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Amino acid residues in Std1 protein required for induction of *SUC2* transcription are also required for suppression of TBPD57 growth defect in *Saccharomyces cerevisiae*

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Abstract

The *STD1* gene of *Saccharomyces cerevisiae* was isolated independently as a multicopy 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 raffinose growth defect of a *std1*$^-$, *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 TBPD57 protein, suggesting that these two genetic screens may be detecting the same biological activity.

Keywords: Mutagenesis; Yeast; TATA binding protein; Invertase

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* 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 raffinose-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

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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 *SUC2* (Hubbard et al., 1994) and *ADH2* (Ganster and Schmidt, unpublished). Increased gene dosage of *STD1* on a 2m plasmid results in a partial derepression of *SUC2*, but overexpression of Std1 protein as a fusion to the bacterial lexA protein from the strong *ADH1* promoter causes an even greater increase in *SUC2* expression (Hubbard et al., 1994). One interpretation for these results is that the increased accumulation of Std1 leads to derepression of *SUC2*, and the enhanced ability of the lexA–Std1 fusion to derepress *SUC2* is due to its higher level of expression. Unfortunately, our antisera directed against recombinant Std1 protein were unable to detect Std1 protein is 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 2m 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* genes was generated and screened for loss of function alleles using growth on raffinose antimycin media for a screen. Alleles found to be defective in this screen were then tested for the ability to suppress the TBPD57 growth defect. Our results suggest that these two genetic screens may in fact be measuring the same activity.

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) (Elledge and Davis, 1988) on a 3.26-kb *Sac*I–*Sph*I fragment. The HA epitope was inserted by replacing the 36-bp *Pst*I 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 (YPYDVPDY). Alleles of *STD1* isolated from the library screen (see below) were subcloned into the YEP351 2m vector (Hill et al., 1986) using the *Sac*I and *Hin*dIII sites.

2.2. Mutagenesis and screening

Plasmid pSTP105 was treated for 0–40 min with 1 M hydroxylamine at 75°C (Sikorski and Boeke, 1991). Reactions were stopped on ice and hydroxylamine 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 ampicilin-resistant colonies were replica-plated to minimal media lacking leucine, DNA from the aliquot treated with hydroxylamine for 20 min yielded ampicilin-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*D*200, ura3–52, leu2–3,−112*) was transformed with the *STD1* library, and approximately 20 000 transformants were selected on glucose media lacking leucine and replica-plated to raffinose media containing 1 mg/ml antimycin. Replicas were grown at both 30 and at 37°C, and *STD1* mutants were identified as slow-growing colonies on raffinose antimycin media. Plasmids from 150 *STD1* mutants were extracted from yeast and amplified in *E. coli*. Only those plasmids that retained the *std1*− phenotype upon retransfomation into MCY2840 were analyzed further.

2.3. Single-stranded conformation polymorphism (SSCP) analysis

SSCP analysis was performed by PCR amplification in the presence of [a-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 0.5× 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 with the nucleotide position of the 5′ base relative to the initiation codon. The primer pairs used in this analysis were P4–P1 (T−324 and B+112), P12–P16 (T−56 and B+368), P13–P10 (T+202 and B+575), P17–P18 (T+483 and B+876), P6–P3 (T+794 and B+1082), P19–P14 (T+970 and B+1386).

2.4. Site-directed mutagenesis

Selected mutations were reconstructed in an expression plasmid based on the two-hybrid vector pGBT9

(Chien et al., 1991). The starting plasmid expresses the DNA binding domain of GAL4 fused to the full-length wild-type STD1 containing three tandem copies of the HA epitope (Tyers et al., 1992) at the C-terminus. Site-directed mutations were created in polymerase chain reactions using the Pfu polymerase and mutagenic oligonucleotide primers. The single amino acid changes L173F (leucine at position 173 to phenylalanine), E225K, P236S, S269L, E274K were made by this method. In addition, the C-terminal deletion of residues 425–444 was constructed such that amino acid 424 was fused to the triple HA tag. N-terminal deletions that removed amino acids 1–96, 1–199, and 1–312 were also constructed by PCR. All plasmids were sequenced to confirm that the intended mutations were present and that no other mutations occurred during the PCR protocol.

