene transcription when fused to a DNA binding domain (Hubbard et al., 1994), nor does Std1p contain a recognizable DNA binding motif. Thus, Std1p does not appear to be
a sequence-specific transcriptional activator. Direct support for a model in which Std1 protein interactions with Snf1 and/or TBP occur in vivo to regulate gene expression is still needed.

Overexpression of Std1 protein causes an induction of some glucose regulated genes, including SU2 (Hubbard et al., 1994) and ADH2 (Gander and Schmidt, unpublished). Increased gene dosage of STD1 on a 2μ plasmid results in a partial derepression of SU2, but overexpression of Std1 protein as a fusion to the bacterial lexA protein from the strong ADH1 promoter causes an even greater increase in SU2 expression (Hubbard et al., 1994). One interpretation for these results is that the increased accumulation of Std1 leads to derepression of SU2, and the enhanced ability of the lexA–Std1 fusion to derepress SU2 is due to its higher level of expression. Unfortunately, our antisera directed against recombinant Std1 protein were unable to detect Std1 protein in yeast cell extracts, thus preventing a direct test of this hypothesis. We report here the use of an epitope tagged Std1 protein expressed from its own promoter on a 2μ plasmid and compare its activity and protein accumulation with a Gal4–Std1 fusion protein driven from the ADH1 promoter.

The goal of this study was to identify the domains and amino acid residues that specify the Std1 protein’s activities. In this manner, we hoped to distinguish whether these two genetic screens were detecting two distinct roles of the Std1 protein or if they were simply two different assays for the same function. Toward that end, a library of randomly mutagenized STD1 gene was generated and screened for loss of function alleles due to its higher level of expression. Unfortunately, our antisera directed against recombinant Std1 protein were unable to detect Std1 protein in yeast cell extracts, thus preventing a direct test of this hypothesis. We report here the use of an epitope tagged Std1 protein expressed from its own promoter on a 2μ plasmid and compare its activity and protein accumulation with a Gal4–Std1 fusion protein driven from the ADH1 promoter.

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2. Materials and methods

2.1. Plasmid construction

Plasmid pSTP105 contains a functional, epitope-tagged version of the STD1 gene inserted into the vector pUN105 (CEN, LEU2) (Ellliedge and Davis, 1988) on a 3.26-kb SacI–Sphi fragment. The HA epitope was inserted by replacing the 36-bp PsI fragment in the STD1 open reading frame (codons 103–114) with a double-stranded oligo nucleotide encoding a single copy of the eight residues comprising the HA epitope (YPYDVPDYD). A alleles of STD1 isolated from the library screen (see below) were subcloned into the YEP351 2μ vector (Hill et al., 1986) using the SacI and HindIII sites.

2.2. Mutagenesis and screening

Plasmid pSTP105 was treated for 0–40 min with 1 M hydroxyamine at 75°C (Sikorski and Boeke, 1991). Reactions were stopped on ice and hydroxyamine removed by dialysis against excess TE. The degree of mutagenesis was determined by measuring the ability of the plasmid encoded yeast LEU2 gene to complement the E. coli leuB600 mutation. When ampicillin-resistant colonies were replica-plated to minimal media lacking leucine, DNA from the aliquot treated with hydroxyamine for 20 min yielded ampicillin-resistant colonies, 7% of which were leucine auxotrophs. This pool of DNA was then amplified in E. coli, and a library of randomly mutagenized STD1 was generated from >10,000 independent E. coli clones. Yeast MCY2840 (MATa, std1::HIS3, mth1::URA3, his3) was generated and screened for loss of function alleles. SSCP analysis was performed by PCR amplification of overlapping PCR fragments were not analyzed further.

