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Identification of *cis*-acting elements in the *SUC2* promoter of *Saccharomyces cerevisiae* required for activation of transcription

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ABSTRACT

We analyzed the effects of site-directed mutations in the *SUC2* promoter of *Saccharomyces cerevisiae*. Analyses were performed in wild-type as well as *mig1* and *tup1* mutant strains after the promoter mutants were reintroduced into the native *SUC2* locus on the left arm of chromosome IX. Mutation of the two GC boxes revealed that these elements play two distinct roles: they are, as expected, required for Mig1-mediated repression but they are also necessary for activation of the *SUC2* promoter in response to glucose limitation. The individual GC boxes are functionally redundant with regard to Mig1-mediated repression, however, only the upstream GC box is essential for high level expression of *SUC2*. Micrococcal nuclease sensitivity of the *SUC2* promoter in derepressed cells was reduced in the GC box mutant promoters, particularly in the vicinity of the TATA box. The difference in nuclease sensitivity between wild-type and GC box mutant promoters was not evident in *tup1*$^{-}$ cells. The formation of nuclease-resistant chromatin does not require the GC boxes, indicating that other *cis*-acting elements can serve to recruit the Ssn6–Tup1 co-repressor complex to the *SUC2* promoter.

INTRODUCTION

The *SUC2* promoter is regulated in response to glucose availability (1). In the presence of high glucose concentrations *SUC2* transcription is repressed by the action of the Ssn6–Tup1 co-repressor complex. These proteins associate in a complex (2,3) and are required for transcription repression of genes regulated by glucose, oxygen, DNA damage and mating type (4). The Ssn6–Tup1 co-repressor complex is recruited to specific promoters through interactions with sequence-specific DNA binding proteins. In the case of the *SUC2* promoter the Ssn6–Tup1 complex is thought to be targeted to the *SUC2* promoter by the sequence-specific DNA binding protein Mig1 (5) and its homologs Mig2 and YER028 (6). Mig1, Mig2 and YER028 each contain two zinc finger motifs that are most closely related to a family of GC box binding proteins that includes the human Wilms' tumor and SP1 proteins (7). Recombinant Mig1 and Mig2 proteins bind *in vitro* to a pair of GC boxes (6,7) present 499 and 442 bp upstream of the *SUC2* translation initiation codon (8). Both proteins show a higher affinity for the upstream GC box, designated SUC2A (6).

In the repressed state the *SUC2* promoter region is present in a condensed chromatin structure as judged by its resistance to nuclease cleavage (9,10). Tup1 interacts directly with the N-terminal tails of histones H3 and H4 and this interaction is inhibited by histone acetylation (11). The Ssn6–Tup1 co-repressor complex may act directly in formation and/or maintenance of repressive chromatin structures (10,12). Current models for repression of the *SUC2* promoter envision the Ssn6–Tup1 complex being recruited to the *SUC2* promoter through interactions with Mig1 protein (or homologs) bound to the GC boxes. One prediction of this model that is tested in this report is that mutation of the GC boxes should interfere with Tup1-mediated repression of *SUC2*.

When glucose becomes limiting, expression of *SUC2* is derepressed by a mechanism that requires the Snf1/Snf4 serine-threonine protein kinase complex. The Mig1 protein is phosphorylated in response to glucose limitation (5) and may be a direct substrate for the Snf1 kinase. In addition, derepression of *SUC2* requires activity of the Swi/Snf ATPase complex (13). The Swi/Snf complex contains at least 11 polypeptides (14,15) and can be isolated in association with RNA polymerase II holoenzyme (16). The purified Swi/Snf complex catalyzes changes in nucleosome structure in an ATP-dependent manner and facilitates transcription factor binding to nucleosomal DNA (17–19). In the presence of a transcriptional activator and its recognition sequence the Swi/Snf complex can catalyze eviction of histones, leaving the activator bound in their place (20). *In vivo* Swi/Snf-mediated changes in the chromatin structure of the *SUC2* promoter have been detected in nuclease sensitivity assays (9,10). However, the mechanism by which the Swi/Snf complex is targeted to the *SUC2* promoter and the requirement for any *cis*-acting promoter sequences are still unknown.

Studies using deletions in the *SUC2* promoter region concluded that the upstream activating sequence (UAS) was located between 418 and 650 bp upstream of the translation start codon (21). In this numbering scheme the transcription start site is at base –40 (8) and the TATA box element is located at –133 (9). The UAS is essential for high level expression of *SUC2* under derepressing conditions. However, none of the deletion mutants

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caused a loss in repression only, suggesting that the sequence elements required for high level expression and those required for repression were closely linked and possibly overlapping. Carlson and colleagues noted a repeated sequence element (AAGAAAT) present five times in the UAS region and showed that multiple copies of a 32 bp fragment containing one copy of this element and its flanking sequences could confer some degree of glucose regulation to a heterologous promoter (22). Identification of the Mig1 binding sites in the *SUC2* promoter (7) revealed that the 32 bp element studied by Carlson and colleagues contained most of the downstream Mig1 site (SUC2B). The fact that this element conferred transcriptional regulation by glucose is consistent with the idea that the repression and activation elements are close together and possibly overlapping.

To determine the role played by individual *SUC2* promoter elements, we constructed a set of site-specific mutations in the *SUC2* promoter and used homologous recombination to reintroduce the mutations into the native *SUC2* locus. We examined the effect of the *SUC2* promoter mutations on *SUC2* expression and nuclease sensitivity in both wild-type and *mig1*$^-$ and *tup1*$^-$ mutant strain backgrounds. Our results indicate that the GC box elements function as negative elements during glucose repression but are essential activation elements during derepression. Furthermore, the GC boxes are the first *cis*-acting promoter elements identified in *SUC2* that affect chromatin structure.