2.5. DNA sequencing

Automated DNA sequencing was performed on an ABI PRISM 377 automated sequencer. The *STD1* open reading frame on all plasmids used here was sequenced on both strands and with a minimum of threefold coverage.

2.6. Invertase assays

Repressed and derepressed cells (Neigeborn and Carlson, 1984) were harvested in mid-log phase and normalized for equal OD_{600}. Cells were harvested and washed in cold 10 mM sodium azide and assayed for invertase activity (Goldstein and Lampen, 1975). Specific activity was defined in terms of milliunits of invertase activity (1 unit being equal to the activity required to release 1 mmol of glucose per minute) per OD_{600} of cells assayed.

2.7. Western blot analysis

Cultures of yeast cells (40 ml) were harvested in log phase (OD_{600} 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 mg/ml 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 mg) 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, 135 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-HA antibody (12CA5; Boehringer Mannheim) at 0.2 mg/ml in 1× TBS for 2 h at room temperature. The membrane was washed and then incubated with sheep anti-mouse-Ig antibody linked with horse-radish peroxidase (Amersham) at a 1:5000 dilution in 1× TBS with 0.1% Tween-20 for 1 h at room temperature and developed according to Amersham's protocol.

2.8. Suppression of the TBPD57 growth defect

Yeast strain BJ1991 (*MATa, ura3–52, leu2 trp1, pep4–3, prb1–1122*) was transformed with plasmids pBM2D-6 or pBM2D-D57 (Ganster et al., 1993) to confer galactose inducible overexpression of wild-type TBP and TBPD57, respectively. Suppression of the TBPD57 growth defect was assayed upon introduction of the wild-type and mutant *STD1* genes in the 2m YEP351 plasmids (see above). Cells transformed with both plasmids were grown overnight in glucose media lacking uracil and leucine. Cultures were then diluted 1:50, 1:500, 1:5000 and 1:50 000 in water, and 5 ml were spotted on to media containing either glucose or galactose as a carbon source but lacking uracil and leucine for plasmid selection. Plates were grown at 30°C for 2–10 days and photographed. For analysis of TBPD57 suppression by the N-terminal deletion mutants, yeast strain BJ5409 (*MATa, ura3–52, leu2*-D*1, trp1, his3*-D*200*) was transformed with wild-type and mutant *STD1* genes cloned by PCR into the 2m vector pRS423.

3. Results

3.1. Creation of a library of mutagenized STD1 genes

A library of mutagenized *STD1* genes was created by treating plasmid pSTP105 (Fig. 1) with hydroxylamine (Sikorski and Boeke, 1991). This mutagen 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 ampicilin resistance. The number of transformed colonies dropped precipitously as the length of reaction time with the mutagen was increased, suggesting that the ampicilin gene was being inactivated by this procedure. After treatment with hydroxylamine for