2.3. Single-stranded conformation polymorphism (SSCP) analysis

SSCP analysis was performed by PCR amplification in the presence of [γ-32P]dATP of six overlapping fragments spanning the entire STD1 open reading frame. Amplification products were heat-denatured, snap-cooled and resolved on non-denaturing 5% acrylamide sequencing gels using two different running conditions. Samples were resolved either on a 5% TBE gel with 5% glycerol run at 4°C or on a 0.5× TBE gel run without glycerol at room temperature. These two different conditions detected overlapping but distinct sets of mutations. Plasmids with mutations in non-overlapping PCR fragments were not analyzed further. Oligonucleotides (18 to 20mers) used for SSCP analysis are listed here as top (T) or bottom (B) strand primers.

2.4. Site-directed mutagenesis

Selected mutations were reconstructed in an expression plasmid based on the two-hybrid vector pGBT9.
invertase activity (1 unit being equal to the activity of 2.6. Invertase assays
Specific activity was defined in terms of milliunits of 3.1. Creation of a library of mutagenized for invertase activity (Goldstein and Lampen, 1975). Cultures of yeast cells (40 ml) were harvested in log phase (OD600 of 0.1–0.4), and protein extracts were prepared by vortexing with glass beads in a solution containing 40 mM HEPES pH 7.3, 350 mM NaCl, 0.1% Tween-20, 10% glycerol, 1 mM PMSF and 1 mM of benzamidine, pepstatin A, leupeptin and aprotinin. The concentration of soluble protein was determined by the Bradford method using bovine serum albumin as a standard, and an equal aliquot (50 ng) from each extract was resolved on a 7.5% SDS–polyacrylamide gel. Gels were either Coomassie-stained, or transferred to Hybond ECL nitrocellulose. The nitrocellulose membrane was blocked with 10% milk and 0.1% Tween-20 in 1× TBS (20 mM Tris–HCl, pH 7.6, 150 mM NaCl) for 1 h at 65°C and washed in 1× TBS with 0.1% Tween-20. The membrane was incubated with monoclonal mouse anti-

3. Results
3.1. Creation of a library of mutagenized STD1 genes
A library of mutagenized STD1 genes was created by treating plasmid p5P105 (Fig. 1) with hydroxylamine (Sikorski and Boeke, 1991). This mutant modifies cytosine residues to N4-hydroxycytosine that can then base-pair with adenosine. After passage through E. coli, this procedure is expected to yield C-to-T and G-to-A transition mutations. In an effort to minimize the creation of alleles with multiple nucleotide changes, pools of plasmid DNA were mutagenized to different extents and then assayed in bacteria for the ability of the yeast LEU2 gene to complement the bacterial leuB600 mutation. Plasmid DNA was treated for increasing periods of time with 1 M hydroxylamine (0–40 min at 75°C), and then individual pools were analyzed for the degree of mutagenesis. Aliquots of each pool were used to transform E. coli JBE181 (pyrF::Tn5, leuB600, trpC9830) to ampicillin resistance. The number of transformed colonies dropped precipitously as the length of reaction time with the mutagen was increased, suggesting that the ampicillin gene was being inactivated by this procedure. After treatment with hydroxylamine for
mately 20,000 clones transformed to LEU + were exam-
ined. Transformants were replica-plated to raffinose-
antimycin media and grown at 30 and 37 °C. MCY 2840
transformed with unmutagenized pSTP105 and plasmid
vector (pUN105) were used as positive and negative
controls, respectively. This initial screen identified over
500 loss of function clones (2.5 %). Clones identified in
the initial screen were subjected to additional testing.
Isolates were streaked for single colonies and retested
on raffinose-antimycin media. A surance that the growth
defect was linked to the library plasmid was obtained
by amplifying the plasmid from the library isolates
in E. coli and retransforming fresh MCY 2840.
Approximately half the plasmids failed to retain a
STD1 loss of function or temperature-sensitive phenotype and
were discarded.