MATERIALS AND METHODS

Strains and genetic methods

The yeast strains used in this study (Table 1) were all constructed in an S288C background using standard genetic methods (23). *SUC2* promoter mutations engineered on plasmids were recombined into the native *SUC2* locus by transforming FY637 (*suc2-101*::*URA3*) with a 1.7 kb *Eco*RI–*Bam*HI fragment that spans the promoter and the 5′-half of the open reading frame. Recombinants were selected on medium containing 5-FOA and the genomic structure of each mutant was confirmed by Southern blot (not shown). The *MIG1* gene was disrupted with the *Sma*I–*Hin*dIII fragment from pJN22 that contains the *MIG1* gene with codons 65–460 replaced by the *LEU2* gene. The *TUP1* gene was disrupted by transformation with the *Hin*dIII fragment of pMC134 that contains the *TUP1* gene with codons 70–597 replaced by the *HIS3* gene. Transformants were selected by histidine prototrophy and by the appearance of the flocculent phenotype caused by *tup1* mutations. The *SKOI* gene was disrupted by transformation with the *Xho*I fragment of pHR92 that contains the *SKO1* gene with codons 312–533 replaced by the *LEU2* gene. All gene replacements were confirmed by Southern blot (not shown). Strains with gene disruptions were then crossed with strains bearing the desired *SUC2* allele to produce the strains listed in Table 1.

Mutagenesis of the *SUC2* promoter

Plasmid pRB60, containing the 1.7 kb *Eco*RI–*Bam*HI fragment of the *SUC2* gene (–907 to +792, relative to the translation start site; 24) cloned into pUC118, was used for oligonucleotide-directed mutagenesis (25). The selective oligonucleotide (5′-GGTTTCTT-AGATATCAGGTGGC), which eliminated the unique *Aat*II site present in these plasmids, and the mutagenic oligonucleotide were hybridized to alkaline-denatured pRB60 and were extended and ligated with T4 DNA polymerase and T4 DNA ligase. The mutagenic oligonucleotides used in this study replaced the promoter elements of interest with a 12 bp sequence containing two restriction enzyme recognition sequences. These clustered point mutations do not cause any changes in spacing between other promoter elements. All mutations were confirmed by DNA sequencing and are shown in Figure 1B.

Invertase assays

Repressed and derepressed cells (26) 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 (27). Specific activity was defined in terms of mU invertase activity (1 U being equal to the activity required to release 1 µmol glucose/min) per OD_{600} of cells assayed. Flocculent cells (*tup1*$^-$) were dispersed by suspension in 20 mM EDTA prior to determination of the OD_{600}.

Micrococcal nuclease sensitivity assay

Chromatin structure of the *SUC2* promoter was assayed by micrococcal nuclease treatment of nuclei using the method described by Hirschhorn *et al*. (9).

Table 1.

Strain[a]	Genotype
FY637	MAT**a**, *suc2-101*::*URA3*, *his4-912*δ, *lys2-128*δ, *leu2*Δ*1*, *ura3-52*
MSY230	MAT**a**, *SUC2*, *his4-912*δ, *lys2-128*δ, *leu2*Δ*1*, *ura3-52*
MSY231	MAT**a**, *suc2-31* (*LS-627*), *his4-912*δ, *lys2-128*δ, *leu2*Δ*1*, *ura3-52*
MSY232	MAT**a**, *suc2-32* (*LS-499*), *his4-912*δ, *lys2-128*δ, *leu2*Δ*1*, *ura3-52*
MSY233	MAT**a**, *suc2-33* (*LS-442*), *his4-912*δ, *lys2-128*δ, *leu2*Δ*1*, *ura3-52*
MSY234	MAT**a**, *suc2-34* (*LS-442/499*), *his4-912*δ, *lys2-128*δ, *leu2*Δ*1*, *ura3-52*
MSY222	MAT**a**, *suc2-22* (*LS-133*), *his4-912*δ, *lys2-128*δ, *leu2*Δ*1*, *ura3-52*
MSY229	MAT**a**, *suc2-29* (*LS-49*), *his4-912*δ, *lys2-128*δ, *leu2*Δ*1*, *ura3-52*
MSY261	MATα, *mig1*::*LEU2*, *SUC2*, *ura3-52*, *leu2*Δ*1*
MSY263	MATα, *mig1*::*LEU2*, *suc2-33*, *ura3-52*, *leu2*Δ*1*
MSY265	MATα, *mig1*::*LEU2*, *suc2-32*, *ura3-52*, *leu2*Δ*1*, *trp1*Δ*63*
MSY269	MATα, *mig1*::*LEU2*, *suc2-34*, *ura3-52*, *leu2*Δ*1*, *lys2-128*δ
MSY271	MAT**a**, *tup1*::*HIS3*, *SUC2*, *ura3-52*, *leu2*Δ*1*, *his3*Δ*200*, *trp1*Δ*63*, *lys2-128*δ
MSY272	MAT**a**, *tup1*::*HIS3*, *suc2-33*, *ura3-52*, *leu2*Δ*1*, *his3*Δ*200*, *trp1*Δ*63*, *lys2-128*δ
MSY273	MAT**a**, *tup1*::*HIS3*, *suc2-32*, *ura3-52*, *leu2*Δ*1*, *his3*Δ*200*, *trp1*Δ*63*, *lys2-128*δ
MSY275	MAT**a**, *tup1*::*HIS3*, *suc2-34*, *ura3-52*, *leu2*Δ*1*, *his3*Δ*200*

[a]All strains are from our laboratory except FY637, which was provided by Fred Winston (Harvard University).

