SIN3 works through two different promoter elements to regulate INO1 gene expression in yeast

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ABSTRACT

The SIN3 global regulatory factor affects expression of many yeast genes, including the phospholipid biosynthetic gene, INO1. Mutations in the SIN3 gene result in elevated levels of INO1 expression under conditions that normally confer full repression of INO1 transcription, indicating that SIN3 is a negative regulator of INO1. In this study, the INO1 promoter was analyzed for sequences that play a role in responding to SIN3-mediated repression. Two distinct promoter elements, the upstream repression sequence (URS1) and the INO1 upstream activation sequence (UASINO) both were found to be involved in enabling SIN3 to repress INO1 expression.

INTRODUCTION

The SIN3 gene encodes a global regulatory factor that has been shown to affect expression of numerous unrelated genes in yeast. Examples of some of the genes that are affected by a sin3 mutation include HO (1,2), TRK2 (3), SPO11, SPO13 (4), IME2 (5), PHO5 (6), and the phospholipid biosynthetic genes INO1, CHO1, CHO2 and OPI3 (7). Generally, expression of these genes is moderately elevated in a sin3 mutant background under conditions that normally allow for their repression. The SIN3 gene encodes a 175 kDa protein containing four paired amphipathic helix (PAH) domains that lack the preceding basic regions thought to be important for DNA binding (6,8). There has been no reported evidence demonstrating direct DNA binding of the Sin3 protein. It has been proposed that the SIN3 gene product may act to control expression of its numerous target genes through protein–protein interactions with more specific DNA binding proteins (6,9), but the precise mode of SIN3 action remains unknown.

There is no single common element that is known to be present in the promoters of all genes that are regulated by SIN3. However, sin3 mutations have been shown to affect expression of several yeast genes that contain an upstream repression sequence (URS1) in their promoters, including INO1 (11), HO (12), SPO13 (13) and IME2 (5). A functional relationship between SIN3 and the URS1 element has been reported. Work by Bowdish and Mitchell (5) suggests a model in which the UME6 and SIN3 gene products work through the IME2 URS1 element to repress IME2 transcription in non-meiotic cells. A separate study demonstrated that a binding activity on the HO promoter, called Sdp1, is missing in cell extracts derived from sin3 mutants (14). Interestingly, the binding site for Sdp1 in the HO promoter shows strong similarity to the URS1 sequence (9). It is not clear whether all genes that are regulated by SIN3 contain a URS1 element in their promoters. The CHO2, OPI3 and CHO1 promoter regions, for example, contain no URS1 consensus element although they may contain URS-like sequences (D. Stillman, personal communication). In addition, it has also been reported that a sin3 mutation has little effect on repression of the CYC1 UAS through URS1 (15,16). Therefore, the precise relationship between SIN3 and the URS1 element in the overall regulation of yeast genes remains unclear at this time.

Regulation of the yeast INO1 phospholipid biosynthetic gene is affected by a mutation in the SIN3 gene. INO1 encodes the enzyme inositol-1-phosphate synthase, which catalyzes a key step in the synthesis of phospholipids. Expression of INO1 is maximal in the absence of the phospholipid precursors inositol and choline (derepressing conditions). When wild-type yeast cells are grown in the presence of inositol and choline (repressing conditions), INO1 expression is repressed at the transcriptional level (17). In sin3 mutant strains this regulation is altered, resulting in elevated levels of INO1 expression under repressing growth conditions (7).

The INO1 promoter region has been extensively analyzed for sequences that may be involved in the control of INO1 expression (11,17–19). A 10 bp element of consensus sequence 5'-CATGTTGAAAAT-3', designated UASINO, is repeated six times in the INO1 promoter (Fig. 1) (17,19). This element is also found upstream of the CHO1, CHO2 and OPI3 phospholipid structural genes (17,19). None of the native UASINO elements located upstream of the phospholipid structural genes are perfect matches to the consensus, as they all represent 7–9 nt matches to the 10 bp consensus. The UASINO element has been identified in the promoters of many other genes involved in phospholipid metabolism in addition to the ones discussed here, including genes that encode enzymes of the Kennedy pathway, the inositol transporter, the INO2 and INO4 regulatory genes, genes involved in fatty acid synthesis, and others (19). In addition, UASINO-like elements have been identified in some genes that are unrelated to phospholipid synthesis (19). There are two known examples of non-phospholipid genes that are regulated by SIN3 and that also contain a UASINO in their promoters: PHO5 (19) and SPO13 (J. Lopes, personal communication; (13)]. In addition