3.3. Detection of mutations by SSCP analysis

In an effort to simplify our analysis, we sought to
eliminate those clones that contained multiple nucleotide
changes in the STD1 gene (Elledge and Davis, 1988) was treated with hydroxylamine and ampli-
fied in E. coli. The library of mutagenized plasmids was then trans-
formed into a std1 −, mth1− yeast strain using selection for leucine
prototrophy. Transformants were then screened for a std1 − phenotype
by replica plating to raffinose-antimycin media at both 30 and 37 °C.
Wild-type STD1 confers a growth advantage to this strain. In this experiment, 21 mutant alleles were exam-
ined, and mutations in the amplified region were detected
in clones 10, 11, 15, 24, 40, 130 and 145. Over 20 loss-
of-function alleles with more than one detectable muta-
tion in non-overlapping regions were not studied further.
Loss-of-function alleles with a single or no detectable
mutations and all temperature-sensitive alleles com-
prised a set of 14 mutant alleles. In this set of 14 mutant
alleles, SSCP analysis detected 14 mutations. DNA
sequence analysis of these clones (see below) identified
19 single base pair changes, yielding an SSCP detection
rate of 74 %, a value that is within the estimates of SSCP
detection limits (Fan et al., 1993).

3.4. DNA sequence analysis

Although our initial screen identified over 500 clones
with raffinose growth defects, secondary screens and
SSCP analysis reduced this number to only 14 that were
likely to be the result of single base pair changes.
Plasmid DNA was amplified in E. coli and subjected to
automated dideoxy sequencing on both strands. The
DNA sequence analysis is presented in Table 1. All
mutations detected were the result of C-to-T transitions
(G to A on the opposite strand) as expected from the
mechanism of hydroxylamine mutagenesis. Nonsense
mutations were detected by sequencing, all within the
STD1 open reading frame. Five of these went undetected
by SSCP. Nonsense mutations were detected in six
clones at codons 213, 219, 231, 331, 423, and 425. Clone
pSTP9 contained both a nonsense mutation in codon

Fig. 1. Mutagenesis scheme. Plasmid pSTP105 containing the epitope
tagged HA-STD1 gene in the centromeric yeast shuttle vector pUN105
(Elledge and Davis, 1988) was treated with hydroxylamine and ampli-
fied in E. coli. The library of mutagenized plasmids was then trans-
formed into a std1 −, mth1− yeast strain using selection for leucine
prototrophy. Transformants were then screened for a std1 − phenotype
by replica plating to raffinose-antimycin media at both 30 and 37 °C.
Wild-type STD1 confers a growth advantage to this strain.

40 min, we were unable to recover any ampicilin-resis-
tant colonies. When transformants from the 0-, 10-, 20-
and 30-min DNA pools were replica-plated to minimal
media lacking leucine, we determined that 0, 0, 7 and
50% of the transformants, respectively, were leucine
auxotrophs. DNA from the 20-min pool (7% leucine
auxotrophs) was amplified by large-scale transformation
onto multiple ampicilin plates. More than 10,000 inde-
pendent colonies were washed from the surface of plates,
grown for an additional 2 h in liquid media and har-
vested. DNA was prepared by alkaline lysis and used
as the library of mutagenized STD1 genes.

3.2. Isolation of STD1 mutant alleles

Yeast strains that lack both STD1 and MTH1 dere-
press invertase poorly (Hubbard et al., 1994) and there-
fore have a significant growth disadvantage on media
that contain raffinose as the sole carbon source and
antimycin to block mitochondrial function. When the
STD1 gene is introduced to such a strain on a centro-
meric plasmid, the growth disadvantage is completely
complemented. We used the ability of plasmid encoded
STD1 to complement the std1 −, mth1− strain for growth
on raffinose-antimycin media as an assay to identify loss
of function alleles of STD1 present in our mutagenized
plasmid library. Yeast strain MCY 2840 (std1 −, mth1−) was transformed with the plasmid library, and approxi-
Fig. 2. SSCP analysis. Plasmids encoding putative std1 alleles were amplified in E. coli and analyzed by SSCP analysis. The set of six overlapping PCR fragments used for this analysis is diagrammed at the top. The thicker lines indicate the open reading frame. A representative autoradiogram of one SSCP gel is shown below. Of the 21 alleles analyzed on this gel, seven show novel conformers (clones 10, 11, 15, 24, 40, 130, and 145), indicated by arrows. DNA sequence analysis of these clones determined that all of the conformers were due to single nucleotide changes in this region. Clone 11 contained two mutations in this region. In addition, subsequent DNA sequencing analysis revealed that two clones (20 and 151) contained single C-to-T transitions that were not detected on this gel.