RESULTS

Mutagenesis of the *SUC2* promoter

In order to identify the *cis*-acting elements that are required for regulation of *SUC2* transcription, we constructed and analyzed a set of linker scan (LS) mutations in the promoter region. Each LS mutation replaced 12 bp of *SUC2* sequence with an unrelated 12 bp sequence containing the recognition sequences of two restriction enzymes. In this way the spacing between unaffected promoter elements was not changed. The sequences of the LS mutations

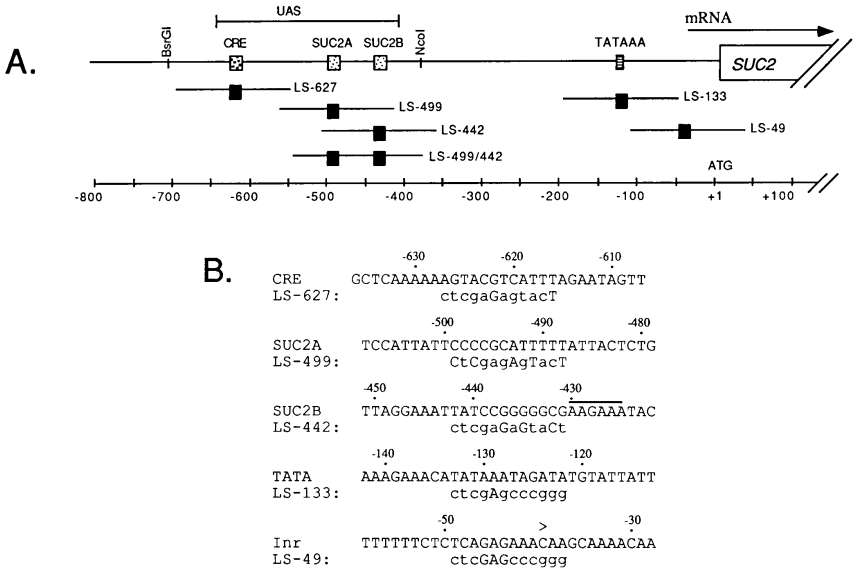

Figure 1. *SUC2* promoter mutations. (**A**) Schematic drawing of the *SUC2* promoter drawn to scale and showing the relative position of the promoter elements that were mutated. (**B**) Sequences of the promoter mutations are shown below the wild-type sequence. Nucleotides that are changed are shown in lower case. Nucleotides that were not altered by the specific mutations are shown in upper case. Numbering is relative to the translation initiation codon. The line drawn over nucleotides –430 to –425 identifies the repeated element noted by Sarokin and Carlson (22). The transcription start site at –40 and direction of transcription are indicated by >.

described here are shown in Figure 1. These mutations were created first in plasmid DNA and were confirmed by restriction analysis and DNA sequencing. Each promoter mutation was then introduced into the *SUC2* locus of chromosome IX by homologous recombination. The recipient strain carried the *suc2-101*::*URA3* allele, allowing recombinants to be selected on medium containing 5-FOA (9). Independent isolates of each construct were obtained and confirmed by Southern blot analysis (not shown).

Core promoter elements of the *SUC2* promoter

The core promoter of mammalian genes contains either a TATA box, an initiator element or both (28). We mutated the TATA element and the sequences surrounding the initiation site of *SUC2* (Fig. 1) and measured the effect of these mutations on invertase expression. Mutation of the TATA box element located at –133 resulted in a reduction of the repressed level of invertase (9 versus 16 mU/OD) and severely reduced the derepressed level (95 versus 948 mU/OD; Fig. 2). The critical role played by this TATA element has been observed previously (9,21).

Initiator elements are defined as sequence elements that span the transcription start site and can direct accurate initiation in the absence of a TATA box (29). To determine whether or not regulation of the *SUC2* gene was influenced by sequences at the transcription start site, we mutated the sequences from –9 to +3 relative to the transcription start site (Fig. 1) and measured invertase expression under repressing and derepressing conditions. This mutation did not significantly affect the level of invertase expression under either condition, indicating that the sequences at the transcription start site are not important for regulation (Fig. 2). However, the sequences flanking the start site do play a role in start site selection, as measured by primer extension analysis (data not shown). Inr mutation caused the 5′-end of the *SUC2* mRNA to become more heterogeneous, with multiple 5′-ends detected. We conclude that the sequences that flank the *SUC2* mRNA start site affect start site selection but do not affect regulation or level of *SUC2* expression.

The CRE motif confers modest repression of the *SUC2* promoter

The SKO1/ACR1 protein is a basic region leucine zipper protein in the ATF/CREB family of transcription factors (30,31). DNA binding studies using *in vitro* translated SKO1 (31) indicated that the SKO1 protein bound with high affinity to oligonucleotides containing the CRE motif (TGACGTCA). SKO1 also bound, although with apparently lower affinity, an oligonucleotide containing a related sequence, AGTACGTCAT, that is present in the *SUC2* promoter 627 bp upstream of the initiating ATG codon (31). We have replaced this sequence with an unrelated 12 bp of DNA (LS-627; Fig. 1) and tested the effect of this mutation on *SUC2* expression when reintroduced into the native *SUC2* locus. Mutation of the CRE motif resulted in slightly increased levels of invertase expression (<1.5-fold) under either repressed or derepressed conditions (Fig. 2), confirming that this promoter element is indeed functional in the native promoter and that it acts as a negative element under both growth conditions. A similar increase in invertase levels under both growth conditions was observed in strains containing the null allele *sko1*::*LEU2* (31; data not shown).