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to the UASINO element, a sequence identical to the URS1 consensus element, AGCCGCCGA, has also been found in the INO1 promoter (Fig. 1) (11). It has been demonstrated that this element plays a functional role in repression of INO1 gene expression (11). The purpose of this work was to identify sequences in the INO1 promoter that are involved in 5/Atf-mediated repression of INO1.

**MATERIALS AND METHODS**

**Strains and plasmids**

The congenic yeast strains used in this study were: SH296, MATa his3 trp1 ura3 sin3::TRP1 leu2 lys2; SH338, MATa his3 trp1 ura3. Plasmids used in this study are listed in Table 1. Plasmid pNB404 (N. Bachhawat, personal communication) was constructed by deleting the CYC1-UAS from plasmid pNG22 (20) and then using the pNG22 CYC1 AUAS promoter region, including the polylinker region, to replace the polylinker region of pJ304 (Table 1), which does not have a polylinker region. The plasmid pNB404, therefore, contains the polylinker region from pNG22, but is based upon pJH304, as is pCON1. To construct pNB503, an oligonucleotide with the sequence:

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ggccgtcCATGTGAAATgc
caAGTACAC TTTAc age t
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was cloned into the polylinker region of pNB404 at the SalI and Eagl sites. The 10 bases in capital letters represent the UASINO consensus sequence as discussed in the Introduction.

**Yeast media**

*Vitamin-defined complete synthetic media.* Three percent glucose, 0.17% YNB Salts (recipe below), 0.0002% trace components (recipe below), 0.5% ammonium sulfate, 1% vitamin mix (21) and supplements (amino acids, uracil and adenine) as described by Culbertson and Henry (22). Where appropriate, medium was supplemented with 75 μM inositol and 1 mM choline (1°C). Unsupplemented medium is designated 1°C. When necessary for plasmid maintenance, uracil was omitted from media. (The YNB salts, trace components and ammonium sulfate, as described in this media recipe, replace 'Yeast Nitrogen Base without Vitamins', formerly manufactured by Difco.)

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**Figure 1.** Analysis of heterologous reporter gene expression driven by fragments of the INO1 promoter. Top line represents 400 bp of INO1 sequence 5' to start of INO1 transcription. Asterisks show positions of UASUMO elements. Open box represents the URS1 element. Black box represents the INO1 TATA box. Each vector (pKS102, pKS101, pMK103, pKS103) contains the designated portion of the INO1 promoter fused to the CYC1-lacFZ chimaera. For specific endpoints of INO1 promoter sequences contained in the vectors, see Table 1. Arrows indicate orientation of the UASiNO elements relative to the start of INO1 transcription. Vector pJH304 contains no INO1 sequences. Black boxes in CYC1-lacFZ represent CYC1 TATA boxes. Each plasmid was transformed into wild-type (SH338) and sin3Δ (SH296) strains, and β-galactosidase activity was assayed under both derepressing (1°C+) and repressing (1°C-) conditions. Numbers shown in the table are β-galactosidase activity units. Each assay was repeated four to six times using three to five independent transformants. Standard deviations are shown. In the last column, activity in the sin3 strain under repressing conditions is divided by activity in the wild-type strain under repressing conditions.
from nucleotides -259 to -154, 5' to the start of INO1 transcription. This 105 bp fragment contains the only URS1 element found in the INO1 promoter and two copies of the 10 bp UASINO element (Fig. 1, expanded section of promoter). Both of these UASINO elements contain a 'C' at the first position of the 10 bp sequence, the significance of which will be addressed in the following section. This 105 bp fragment from the INO1 promoter was previously shown to provide full regulated expression to the heterologous reporter gene CYC1-lacZ (18). This result was confirmed by transforming plasmid pKS102 (contains sequences -259 to -154) into wild-type strain SH338, and measuring β-galactosidase production under both derepressing (I^c+) and repressing (I^c−) conditions (Fig. 1). In the wild-type strain used for these studies, the INO1 promoter sequences present on plasmid pKS102 were capable of providing >600-fold repression in response to inositol and choline (Fig. 1). However, when the same experiment was performed in the sin3 disruption strain SH296, expression of this construct was elevated under repressing growth conditions, relative to wild-type levels. Under repressing conditions, plasmid pKS102 supported ~34-fold more β-galactosidase production in the sin3 mutant than it did in the wild-type strain, expressed as a ratio of sin3/WT (Fig. 1).