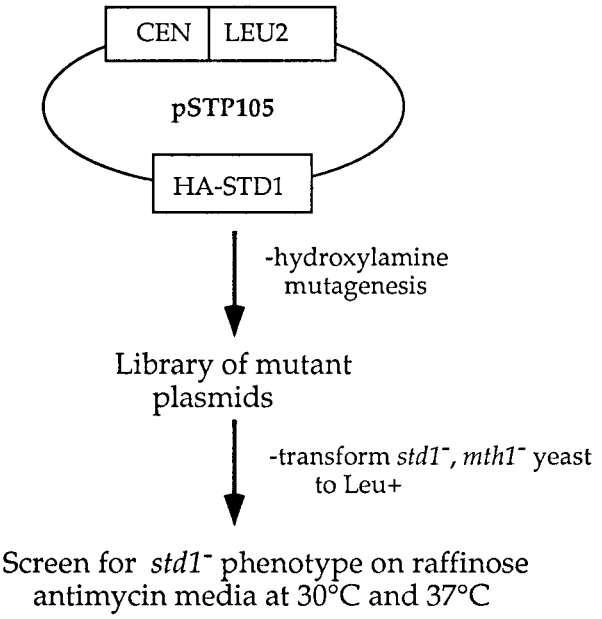

Fig. 1. Mutagenesis scheme. Plasmid pSTP105 containing the epitope tagged HA-STD1 gene in the centromeric yeast shuttle vector pUN105 (Elledge and Davis, 1988) was treated with hydroxylamine and amplified in *E. coli*. The library of mutagenized plasmids was then transformed into a *std1*$^-$, *mth1*$^-$ 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-resistant 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 on to multiple ampicilin plates. More than 10 000 independent colonies were washed from the surface of plates, grown for an additional 2 h in liquid media and harvested. 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* derepress invertase poorly (Hubbard et al., 1994) and therefore 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 centromeric 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 MCY2840 (*std1*$^-$, *mth1*$^-$) was transformed with the plasmid library, and approximately 20 000 clones transformed to LEU + were examined. Transformants were replica-plated to raffinose-antimycin media and grown at 30 and 37°C. MCY2840 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. Assurance 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 MCY2840. 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. Mutations in the *STD1* gene were detected by SSCP analysis using a set of six overlapping PCR fragments ranging in size from 291 to 438 bp. A representative autoradiogram is shown in Fig. 2. In this experiment, 21 mutant alleles were examined, 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 mutation in non-overlapping regions were not studied further. Loss-of-function alleles with a single or no detectable mutations and all temperature-sensitive alleles comprised 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. Nineteen 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