Role of the GC box elements

The *SUC2* promoter UAS contains two GC box sequences that are bound by the Mig1 (7) and Mig2 (6) proteins *in vitro*. The two GC box elements at –499 and –442 are located within a region (–650 to –418) that promoter deletion studies had shown was required for derepression of *SUC2* expression (21). These elements have been denoted SUC2A and SUC2B respectively (6,32). In order to directly test the role of the GC box elements in regulated expression of *SUC2*, we replaced the sequences that

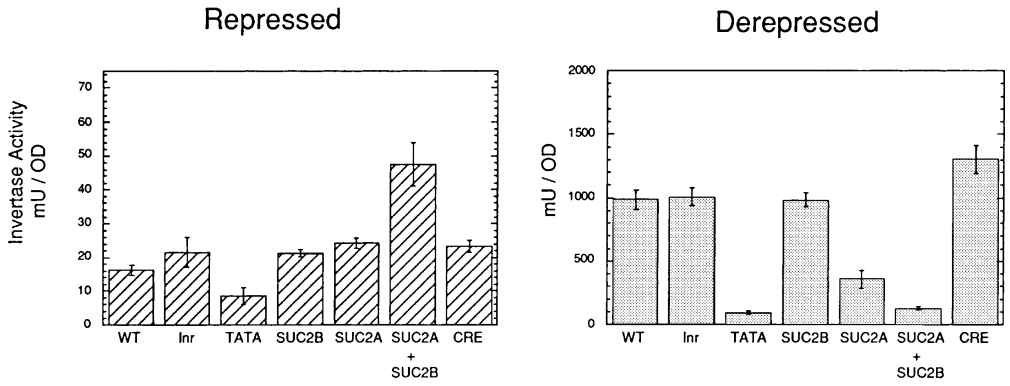

Figure 2. Effect of *SUC2* promoter mutations on invertase expression. Yeast cells from repressed and derepressed cultures were harvested and assayed for invertase activity. The strains used in this experiment, containing either the wild-type or the indicated *SUC2* promoter mutation, were MSY230, MSY229, MSY222, MSY233, MSY232, MSY234 and MSY231. The mean value from at least three independent determinations is plotted with the error bar representing 1 SD.

comprise the downstream GC box (SUC2B) and the upstream GC box (SUC2A) or both with an unrelated 12 bp of sequence (Fig. 1). These mutations eliminate both the GC box sequence and the flanking AT-rich sequence that define high affinity Mig1 sites (32). The individual SUC2A and SUC2B mutations and the double mutation were reintroduced into the native *SUC2* locus and their effect on *SUC2* expression was determined (Fig. 2).

Mutation of either SUC2A or SUC2B resulted in a consistent but slight increase (<2-fold) in the level of *SUC2* expression under repressed conditions. Therefore, neither GC box element was by itself essential for repression. However, mutation of both GC boxes did result in a more significant increase in *SUC2* expression (3-fold), suggesting that these elements are partially redundant with respect to repression of *SUC2*. Since our data with repressed cells indicated that the GC boxes were required for full repression, we were intrigued to find that these same elements were also important promoter elements during derepression (Fig. 2). Mutation of SUC2B by itself had no effect on the derepressed level of invertase expression, indicating that SUC2B is not required for high level expression of *SUC2*. In contrast, mutation of SUC2A by itself resulted in a >2-fold reduction in the derepressed level of *SUC2*. Therefore, SUC2A and SUC2B are functionally distinct and SUC2A by itself is essential for normal derepression. Mutation of both SUC2A and SUC2B elements resulted in a greater defect than either single GC box mutation and was comparable in severity to the TATA box mutation. Thus SUC2B is also a functional activation sequence when SUC2A is absent.

Mig1-mediated repression requires the GC boxes

In order to assess the connection between the GC boxes and Mig1-mediated repression, a *mig1* null allele (*mig1*::*LEU2*) was introduced by one step gene replacement into each of the strains bearing mutations in the GC boxes. Mutation of the *MIG1* gene results in partial derepression of the wild-type *SUC2* gene (Fig. 3), a result that has been observed by others (7,33). The partial derepression of *SUC2* caused by the *mig1* mutation is observed in both the SUC2A and SUC2B mutant promoters, indicating that either GC box element is sufficient for Mig1-mediated repression of *SUC2*. However, mutation of both GC boxes completely abrogates any effect of Mig1 on the *SUC2* promoter, indicating that the ability of the Mig1 protein to confer repression on the *SUC2* promoter is absolutely dependent on the presence of either SUC2A or SUC2B. The Mig1 protein also represses *SUC2* expression during derepression, an effect that is also GC box dependent. Differences in the absolute value of invertase activity observed between the experiments shown in Figures 2–4 (for instance 1000 versus 1500 mU/OD for the wild-type derepressed value) can be attributed to the use of rich medium (YEPD) in Figure 2 and synthetic complete medium in Figures 3 and 4. However, in all experiments reported here multiple independent cultures were assayed and 1 SD is indicated for all values.