The INO1 promoter sequences present on plasmid pKS103 were also capable of driving expression that was regulated response to inositol and choline (Fig. 1) (18). This construct contains INO1 promoter sequences -259 to -219, including the second UASINO element and the first URS1 element. The level of expression driven by this promoter fragment was ~15-fold greater in the sin3Δ mutant than it was in the wild-type strain, when grown under repressing conditions (Fig. 1).

When the URS1 element is removed from pKS101 to create plasmid pKS103 (-246 to -220), a much higher level of reporter gene expression was observed in the wild-type strain, relative to expression from pKS101 (Fig. 1). This is consistent with previous studies by Lopes et al. (11), who conclude that the INO1 URS1 element is a functional repression sequence. Construct pKS103, which contains the first UASINO but lacks URS1, supported reporter gene expression that was not appreciably different in sin3Δ and wild-type strains, under repressing conditions (Fig. 1).

Plasmid pMK103 contains INO1 sequences -219 to -154, including the second UASINO element. Reporter gene expression driven by these promoter sequences is regulated by inositol and choline, in a wild-type strain (18). In the sin3Δ mutant, this plasmid supported ~3-fold more β-galactosidase production than wild-type, under repressing conditions (Fig. 1).

Vector pKH304 contains CYC1 TATA boxes, fused to lacZ, but lacks any INO1 promoter sequences. There is no appreciable difference in pKH304 expression in the wild-type and sin3Δ strains (Fig. 1).

Analysis of UASINO in SIN3-mediated control of INO1 expression

The 10 bp UASINO element alone has been shown to be sufficient to drive expression of a heterologous reporter gene when a 'C' or an 'A' residue is present at the first nucleotide position of the element (J. Koipally and J. Lopes, personal communication). This expression is regulated in response to inositol and choline. These results are illustrated in Table 2, where reporter gene expression driven by construct pCON1 was examined in wild-type strain SH338. In this experiment, an oligonucleotide consisting of a
perfect match to the UASINO 10 bp consensus sequence was sufficient to support expression of the heterologous reporter gene CYC1-lacZ, and this expression was repressed 20-fold in response to inositol and choline. When this same construct was tested in sin3A mutant strain SH296, expression was ~2-fold greater than wild-type levels, under repressing conditions (Table 2).

As discussed previously, none of the native UASINO elements are perfect matches to the 10 bp consensus sequence 5'-CATGTGAAAT'. In order to study the native UASINO elements present on the -259 to -154 fragment of the INO1 promoter, oligonucleotides that matched the two native 10 bp elements in question were placed upstream of the heterologous reporter gene CYC1-lacZ to create the vectors pKH200 and pKH201. The construct pKH200 contains sequences identical to the first native 10 bp element (corresponding to the first UASINO on the pKS102 fragment, Fig. 1). This construct differs from the pCON1 construct discussed above only by the single base change of T to A at the 10th position of the inserted UASINO. Levels of pKH200 expression were similar to levels of pCON1 expression in the wild-type strain (Table 2). However, like the result with pCON1, expression of pKH200 was ~2-fold greater in the sin3 mutant than it did in the wild-type strain (Table 2).