Table 1

<table>
<thead>
<tr>
<th>pSTP clone</th>
<th>Allinose phenotype</th>
<th>Region with SSCP conformer</th>
<th>Nucleotide changes</th>
<th>Amino acid changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>std1−</td>
<td>-56 to 368</td>
<td>C131T</td>
<td>S44F</td>
</tr>
<tr>
<td>5</td>
<td>std1−</td>
<td>970 to 1386</td>
<td>C1082T</td>
<td>P361I</td>
</tr>
<tr>
<td>9</td>
<td>std1−</td>
<td>56 to 368</td>
<td>C991T</td>
<td>Q331Stop</td>
</tr>
<tr>
<td>10</td>
<td>std1−</td>
<td>483 to 876</td>
<td>C1273T</td>
<td>Q425Stop</td>
</tr>
<tr>
<td>11</td>
<td>std1−</td>
<td>262 to 575</td>
<td>C1131T</td>
<td>Q331Stop</td>
</tr>
<tr>
<td>15</td>
<td>std1−</td>
<td>483 to 876</td>
<td>C655T</td>
<td>R219Stop</td>
</tr>
<tr>
<td>18</td>
<td>std1−</td>
<td>483 to 876</td>
<td>C706T</td>
<td>P236S</td>
</tr>
<tr>
<td>20</td>
<td>std1 ts</td>
<td>794 to 1082</td>
<td>G692A</td>
<td>W231Stop</td>
</tr>
<tr>
<td>23</td>
<td>std1−</td>
<td>734 to 1132</td>
<td>G692A</td>
<td>W231Stop</td>
</tr>
<tr>
<td>24</td>
<td>std1−</td>
<td>483 to 876</td>
<td>G675A</td>
<td>E225K</td>
</tr>
<tr>
<td>34</td>
<td>std1 ts</td>
<td>794 to 1082</td>
<td>G673A</td>
<td>E225K</td>
</tr>
<tr>
<td>130</td>
<td>std1 ts</td>
<td>262 to 575</td>
<td>C508T</td>
<td>L175F</td>
</tr>
<tr>
<td>145</td>
<td>std1 ts</td>
<td>483 to 876</td>
<td>C508T</td>
<td>L175F</td>
</tr>
<tr>
<td>151</td>
<td>std1−</td>
<td>483 to 876</td>
<td>G820A</td>
<td>E274K</td>
</tr>
</tbody>
</table>

All nucleotide numbering is relative to the STD1 initiation codon with the adenosine residue defined as +1.

Amino acid changes (e.g. E225K) are denoted as the amino acid in the wild-type protein (glutamic acid), followed by the codon number (225) and amino acid in the mutant allele (lysine).
331 as well as a missense mutation in codon 44 (serine to phenylalanine at codon 44, S44F). The remaining eight clones contained one or two missense mutations scattered throughout the protein. Clone pSTP23 contained two missense mutations including one in the initiating codon and was not studied further. Four mutants whose phenotype was due to a single amino acid change were detected. Three of the missense mutations were found to contain mutations at two positions. All of the missense alleles caused changes in amino acid residues that are conserved between Std1 and Mth1 proteins, consistent with the idea that these are residues that are essential for Std1 protein function.