Activation of *SUC2* in the absence of TUP1-mediated repression

The predominant effector of glucose repression of *SUC2* expression is the Ssn6–Tup1 co-repressor complex. In addition to counteracting the repression by Ssn6–Tup1, full derepression of *SUC2* involves an activation step that is most easily discernible in *tup1* mutants (33). We investigated in *tup1*$^-$ cells the *cis* elements needed for activation of *SUC2* in response to glucose limitation (Fig. 4). Cells with a wild-type *SUC2* promoter in a *tup1*$^-$ background have high levels of invertase expression under repressing conditions indicative of the loss of Tup1-mediated repression. Shifting these cells to low glucose medium causes an additional 2-fold increase in invertase levels (2230 versus 1230 mU/OD) that represents the activation pathway. The effects of introducing mutations in the GC boxes were determined in wild-type and *tup1*$^-$ strains. In *tup1*$^-$ cells activation of *SUC2* expression by glucose limitation was largely dependent on the upstream GC box, SUC2A. In the absence of the SUC2A site there was no significant difference between invertase expression under high or low glucose conditions. In contrast, mutation of the downstream GC box, SUC2B, did not abrogate induction of invertase by glucose limitation. These results underscore our earlier conclusion that the two GC boxes are functionally distinct during derepression and that the upstream GC box, SUC2A, plays a critical role in activation of *SUC2* transcription. The mutation of both GC boxes greatly impairs expression of *SUC2* even in a *tup1*$^-$ background. This result demonstrates that relief from Tup1-mediated repression by itself is not sufficient for high level expression of *SUC2*. High levels of *SUC2* transcription requires both

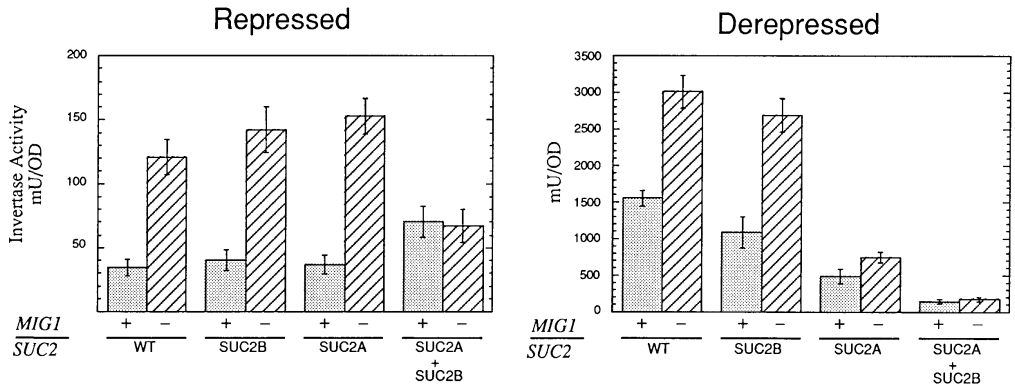

Figure 3. Mig1-mediated repression requires the SUC2A and SUC2B elements. Yeast cells from repressed and derepressed cultures were harvested and assayed for invertase activity. The strains used in this experiment, containing either a wild-type *MIG1* gene (+) or the *mig1*::*LEU2* null allele (–) and either the wild-type *SUC2* gene or the promoter mutations indicated, were MSY230, MSY261, MSY233, MSY263, MSY232, MSY265, MSY234 and MSY269. The mean value from at least three independent determinations is plotted with the error bar representing 1 SD.

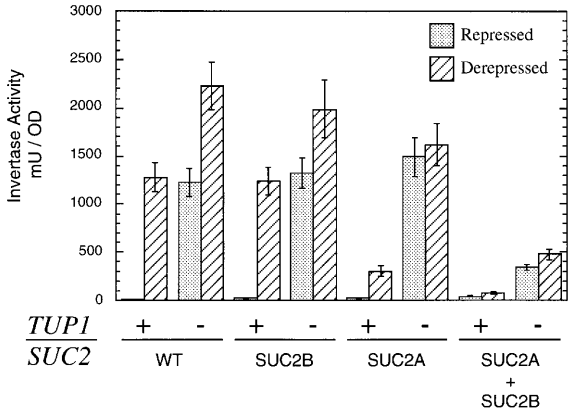

Figure 4. Activation of *SUC2* by glucose limitation requires the SUC2A and SUC2B elements. Yeast cells from repressed and derepressed cultures were harvested and assayed for invertase activity. The strains used in this experiment, containing either a wild-type *TUP1* gene (+) or the *tup1*::*HIS3* null allele (–) and either the wild-type *SUC2* gene or the promoter mutations indicated, were MSY230, MSY271, MSY233, MSY272, MSY232, MSY273, MSY234 and MSY275. The mean value from at least three independent determinations is plotted with the error bar representing 1 SD.

relief from Tup1-mediated repression and also GC box-dependent activation.

GC box mutations affect chromatin structure

Having identified the GC boxes as *cis*-acting elements essential for high level *SUC2* expression, we next investigated whether loss of these elements affects nuclease sensitivity of the *SUC2* promoter in isolated yeast nuclei. In this experiment micrococcal nuclease (MNase) digestion of nuclei from derepressed cultures (9) was analyzed in wild-type and various mutant yeast strains (Fig. 5). Nuclease sensitivity of sites surrounding the TATA element is diagnostic of active chromatin (9,10) and is readily detected in wild-type derepressed cells (Fig. 5, lanes 2–4). Mutation of both SUC2A and SUC2B results in a reduction in MNase cleavage relative to the wild-type *SUC2* promoter. In particular, three sites of cleavage (marked by asterisks) in the wild-type pattern are greatly reduced or missing in the SUC2A+B mutant. Conversely, two novel sites of MNase cleavage appear in the SUC2A+B mutant promoter (marked by <, lanes 6–8). We conclude that mutation of SUC2A+B results in distinct changes in the MNase cleavage pattern. The altered cleavage pattern is not likely due to changes in the promoter DNA sequence itself, since changes in the cleavage pattern occur as far as 300 bp downstream of the SUC2A+B mutations. This result suggests that the changes in MNase cleavage reflect changes in proteins bound to the *SUC2* promoter. Indeed, this suggestion is supported by the finding that the differences between the wild-type and SUC2A+B mutant cleavage patterns are largely eliminated in a *tup1*$^{-}$ background (lanes 9–16). In addition, comparison of the cleavage patterns of the SUC2A+B mutant in *TUP1*$^{+}$ and *tup1*$^{-}$ cells reveals distinct changes in cleavage pattern, indicating that the Ssn6–Tup1 co-repressor complex can act directly at the *SUC2* promoter in the absence of the SUC2A and SUC2B elements. Therefore, additional *cis*-acting elements must be present in the *SUC2* promoter that act to recruit the Ssn6–Tup1 complex.