The construct pKH201 contains sequences that are identical to the second native 10 bp element in question (corresponding to the second UASINO on the pKS102 fragment, Fig. 1). Plasmid pKH201 differs from pCON1 only by the single base change of A to T at the ninth position of the inserted UASINO. Levels of pKH201 expression in the wild-type strain were similar to levels of pCON1 and pKH200 expression (Table 2). Under repressing conditions, pKH201 supported more β-galactosidase production in the sin3 mutant than it did in the wild-type strain (Table 2). Construct pKH201 differs from its corresponding native UASINO element in one respect: orientation relative to the start of transcription. To test the effect of orientation on expression driven by this sequence, vector pKH202 was constructed, which differs from pKH201 only by the reversed orientation of the inserted sequence. Thus, pKH202 contains its corresponding UASINO element in the ‘native’ orientation. As predicted by other studies (J. Koipally and J. Lopes, personal communication), expression driven by the 10 bp element was not affected by orientation in a wild-type strain (Table 2, compare pKH201 with pKH202). When pKH202 was tested in the sin3 mutant strain under repressing conditions, expression levels were ~2-fold greater than wild-type (Table 2).

It has been demonstrated that 5' sequences immediately flanking the 10 bp UASINO element are also critical for expression. That is, modifying the sequences that flank the 10 bp oligonucleotide caused a change the level of expression of a heterologous reporter gene driven by the UASINO (N. Bachhawat, personal communication). For example, basal expression driven by plasmid pNB503 was ~7-fold lower than that of plasmid pCON1, in a wild-type strain (Table 2 and N. Bachhawat, personal communication). Both pCON1 and pNB503 contain the same 10 bp oligonucleotide, identical to the UASINO consensus sequence, but the sequences that flank the element are different in the two vectors. However, expression of both constructs was affected in the same manner in a sin3 mutant background. Plasmid pNB503 supported reporter gene expression at levels
2-fold higher than wild-type levels in the sin3 mutant, under repressing conditions (Table 2).

**DISCUSSION**

To learn how the SIN3 gene product may function to control phospholipid gene expression, experiments were carried out to define the regions of the INO1 promoter that were critical for responding to SIN3-mediated repression. Expression of reporter constructs containing different fragments of the INO1 promoter fused to CYCI-lacZ was analyzed in wild-type and sin3A strains. The contribution of both the URS1 and the UASINO promoter elements to SIN3-mediated repression of INO1 was examined.

In this study, reporter gene expression driven by promoter sequences on both pKS102 (contains URS1 and two ‘C’-containing UASINO elements) and pKS101 (contains URS1 and the first of the two UASINO elements) was elevated in a sin3A mutant strain under repressing conditions, relative to wild-type (Fig. 1). Therefore, the SIN3 gene product is required to mediate the repression conferred by these INO1 promoter sequences. Expression of plasmid pKS103 (contains the first UASINO element) was not affected by a sin3 mutation (Fig. 1). Since the only difference between constructs pKS103 and pKS101 is the presence of the INO1 URS1 element in pKS101, this result strongly implicates the URS1 element as being involved in SIN3-mediated repression of INO1.

A comparison of pKS101 with pKS103 expression in the sin3 strain (Fig. 1) indicates that the SIN3 gene product is not absolutely required for all repression from the URS1 element. If SIN3 were absolutely required for URS1-mediated repression, then one would expect to find that pKS101 and pKS103 each supported similar levels of reporter gene expression in the sin3 mutant strain. However, this was not the case, as removing the URS1 element resulted in a 4-fold increase in expression in the sin3 strain under repressing conditions, and a 2.5-fold increase under derepressing conditions (Fig. 1). A comparison of pKS101 with pKS103 expression in the wild-type strain indicated that removal of the URS1 element resulted in a 55-fold increase in expression under repressing conditions, and an 8.6-fold increase in expression under derepressing conditions (Fig. 1); a much greater effect than that seen in the sin3 mutant strain. Therefore, the URS1 element is not capable of mediating wild-type levels of repression without the SIN3 gene product, but does remain partially functional when SIN3 is absent.