3.5. Gene-inductive activity of STD1 mutants

The STD1 mutants were identified by their inability to ameliorate the std1− mth1− growth defect on raffinose-antimycin media. The underlying cause of this phenotype is most likely due to defects in the derepression of the SUC2 gene encoding invertase. Wild-type STD1 present on a high-copy-number plasmid causes the partial derepression of invertase under repressing growth conditions (2% glucose) (Hubbard et al., 1994). The mutant alleles of STD1 were subcloned into YEP351 (Hill et al., 1986), a 2m plasmid vector, and tested for the ability to induce expression of SUC2 in wild-type cells grown under repressing conditions. All of the nonsense alleles analyzed (Fig. 3) showed a complete loss of SUC2 gene inductive activity. The smallest truncation in mutant number 9 (Q425stop) causes the deletion of the last 20 amino acid residues and a complete loss in SUC2 induction activity. Therefore, we conclude that the C-terminal 20 residues of Std1 are essential for its gene inductive activity. Since all the other nonsense mutations also delete this essential region, no additional conclusions can be made from this set of mutant alleles. One missense allele of STD1 that also had lost all of its SUC2 induction activity at 30°C was identified. This allele replaces a proline residue at position 236 with a serine residue (P236S).

3.6. Temperature-sensitive alleles of STD1

A number of temperature-sensitive alleles of STD1 were identified by their ability to confer a growth advantage to std1− mth1− cells on raffinose-antimycin media at 30°C but not at 37°C. Sequence analysis of this set of mutants (Table 1) revealed that all contained missense mutations in the central portion of the protein (residues 170–280). Of the four temperature-sensitive alleles studied here, three were the result of single amino acid changes (E225K, L173F, and S269L), and one allele contained two amino acid changes from the wild-type sequence (E274K, G367S). Subsequent analysis of this allele indicates that the E274K mutation by itself is sufficient to confer a ts phenotype (see below). The temperature-sensitive alleles were subcloned into a 2m vector and tested for their ability to induce SUC2 expression at 30 and 37°C (Fig. 4). All four alleles confer differing levels of gene induction activity at 30°C, with the E225K mutant having the most activity at 30°C (80% of wild-type function) and S269L having the least (30% of wild-type function). When cells were grown at 37°C, a significant drop in the SUC2 inductive activity was observed for all the ts alleles.

3.7. Effect of STD1 mutations on protein accumulation

The STD1 mutations isolated here could result in a loss of STD1 function due to defects in protein-protein interactions. Alternatively, it is possible that these mutations cause defects in Std1 protein accumulation. In order to test this possibility, a direct assay for protein levels was needed. The original mutagenesis was performed on a centromeric plasmid encoding an epitope tagged version of the Std1 protein designated HA-STD1. This construct was functional in our raffinose-antimycin growth assays but was not detectable by Western blot. Indeed, the HA-STD1 construct was only weakly and inconsistently detectable when the gene was present on a high copy plasmid vector. However, while these experiments were ongoing, an additional epitope tagged version of STD1 containing three tandem copies of the HA epitope (Tyers et al., 1992) fused to the C-terminus of Std1 protein was constructed (STD1-3HA). This fusion protein was readily detectable by Western blot (Fig. 5A). To assay protein accumulation directly and for future two-hybrid analyses, the STD1-3HA gene was inserted into the two-hybrid vector pGB75. This construction
for protein accumulation by Western blot and for SUC2 induction (Fig. 5A). Although all of the mutant proteins were defective for SUC2 induction at either 30 or 37°C, none of the mutations resulted in a decrease in protein accumulation. In fact, one mutant, P236S, that is completely defective at inducing SUC2 at 30°C, actually accumulates to higher levels than the wild-type protein. Thus, changes in protein levels cannot account for the loss of Std1 protein’s gene inductive activity. The mutants’ ability to induce SUC2 expression was not grossly affected by the GAL4 fusion background. For instance, the mutants with the least and most severe defects at 30°C in the absence of GAL4 (E225K and P236S, respectively), likewise showed the least and most severe defects, respectively, in the GAL4 fusion background.

One of the original mutant alleles (pSTP145) contained two mutations, resulting in two changes in the amino acid sequence (E274K, G367S). The E274K/G367S mutations conferred a temperature-sensitive phenotype when tested for SUC2 induction. We hypothesized that the mutant phenotype was likely due to the non-conservative E274K change, rather than the G367S change. The E274K mutation was engineered into the GAL4–STD1–3HA construct and tested for SUC2 induction and protein accumulation (Fig. 5A). The E274K mutation resulted in a temperature-sensitive phenotype with the mutant protein exhibiting 46% of the wild-type activity at 30°C and only 6% at 37°C. The G367S mutation has not been tested by itself. Although it is possible that the G367S mutation contributes to the mutant phenotype, we conclude that the E274K mutation by itself is sufficient to confer a temperature-sensitive phenotype to STD1.