DISCUSSION

Core promoter elements

Two core promoter elements of the *SUC2* promoter were analyzed by mutation. Mutation of the TATA box sequence at –139 causes a severe defect in the derepression pathway. However, the promoter bearing the TATA mutation is still capable of a 10-fold induction in response to glucose limitation (9.5 versus 95 mU/OD; Fig. 2). Alternative TATA-like sequences present upstream of the start site may be utilized when the cognate TATA is mutated. The sequences TATAAT at –160 and TATTATT at –119 are candidates for cryptic TATA elements that may become functional in the absence of the cognate TATA element at –139. The second core promoter element characterized in this study was the sequences surrounding the transcription start site. The start site of transcription for the secreted form of invertase has been mapped to a cytosine residue 39 bp upstream of the initiating ATG codon (8). Our primer extension assays of *SUC2* mRNA are in agreement with this result (34). The *SUC2* initiation site (AACAA) conforms with the start site consensus, RRYRR, first proposed by Hahn *et al*. (35) and subsequently supported by analysis of a series of point mutations in the *TRP4* promoter (36). The initiator mutation altered the sequences at and surrounding the start site to eliminate the fit to the consensus sequence. In the

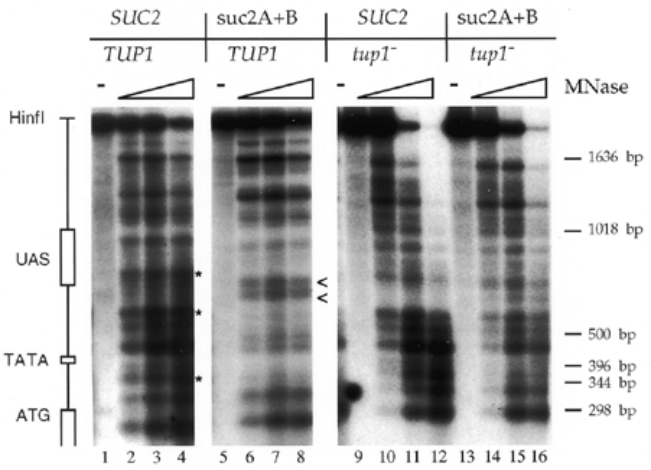

Figure 5. Requirement of the GC boxes for chromatin remodeling. Yeast were grown under derepressing conditions. Nuclei were isolated and treated with increasing concentrations of micrococcal nuclease (0–50 U/ml) as indicated by the ramps. DNA was purified and digested to completion with *Hin*fI, which cleaves the *SUC2* gene at –1753 and +291 relative to the ATG. Sites of MNase cleavage were determined by indirect end-labeling. Cleavages present in the wild-type *SUC2* promoter but reduced in the SUC2A+B mutant promoter are indicated by asterisks; cleavages enhanced in the SUC2A+B mutant promoter are marked by <. Chromatin was prepared from wild-type cells or *tup1*⁻ cells, each containing either wild-type *SUC2* promoter or the SUC2A+B mutant promoter as indicated. The strains used in this experiment were MSY230, MSY234, MSY271 and MSY275.

absence of the preferred start site sequence transcription was initiated at multiple positions, including the nearest base downstream (+3) that conformed to the RRYRR consensus (data not shown). However, the alterations in start site selection did not have any significant effect on the level of invertase expression. Therefore, the sequences surrounding the *SUC2* start site do not play a significant role in *SUC2* regulation.

Dual function elements

Identification of the GC boxes in *SUC2* that bind the Mig1 and Mig2 proteins (6,7) allowed us to test directly whether these elements function only in repression or whether they are also required for derepression. Our results show quite clearly that the GC boxes play two distinct roles in regulation of *SUC2* expression. In the repressed state the GC boxes are necessary for Mig1-mediated repression. Either SUC2A or SUC2B is sufficient for Mig1-mediated repression, indicating that these two elements are functionally redundant with regard to Mig1 function. The requirement for SUC2A and SUC2B for Mig2 or YER028 function has not been tested (7,32).

The two GC box elements are functionally distinct during derepression, with only SUC2A being essential for high level *SUC2* expression. Mutation of both GC boxes results in a severely defective promoter that is unable to efficiently express high levels of *SUC2* mRNA. These data suggest that either the SUC2A element has a greater affinity for the *SUC2* activator(s) or that its position in the promoter leads to more efficient activation of transcription. This prediction can be tested by exchanging the GC box elements or by direct measurement of binding affinity of the activator(s) for the two GC boxes. A precedent for dual function GC boxes has been observed in mammalian cell transfection experiments where the GC box binding repressor WT-1 and the GC box binding activator EGR-1 compete for the same promoter elements (37,38). Indeed, the finding that Mig1 expression represses *SUC2* during derepression can be explained if there were direct competition between the Mig1 protein and the *SUC2* activator(s) for binding to the GC boxes. Repression mediated by both WT-1 (39) and Mig1 (40) is dependent on an effector domain present outside the DNA binding domain. In the case of Mig1 this domain is thought to recruit the Ssn6–Tup1 co-repressor complex to the promoter (5). In the case of WT-1 the proteins it presumably recruits to the promoter have not yet been identified. Recently a GC box element in the yeast *CAT8* promoter, termed URECAT8, has been shown to act as a dual function element (41). In the presence of glucose this element acts as a Mig1p-dependent repressor. However, in medium containing ethanol as the carbon source this same element is required for activation of transcription. It is possible that the same activator(s) that responds to glucose levels and binds the Mig1p sites in *SUC2* may also bind and activate transcription at the Mig1p site in the *CAT8* promoter.