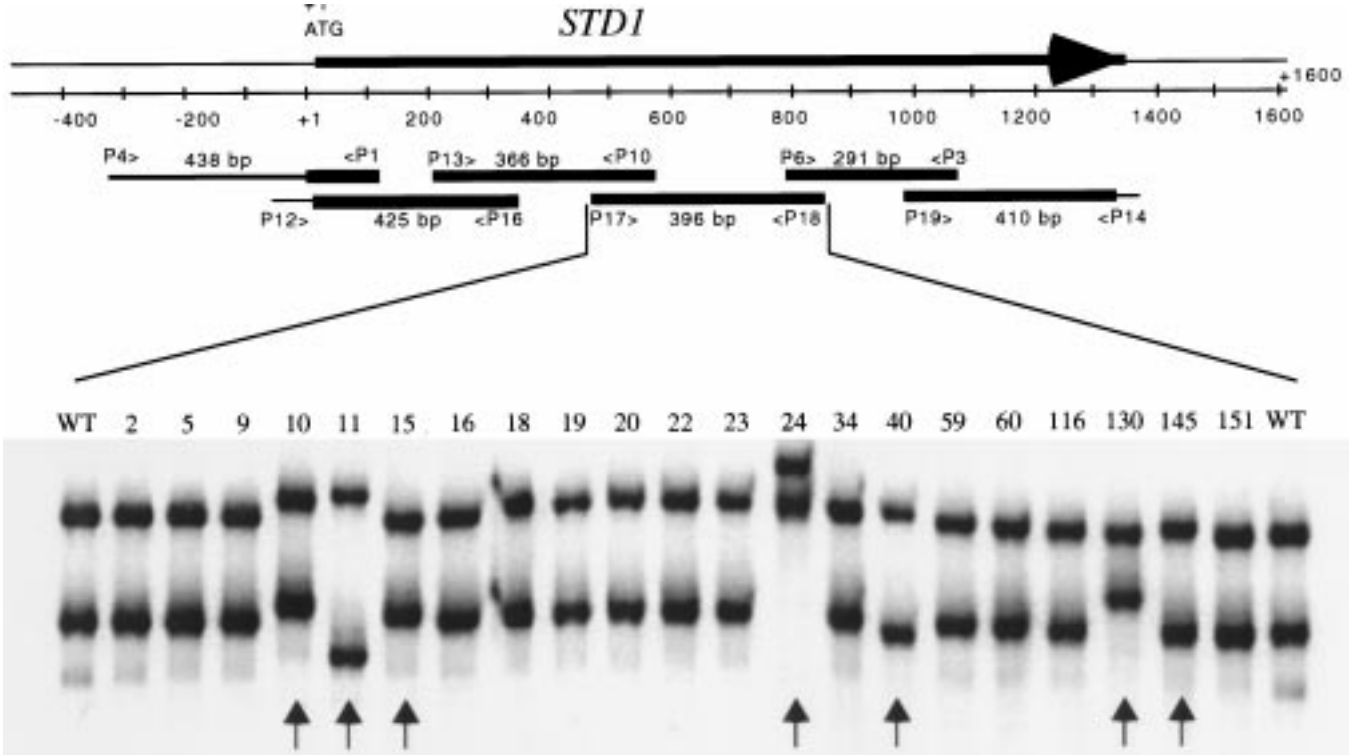

Fig. 2. SSCP analysis. Plasmids encoding putative *std1*$^-$ alleles were amplified in *E. coli* and analyzed by SSCP analysis. The set of six overlapping PCR fragments used for this analysis is diagrammed at the top. The thicker lines indicate the open reading frame. A representative autoradiogram of 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
Position of *STD1* mutations

pSTP clone	Raffinose phenotype	Region with SSCP conformer	Nucleotide changes[a]	Amino acid changes[b]
2	*std1*$^-$	Not detected	G547A	D183N
		970 to 1386	C1082T	P361L
5	*std1*$^-$	−56 to 368	C131T	S44F
		Not detected	C991T	Q331stop
9	*std1*$^-$	970 to 1386	C1273T	Q425stop
10	*std1*$^-$	483 to 876	G692A	W231stop
11	*std1*$^-$	202 to 575	C643T	(silent)
		483 to 876	C655T	R219stop
15	*std1*$^-$	483 to 876	C706T	P236S
18	*std1*$^-$	Not detected	G1269 A	W423stop
20	*std1* ts	794 to 1082	G673A	E225K
23	*std1*$^-$	−324 to 112	G3A	M1I
24	*std1*$^-$	483 to 876	G639A	W213stop
34	*std1* ts	794 to 1082	G673A	E225K
130	*std1* ts	202 to 575	C508T	L173F
		483 to 876		
145	*std1* ts	483 to 876	G820A	E274K
151	*std1*$^-$	Not detected	C806T	S269L

[a]Nucleotide numbering is relative to the STD1 initiation codon with the adenosine residue defined as +1.
[b]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 mutants 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).

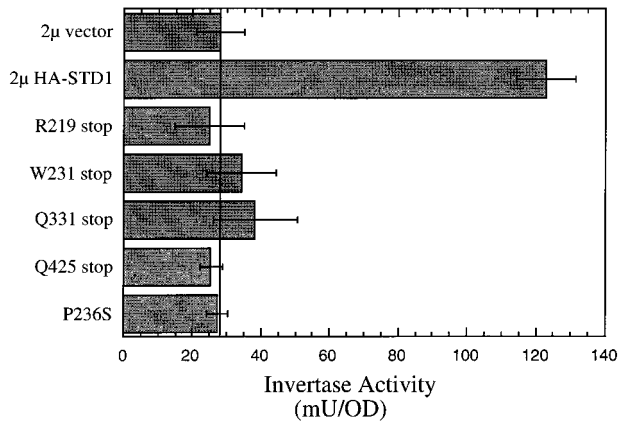

Fig. 3. *SUC2* induction by 2m *STD1*. Induction of *SUC2* expression was measured by assaying invertase enzyme activity levels in a wild-type yeast strain transformed with 2m plasmids containing either wild type, mutant or no HA-STD1 gene. Invertase assays were performed on at least three independent transformants of each construct. The mean value is plotted with each error bar representing one standard deviation. The line marks the mean invertase activity of the negative control transformed with the plasmid vector containing no *STD1* gene.