3.8. Deletion mutants

Our analysis of STD1 protein domains was extended by the creation of a set of deletion constructs in the GAL4–STD1–3HA hybrid. Precise deletion of the C-terminal residues 425–444 was constructed such that codon 424 was fused in frame with the 3HA tag. This protein accumulates to a slightly higher level than the wild-type construct and yet has no gene inductive activity at all (Fig. 5B). This result clearly demonstrates that the C-terminal 20 residues of Std1 protein are essential for its activity and not its accumulation. Since these C-terminal 20 residues are essential, further C-terminal truncations were not constructed. However, we did make a set of N-terminal deletions that removed the first 96, 199 and 312 residues of STD1. These constructs were assayed for protein accumulation and for SUC2 induction. None of the deletions had a significant effect on protein accumulation by Western blot and for SUC2 induction (Fig. 5A).
Fig. 5. Effect of STD1 mutations on protein accumulation. The effect of the STD1 mutations on protein accumulation was measured by Western blot analysis of cells transformed with plasmids expressing Std1 protein fused to the Gal4 protein DNA binding domain at the N-terminus and three copies of the HA epitope at the C-terminus (Tyers et al., 1992). (A) Proteins containing missense mutations were analyzed at both 30 and 37°C. Induction of SUC2 by these same constructs was determined separately and is shown below each lane as the percentage of induction conferred by the wild-type Gal4–Std1–3HA construct. Invertase assays were performed in triplicate, and the mean value was used for this calculation. The standard deviations were all less than 15% of the mean. The invertase activities for the wild-type Gal4–Std1–3HA construct were 260 and 75 mU/OD at 30 and 37°C, respectively. (B) Deletion constructs were generated in the Gal4–Std1–3HA backbone and were assayed for effects on protein accumulation and on SUC2 induction as above.

Deletion of the first 96 residues resulted in a protein with wild-type levels of SUC2 induction. However, deletion of the first 199 or 312 residues resulted in a complete loss of SUC2 induction. Thus, in contrast to the C-terminus, the N-terminal 96 residues of Std1 protein are completely dispensable for gene inductive activity.

3.9. Suppression of the TBP057 growth defect

The STD1 gene was first isolated in our lab as a high-copy suppressor of the growth defect caused by overexpression of a dominant negative mutation in the TATA binding protein, TBP057 (Ganster et al., 1993). In this assay, the TBP057 protein is expressed from a CEN
plasmid under the control of the GAL1 promoter such that cells grown on galactose overexpress TBP0.57 and show a severe growth defect. The presence of wild-type STD1 on a high-copy-number plasmid partially suppresses this defect. When each of the mutant Std1 proteins was tested in this assay, the ability to suppress the TBP0.57 growth defect correlated with the ability to induce SUC2 expression (Fig. 6). Thus, deletion of the C-terminal 20, 114 or 214 residues results in a complete loss of SUC2 induction as well as a complete loss of TBP0.57 suppression. Likewise, the P2365 mutation results in a complete loss of function phenotype in both the SUC2 induction and in TBP0.57 suppression. Analysis of the temperature-sensitive alleles is complicated by the finding that even wild-type Std1 protein is not able to suppress the TBP0.57 growth defect at 37°C (data not shown). However, when the temperature-sensitive alleles were tested at 30°C, all of the ts alleles showed a reduced, but detectable, level of TBP0.57 suppression, consistent with the finding that all the ts alleles have a reduced, but detectable, level of SUC2 inductive activity at 30°C.