Identity of the *SUC2* activator

The GC boxes in the *SUC2* promoter are essential for high level expression. The identity of the transcriptional activator(s) that binds to these elements was not determined in this study. However, some conclusions can be drawn concerning the identity of the *SUC2* activator(s). The activator(s) cannot be Mig1 protein. This is evident from the data reported here (Fig. 3) and elsewhere that shows efficient derepression of *SUC2* in the absence of Mig1 protein (33). This conclusion appears obvious, but is important to note since recent studies of Mig1 indicate that in an *ssn6*⁻ background Mig1 can act as an activator of transcription (5). Furthermore, the activator cannot be Mig2 or YER028, since mutations in these proteins do not interfere with activation of *SUC2* in response to low glucose (6). Searches of the yeast genome identified >30 zinc finger proteins that may possess GC box binding activity. Two in particular, Msn2p and Msn4p, identified previously as potential activators of *SUC2*, contain zinc fingers related to those in Mig1 (42). However, these proteins have recently been shown to regulate transcription through stress response elements (STRE), whose sequences are distinct from both SUC2A and SUC2B (43,44). Therefore, it is unlikely that Msn2p and Msn4p are the *SUC2* activators which function through the SUC2A and SUC2B elements. Their effect on *SUC2* expression may be indirect or may be due to the presence of two cryptic STRE elements (AGGGG) present in the *SUC2* promoter region at –806 and –719. Inactivating mutations in the *SUC2* activator have never been isolated in genetic screens for a sucrose non-fermenting phenotype. One possible explanation for this may be the presence of more than one activator gene. Lastly, there is no reason *a priori* to assume that the activator of *SUC2* is necessarily a zinc finger protein. Further studies with these mutant *SUC2* promoters and other reporter constructs will be needed to unambiguously identify the *SUC2* activator(s).

Mig1-independent repression by TUP1

Mutation of the *MIG1* gene has a much smaller effect on *SUC2* repression than does mutation of the *TUP1* gene (33). Thus recruitment of Ssn6–Tup1 to the promoter by Mig1 is only part of the Tup1 repression activity at *SUC2*. The ability of Tup1 to repress *SUC2* expression in the absence of Mig1 might be