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 pGBT9. This construction

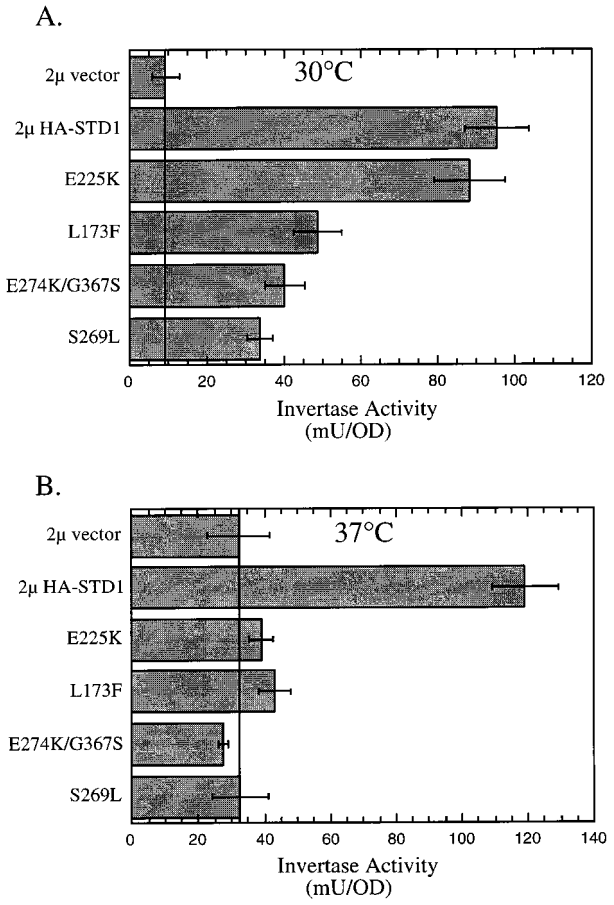

Fig. 4. *SUC2* induction by 2m *STD1* at 30 and 37°C. Induction of *SUC2* expression at 30 and 37°C was measured by assaying invertase enzyme activity levels in a wild-type yeast strain transformed with 2m plasmids containing either wild type, mutant or no HA-STD1 gene. Invertase assays were performed on at least three independent transformants of each construct. The mean value is plotted with each error bar representing one standard deviation. The line marks the mean invertase activity of the negative control transformed with the plasmid vector with no *STD1* gene.

results in the expression of the wild-type Std1 protein fused to the DNA binding domain of GAL4 at the N-terminus and the triple HA epitope at the C-terminus. This hybrid construct is more efficient than wild-type 2m *STD1* at inducing *SUC2* expression, a result also observed with a lexA–STD1 fusion (Hubbard et al., 1994). Indeed, the GAL4–STD1–3HA construct induces twice as much *SUC2* activity as the STD1–3HA construct (250 mU/OD versus 120 mU/OD, respectively) and yet accumulates to a lower level than the STD1–3HA construct. The greater efficiency of the Gal4–STD1–3HA construct at *SUC2* induction clearly indicates that other factors besides protein accumulation determine the gene inductive activity of the Std1 protein.

In order to test the effects of the various *STD1* mutations on protein levels, the mutations were remade by PCR in the GAL4–STD1–3HA fusion protein. Each construct was confirmed by DNA sequencing and tested 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 original allele with both mutations in the 2m HA–STD1 construction exhibits 37% and 0% of wild-type activity at 30°C and 37°C, respectively. 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