A analysis of a set of N-terminal deletion mutants fused to the Gal4 DNA binding domain showed that the first 96 residues of Std1p were not essential for its ability to induce SUC2 expression. In order to test whether the N-terminus of Std1p was required for suppression of TBP0.57, we first constructed this same set of deletions but without the Gal4 domain. This new set of N-terminal deletion mutants was tested for SUC2 induction and protein accumulation by Western blot. In both assays, this new set of N-terminal deletions behaved in the same manner as those expressed as a fusion to Gal4 DNA binding domain (Fig. 5B). Deletion of the first 96 amino acids of Std1p does not reduce its ability to induce SUC2, and all deletion mutants were expressed at equivalent levels as judged by Western blot (data not shown). Deletion of residues 2–199 or 1–312 resulted in complete loss of function. When this set of N-terminal deletion mutants was tested for suppression of TBP0.57, the

A.

B.

Fig. 6. TBP0.57 suppression. Expression of TBP0.57 under the control of the GAL1 promoter results in a severe growth defect (Ganster et al., 1993) that is partially suppressed by the increased gene dosage of STD1. (A) Yeast cells (B) 1993) were transformed with the TBP0.57 expression plasmid pBM2D0.57 and by 2μ plasmids bearing different STD1 alleles as indicated. Serial dilutions of cultures grown on glucose containing media were spotted on to plates with either glucose or galactose as the carbon source as indicated. Plates were photographed after 3–10 days of growth at 30°C. (B) N-terminal deletion mutants were analyzed in BJ5409 transformed with the either plasmid pBM2D0.57 or pBM2D0.57, expressing under GAL control the wild-type TBP or TBP0.57, respectively.
amino terminal 96 residues were found to be non-

essential (Fig. 6B), but deletion of the N-terminal 199 or 312 residues abolished all TBP057 suppression activ-

ity. Analysis of these protein constructs by Western blotting revealed that all wild-type and N-terminal dele-
tion constructs all accumulated to similar levels in vivo (data not shown). Therefore, all the mutant forms of Std1 protein that were defective at 30°C for SUC2 induction were also defective for suppression of TBP057.

4. Discussion

Overexpression of Std1 protein suppresses growth defects caused by mutations in two distinct protein complexes, the general transcription factor TBP with its associated factors (Poon et al., 1996) and in Snf4 protein, a regulatory subunit of the Snf1 protein kinase complex (Jiang and Carlson, 1996, 1997). The exact role played by the Std1 protein either in transcription or protein phosphorylation is not known. Furthermore, Std1 protein and its homologue Mth1 are not closely related to any other proteins in the current databases and have no recognizable amino acid sequence motifs that might suggest a biochemical function. Thus, it has been difficult to ascribe an exact role for the Std1 protein. Deletion of both STD1 and MTH1 results in defects in the induction of SUC2, suggesting that these proteins play a positive role during the derepression of SUC2. However, both the TATA binding protein and the Snf1 kinase complex are needed for SUC2 derepres-
sion, making it difficult to determine which interaction is biologically relevant.

In order to define the domains of Std1 protein that specify its biological activities, we screened a library of randomly mutagenized STD1 genes for loss of function alleles. Five missense alleles with complete or temper-

ture-sensitive loss of function were isolated. All five alleles contained changes in amino acid residues that were conserved between Std1 and Mth1 proteins, consistent with the idea that these are critical residues. All five missense alleles were located in the central portion of

the protein, between residues 173 and 274. None of these mutations caused significant reductions in protein accumulation. The one missense allele with complete loss of function at 30°C, P236S, actually accumulates to a higher level than the wild-type protein. Despite the increased level of accumulation, the P236S protein is completely non-functional for both SUC2 induction and for TBP057 suppression. A second class of loss of function alleles was nonsense mutations. Six nonsense alleles, sequenced in their entirety, contained stop codons at positions 213, 219, 231, 331, 423, and 425. Since all were complete loss of function alleles, we can only conclude that the C-terminal 20 residues of Std1 protein are essential for its activity. Deletion of this region did not affect accumulation. In fact, the Q425 nonsense allele accumulates to an even higher level than the wild-type construct and yet is completely non-

functional in both genetic screens. The C-terminal 20 residues of Std1 protein are highly conserved with Mth1 with 15 of the final 20 residues being identical. In contrast, the N-terminus of Std1, which is not required for SUC2 induction, is not highly conserved with Mth1, showing only 39% identity over the first 100 residues. These findings support the idea that the residues con-
served between the Std1 and Mth1 proteins are impor-
tant for Std1 protein function.