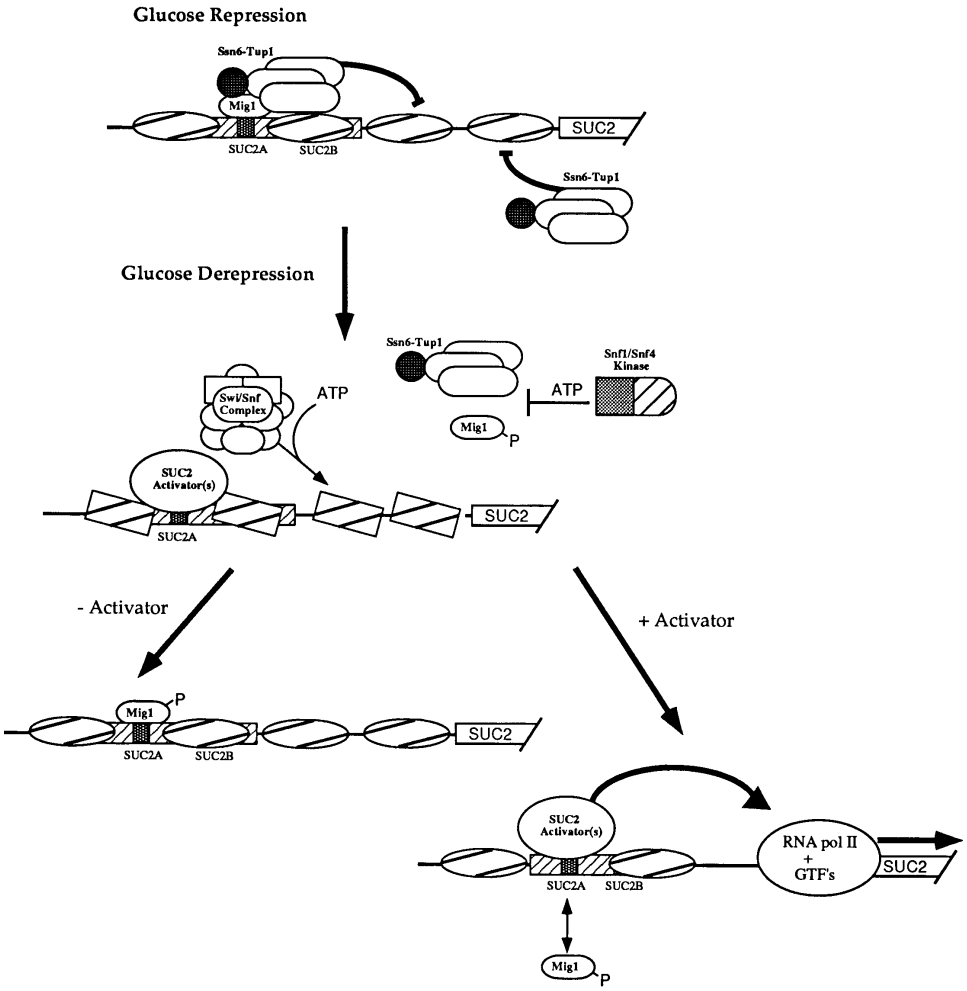

Figure 6. Model of *SUC2* regulation. The yeast *SUC2* gene is shown in the repressed state with nucleosomes (ovals) positioned over the core promoter region and stabilized by the Ssn6–Tup1 complex using both Mig1-dependent and -independent mechanisms. During derepression the Mig1 protein becomes phosphorylated in a Snf1-dependent manner. Positioned nucleosomes are activated (rectangles) by the Swi/Snf complex. In the absence of activator binding, as is the case in the SUC2A SUC2B double mutant, the activated nucleosomes are not stable and revert to the relaxed state found during repression. In the presence of activator binding the activated nucleosomes are displaced, allowing RNA polymerase II and the general transcription factors (GTFs) to bind.

explained by the recent discovery of two Mig1 homologs, Mig2 and YER028 (6). Alternatively, it is possible that Mig1-independent repression by Tup1 is due to effects of Tup1 at other genes whose products are themselves regulators of *SUC2*. If Ssn6–Tup1 is recruited to *SUC2* by Mig2 and/or YER028 then one would expect these highly related proteins to use the SUC2A and SUC2B elements as binding sites. Indeed, the Mig2 protein has been shown to bind to SUC2A *in vitro* (6). Our studies show that the Tup1 protein has a large effect on the MNase cleavage pattern of the *SUC2* promoter even in the absence of the SUC2A and SUC2B elements. Thus Tup1-mediated repression of *SUC2* must be targeted by some additional GC box-independent sequence element. A good candidate for the GC box-independent pathway of Tup1 repression is one which utilizes the recently identified URS element located between the GC boxes and the TATA box of the *SUC2* promoter (1). Our data are consistent with a model in which the Ssn6–Tup1 complex can be recruited to the *SUC2* promoter by either GC box- or URS-dependent pathways.

SUC2 as a paradigm for regulation by chromatin structure

The discovery of the nucleosome remodeling activity of the yeast Swi/Snf complex (19) has led to intensive study of this complex and its metazoan homologs (17,45). Large portions of metazoan DNA are packaged in transcriptionally inactive heterochromatin and complexes with chromatin remodeling activity are likely to determine patterns of gene regulation. Studies of *SUC2* regulation demonstrated that chromatin remodeling by the Swi/Snf complex precedes and is independent of increased transcription of *SUC2* (9). Our data identify *cis*-acting elements in the *SUC2* promoter that are required for formation of active chromatin. The simplest explanation for our findings is that an activator protein present in derepressed cells plays an active and essential role in chromatin remodeling. Based on the data presented here and elsewhere, we propose the following model for *SUC2* regulation (Fig. 6). In the presence of high glucose concentrations (repressing conditions) *SUC2* expression is repressed by the action of the Ssn6–Tup1

complex. This complex, found in a stoichiometry of one Ssn6p bound to three subunits of Tup1p (3), is not required for nucleosome postioning over the critical core promoter elements but may act to increase the stability of the repressive chromatin (10). Interestingly, the SUC2A element is found in a linker region between nucleosomes (10), thereby allowing access to DNA binding proteins that may participate in either repression or activation. The finding that SUC2A is present in linker DNA while SUC2B is packaged in a nucleosome might explain our finding that SUC2A plays a more prominent role in derepression. The Ssn6–Tup1 complex is most likely recruited to the *SUC2* promoter by the Mig1 protein bound at SUC2A, since deletion of *MIG1* but not of *MIG2* or YER028 produces an increase in *SUC2* expression under repressing conditions (6). Our nuclease sensitivity data indicate that the Ssn6–Tup1 complex can also be recruited to the *SUC2* promoter by a GC box-independent mechanism, presumably through interactions with other DNA binding proteins. When cells encounter medium with low glucose concentrations (derepressing conditions) the Snf1/Snf4 protein kinase complex inactivates the Ssn6–Tup1 complex, possibly by direct phosphorylation of the Mig1 protein (5), and possibly other proteins. Phosphorylation of the Mig1 protein may disrupt its interaction with the Ssn6–Tup1 complex, thereby destabilizing the repressive chromatin. The Swi/Snf complex is recruited to the *SUC2* promoter, possibly through interactions with the *SUC2* activator bound at SUC2A, and it then destabilizes the nucleosomes covering the promoter. This change in nucleosome structure is not stable. In the absence of activators or their binding sites (i.e. the SUC2A and SUC2B elements) the nucleosomes can revert to their normal structure and position, with consequent inhibition of preinitiation complex formation. When activator(s) is present Swi/Snf facilitates nucleosome eviction (20), leading to recruitment of RNA polymerase II and general transcription factors to the core promoter. Since the Swi/Snf complex can be found associated with RNA polymerase II holoenzyme (16), it is possible that the *SUC2* activator may recruit both the polymerase and the Swi/Snf complex together. During derepression phosphorylated Mig1 protein still inhibits *SUC2* expression, though we suggest that this is not through interactions with Ssn6–Tup1, but by direct competition with the activator(s) for GC box occupancy. Our data support this model and suggest that one or more activators binding to the GC boxes are required to stabilize changes in chromatin structure. However, chromatin remodeling by itself is not sufficient for transcriptional induction (9). Gene transcription requires two conditions be met: both an open chromatin structure and also recruitment of RNA polymerase. The *SUC2* activator protein(s) is likely to participate in both steps of gene activation.

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