Under repressing conditions, plasmid pMK103 supported expression that was 3-fold elevated in a sin3 mutant strain, relative to the basal expression in wild-type (Fig. 1). Therefore, the SIN3 gene product is required to achieve full levels of repression of the INO1 promoter fragment present on pMK103. This 65 bp fragment contains a UASINO element, suggesting that this element may also be involved in SIN3-mediated repression. However, expression of plasmid pKS103, which contains a UASINO element, was not affected by a sin3 mutation.

Additional experimentation proved to be successful in defining the role of the UASINO element in SIN3-mediated repression. Reporter gene expression driven only by the UASINO elements present in vectors pCON1, pKH200, pKH201, pKH202 and pNBS03 was consistently elevated 2-fold in the sin3A strain, under repressing conditions (Table 2). These independent experiments, involving a study of five separate vectors, yielded results that were extremely reproducible. Therefore, the SIN3 gene product must be required to achieve wild-type levels of repression of the UASINO element. These results demonstrate that SIN3 is capable of mediating repression of the consensus UASINO element, as well as both of the native UASINO elements present on the INO1 promoter fragment between residues –259 and –154. This result is not affected by the orientation of the 10 bp element relative to the start of transcription. A puzzling result was that expression of pKS103, which contains the first UASINO, was not affected by a sin3 mutation (Fig. 1). However, when the same UASINO element was isolated in vector pKH201 without any native flanking DNA, expression was elevated in a sin3 background (Table 2). Most probably, sequences flanking the UASINO that are present on the 26 bp fragment in pKS103 were responsible for modifying its response to a sin3 mutation.

In a previous study, it was demonstrated that regulation of the CHO1, CHO2 and OP13 genes, which also contain UASINO elements, was affected by a sin3 mutation (7). The repression of CHO1, CHO2 and OP13 appeared to be affected to a lesser degree by a sin3 mutation than was repression of INO1 (7). Unlike INO1, these three genes contain no consensus URS1 element. However, David Stillman (personal communication) recently re-evaluated the CHO2, OP13 and CHO1 promoters and found URS1-like elements that have some homology to the functional URS1 element as determined by Luche et al. (20). None of the elements detected by Stillman, however, is a perfect match for the URS1 consensus and none has been tested for functionality.

In summary, the results reported here implicate both the URS1 element and the UASINO element in SIN3-mediated repression of INO1. A UASINO element was responsible for ~2-fold repression by the SIN3 gene product, while the INO1 URS1 contributed significantly more to SIN3-mediated repression.

The SIN3 gene product may act directly or indirectly (or both) at INO1 and the promoters of co-regulated genes of phospholipid metabolism. Evidence for an indirect, as well as a direct, effect of SIN3 on the structural genes INO1, CHO2, OP13 and CHO1, comes from a recent study demonstrating that a sin3 mutation resulted in increased expression of the INO2 regulatory gene (J. Jackson and J. Lopes, personal communication). The INO2 gene has been shown to be autoregulated via a UASINO element located in its own promoter (24). INO2 encodes a protein of the basic helix–loop–helix (bHLH) class that is a positive regulatory factor required for INO1 expression. The INO2 gene product is also required for maximal derepression of the CHO1, CHO2 and OP13 genes (19). INO2 and several other regulatory factors that are critical for controlling INO1 expression have been shown, like SIN3, to work through the UASINO element. For example, in vitro studies have suggested a model in which Ino2p and Ino4p are both bHLH proteins, dimerize and bind to the UASINO element to activate INO1 transcription (25). Thus, any effect of SIN3 on INO2 expression might cause an indirect affect on INO1 expression. Presumably, however, the effect of SIN3 on INO2 would also occur through the UASINO located in the INO2 promoter since INO2 is autoregulated (24). Thus, modulation of the expression of the structural genes, INO1, CHO1, CHO2 and OP13, might occur directly through UASINO, as well as through modulation of regulatory gene products interacting with UASINO. It is interesting in this context, and perhaps significant, that two recent reports have described direct interaction of a SIN3 mammalian homologue with regulatory proteins of the bHLH category (26,27). The ultimate goal of future experiments will be...
to understand the interplay between SIN3 and the specific regulatory factors, such as the INO2 gene product, in controlling INO1 gene expression.

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