The STD1 gene was isolated in two very different high-copy suppressor screens (Garster et al., 1993; Hubbard et al., 1994). Genetic and biochemical inter-

actions between Std1 and both the Snf1 protein kinase and the TATA binding protein can be detected, and an argument for either one or both of these interactions being important for glucose derepression can be made. In this study, we wanted to determine whether these two genetic screens were assaying different biological functions of the Std1 protein or whether they were in fact measuring the same biological activity. In order to distinguish these possibilities, we screened for loss of function alleles in the ability to promote SUC2 derepres-
sion and then assayed those same alleles for the ability to suppress the TBP057 growth defect. If alleles that lost function in the first screen were functional in the second, it would argue that these two genetic screens represent distinct biological activities. However, the opposite result was found. All of the alleles that lost the ability to promote SUC2 derepression were also unable to suppress the TBP057 growth defect. These results are consistent with a model in which a single Std1 function can be detected by these two distinct genetic screens.

SUC2 gene expression is repressed in cells grown on glucose but can be partially induced by cloned STD1 present on high copy 2μm vectors. When present on a single copy centromeric vector, the STD1 gene does not induce SUC2 expression and accumulates to a 10- to 20-fold lower level than high-copy vectors as determined by Western blotting (data not shown). This observation suggested that increased Std1 protein accumulation leads to an increase in SUC2 expression. Vectors that express recombinant Std1 protein as a fusion with the LexA (Hubbard et al., 1994) or Gal4 (Fig. 5) DNA binding domains have proven to be two- to 10-fold more efficient at SUC2 induction. It seemed logical that these con-

structs were more efficient at inducing SUC2 because the fusion genes were transcribed under the control of the strong ADH1 promoter and were therefore likely to accumulate to higher levels in vivo. However, this assumption was directly tested in this study and found to be incorrect. In the experiment reported here,
Gal4-Std1–3HA induced twofold more invertase activity than did Std1–3HA and yet accumulated to a lower level. This incongruence suggests that factors other than accumulation must affect Std1’s ability to induce SUC2.

One possibility is that fusion of Std1 protein to DNA binding domains with or without yeast nuclear localization signals (Gal4 and lexA, respectively) leads to changes in subcellular localization. The ability of Std1 protein to induce expression of the SUC2 gene may be limited by its ability to localize to the cell nucleus, a property that may be facilitated by the Gal4 and lexA DNA binding domains.

Our results strongly suggest that both genetic screens that identified STD1 reflect the same biological activity. We provide two possible explanations to account for this finding. First, it is possible that Std1 protein directly interacts with TBP or TFB and acts to limit its deleterious effects on gene expression (Zhou et al., 1991). Since the SUC2 gene is regulated at the level of transcription, it is possible that the Std1 mutants that have lost the ability to interact with TBP or TFB thereby lose their ability to affect SUC2 expression. An alternative possibility is that Std1 acts upstream in the glucose signaling pathway. Consistent with this possibility is the finding that Std1p interacts with the Snf1 kinase (Hubbard et al., 1994). In this model, Std1p may modulate Snf1 kinase activity and thereby affect glucose-regulated gene expression. It is worth noting that the STD1 gene was isolated by its ability to suppress the effects of TBP or TFB expressed from the GAL1 promoter under growth conditions (galactose media) that require a functional Snf1 kinase. Thus, it is possible that Std1p acts upstream in the glucose signaling pathway by affecting Snf1 kinase, downstream in the pathway by affecting TBP function or that Std1 interacts with both Snf1 kinase and TBP, thereby coupling the glucose signal to the transcriptional apparatus. The Std1 mutants reported here will certainly help with future studies to map the functional domains of Std1p and to determine its molecular role in gene regulation.

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References