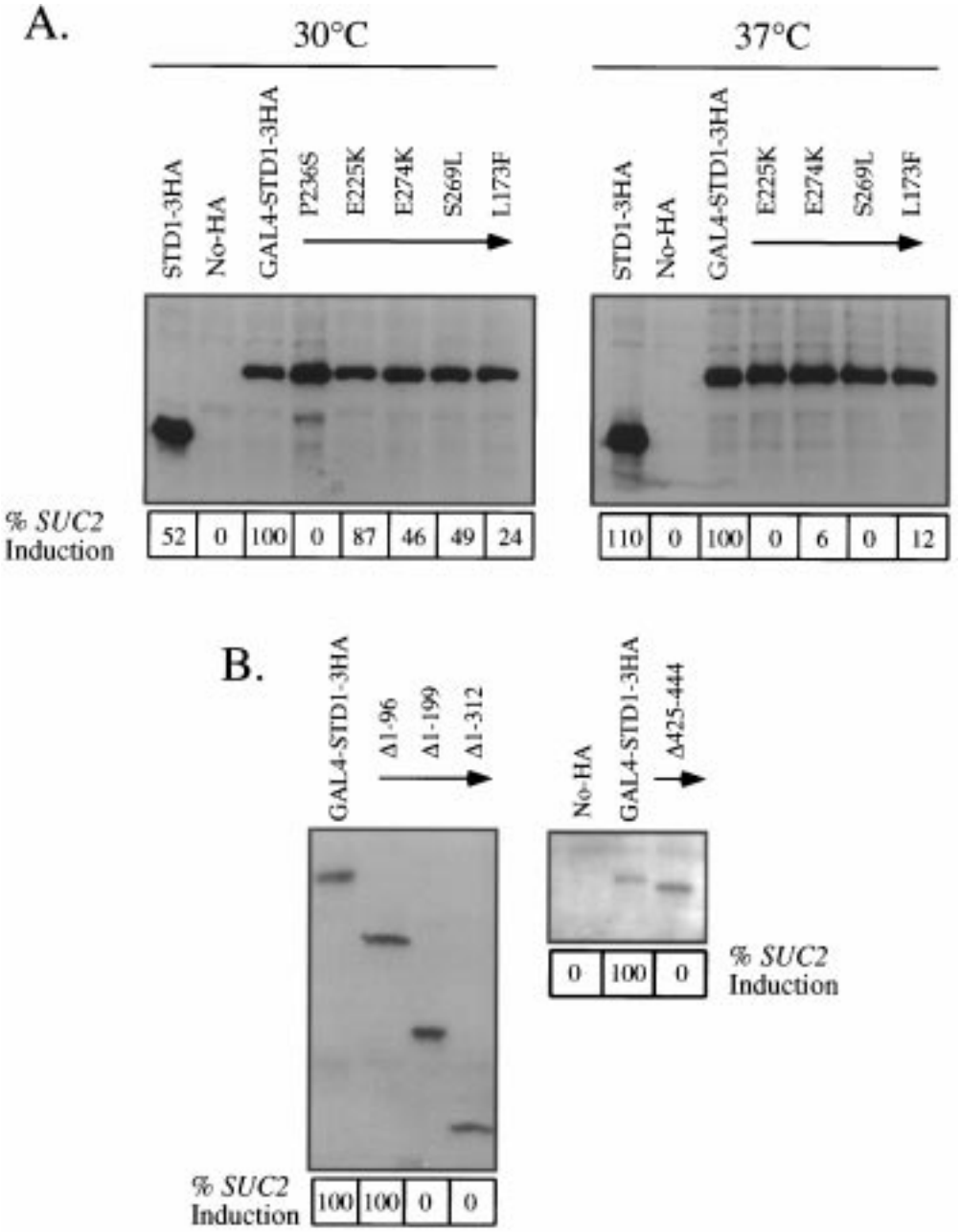

Fig. 5. Effect of *STD1* mutations on protein accumulation. The effect of the *STD1* mutations on protein accumulation was measured by Western blot analysis of cells transformed with plasmids expressing Std1 protein fused to the Gal4 protein DNA binding domain at the N-terminus and three copies of the 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.

on protein accumulation. 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 TBPD57 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, TBPD57 (Ganster et al., 1993). In this assay, the TBPD57 protein is expressed from a CEN

plasmid under the control of the *GAL1* promoter such that cells grown on galactose overexpress TBPD57 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 TBPD57 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 TBPD57 suppression. Likewise, the P236S mutation results in a complete loss of function phenotype in both the *SUC2* induction and in TBPD57 suppression. Analysis of the temperature-sensitive alleles is complicated by the finding that even wild-type Std1 protein is not able to suppress the TBPD57 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 TBPD57 suppression, consistent with the finding that all the ts alleles have a reduced, but detectable, level of *SUC2* inductive activity at 30°C.

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 TBPD57, 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 2–312 resulted in complete loss of function. When this set of N-terminal deletion mutants was tested for suppression of TBPD57, the

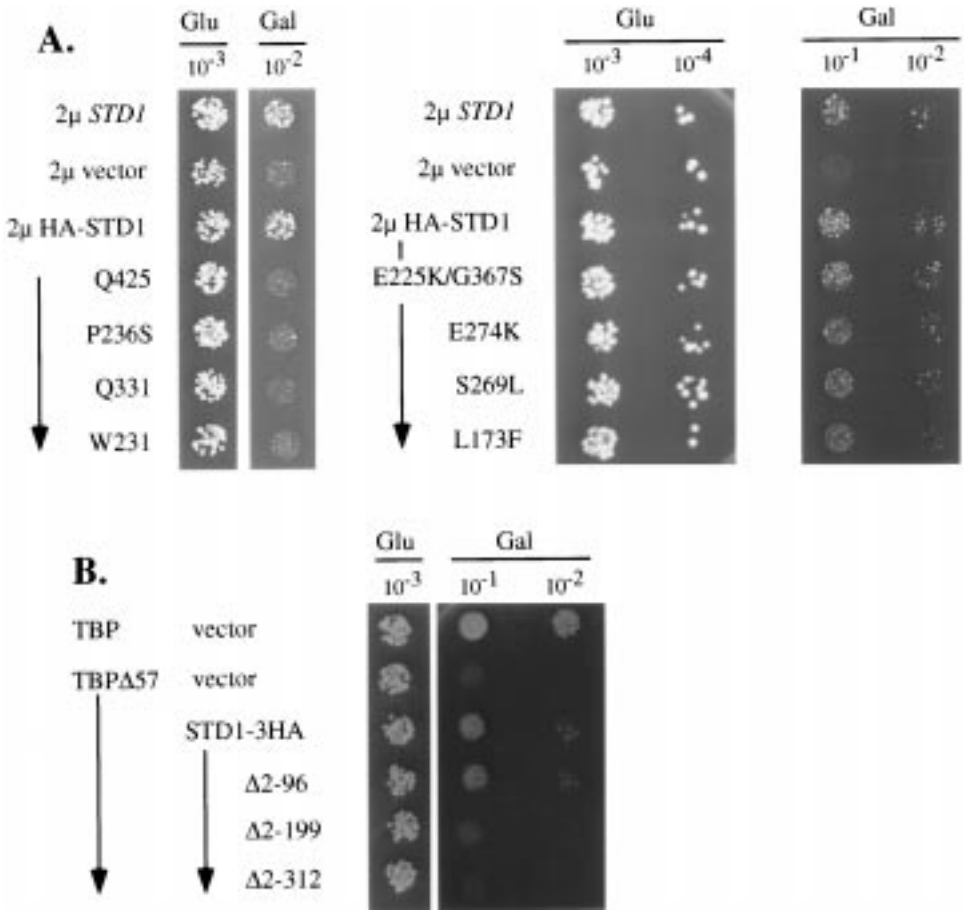

Fig. 6. TBPD57 suppression. Expression of TBPD57 under the control of the *GAL1* promoter results in a severe growth defect (Ganster et al., 1993) that is partially suppressed by the increased gene dosage of *STD1*. (**A**) Yeast cells (BJ1991) were transformed with the TBPD57 expression plasmid pBM2D-D57 and by 2m 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 pBM2D or pBM2DD57, expressing under GAL control the wild-type TBP or TBPAD57, 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 TBPD57 suppression activity. Analysis of these protein constructs by Western blotting revealed that all wild-type and N-terminal deletion 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 TBPD57.

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* derepression, 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 temperature-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 TBPD57 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 conserved between the Std1 and Mth1 proteins are important for Std1 protein function.

The *STD1* gene was isolated in two very different high-copy suppressor screens (Ganster et al., 1993; Hubbard et al., 1994). Genetic and biochemical interactions 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* derepression and then assayed those same alleles for the ability to suppress the TBPD57 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 TBPD57 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 2m 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 constructs 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 TBPD57 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 TBPD57 or TBP thereby lose their ability to affect *SUC2* expression. An alternative possibility is that Std1p 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 TBPD57 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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