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Interaction of *trans* and *cis* regulatory elements in the *INO1* promoter of *Saccharomyces cerevisiae*

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ABSTRACT

Electrophoretic mobility shift assays (EMSA) were used to define the regions of the *INO1* promoter that interact with factors present in extracts prepared from the yeast, *Saccharomyces cerevisae*. These experiments identified three different types of protein:DNA complexes that assemble with the *INO1* promoter. Formation of one type of complex depended on functional alleles of previously described regulatory genes, *INO2* and *INO4*, that encode positive regulatory factors. Formation of the *INO2/INO4*-dependent complexes was increased when extracts prepared from cells grown under derepressing conditions (i.e. absence of inositol and choline). A second type of complex was dependent on the *OPI1* gene, that encodes a negative regulator. Computer-aided sequence analysis of the promoters of several genes encoding phospholipid biosynthetic enzymes revealed a highly conserved nine basepair element (5'-ATGTG-AAAT-3'), designated 'nonamer' motif, that is similar to the octamer motif identified in the promoters of mammalian immunoglobulin genes. The nonamer motif was shown to form a specific complex with a factor, designated nonamer binding factor (NBF). Extracts prepared from *Schizosaccharomyces pombe* formed a nonamer-specific complex.

INTRODUCTION

Biosynthesis of membrane phospholipids in the yeast *Saccharomyces cerevisiae* is regulated in a complex manner (1, 2). Five genes that encode phospholipid biosynthetic enzymes have been cloned and sequenced (2). One of these genes, *INO1*, encodes inositol-1-phosphate synthase that is required for the conversion of glucose-6-phosphate to inositol-1-phosphate, the first committed step in the formation of inositol-containing phospholipids (1, 2). Expression of four of these genes is regulated at the transcriptional level (3, 4, T.Gill and S.Toutenhoofd unpublished data) in response to inositol and choline. Computer-aided DNA sequence analysis of the promoter regions of these genes identified a repeated 9 bp element, 5'-ATGTGAAAT-3' (1), designated 'nonamer' motif. This element is similar to the octamer motif (5'-ATGCAAAT-3') initially identified in the promoter region of immunoglobulin genes (5, 6). Recently, a detailed analysis of the *INO1* promoter defined a minimum sequence that retained *INO1*-specific UAS activity (7). A fragment of the *INO1* promoter spanning 105 nucleotides from −259 to −154 conferred complete *INO1*-specific regulation upon a heterologous reporter gene. Furthermore, each fragment of the *INO1* promoter that conferred *INO1*-specific regulation contained at least one copy of the nonamer motif. However, the nonamer motif (by itself) does not appear to define the *INO1* UAS since it failed to activate transcription of a heterologous reporter gene (7).

Transcription of *INO1* is affected by mutations in three regulatory genes. Mutations in the *INO2* and *INO4* genes reduce expression of the *INO1* structural gene (3) resulting in inositol auxotrophy (8). These two genes are believed to encode activators that function in concert (9, 10). Previous analysis also identified *opi1* mutations (11) that cause constitutive overexpression of the *INO1* gene (3). Strains harboring *opi1* mutations have been shown to express the *INO1* and *CHO1* transcripts constitutively at a level 2-fold higher than wild type derepressed levels (3, 4). The *OPI1* gene is believed to encode a negative regulator of gene expression, and its DNA sequence predicts a gene product that contains characteristics previously identified in several DNA-binding proteins (12).

In the present study, regions of the *INO1* promoter involved in binding of *trans*-acting factors have been defined. Fragments of the *INO1* promoter have been used as templates for protein binding in a series of electrophoretic mobility shift assays (EMSA). These experiments identify several protein:DNA complexes, including some that are dependent on the products of previously identified regulatory loci.

MATERIALS AND METHODS

Yeast strains

Strains of *S.cerevisiae* used to generate whole cell extracts were: BRS1001 (*MATa, ade2, his3, leu2, can1, trp1, ura3*); BRS1017 (*MATa, ino2, ura3, lys2*); BRS1021 (*MATa, opi1, ade5, leu2, trp1, ura3*); BRS1023 (*MATa, opi1::LEU2, leu2, his3*); BRS1028 (*MATa, ino4, leu2, ura3*); BRS1030 (*MATα*, ade2, his3, leu2,

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can1, trp1, ura3, ino4::URA3); BRS1141 (*MATa, ura3, his3Δ200, leu2, ade2, lys2Δ201*). With the exception of BRS1141 (previously called N460; 13) all of the strains used in this study are related to each other by extensive backcrossing of BRS1001 to strains containing certain regulatory mutations affecting phospholipid biosynthesis (8, 11). BRS1030, which harbors the *ino4* disruption allele (10), is isogenic to BRS1001. In addition, *S.pombe* strain 972 (h^{-S}), generously provided by Dr. J.E.Hill, was also employed to generate cellular extracts.

Plasmid constructions

Two plasmids were constructed to serve as source of DNA templates for mobility shift assays. An 84 bp *Hin*dIII- *Hin*PI restriction fragment from pJH339 (7) was inserted into the *Hin*dIII-*Acc*I sites of pGEM1 (Promega, Madison, WI) to yield pJL104. This construction was used to generate template A which contains sequences −333 to −258 of the *INO1* promoter (Figure 1). Likewise, a 105 bp *Hin*PI restriction fragment from pJH339 was inserted into the *Acc*I site of pGEM1 to create pJL105. This plasmid contains sequences −259 to −154 of the *INO1* promoter (template B; Figure 1) . In either case, *INO1* promoter sequences were isolated by digestion with *Hin*dIII and *Eco*RI (Figure 1). In addition, template C was prepared directly from pJH339 by digestion with *Fok*I which yields a 77 bp fragment (−295 to −219; Figure 1).

Derivatives of templates A and B, that contained a single copy of the nonamer, were generated by initially linearizing pJL104 and pJL105 (templates A and B, respectively; Figure 1) by digestion with *Hin*dIII and 5′ end-labelling at that site (*). Linearized plasmids were digested with *Eco*RI and pertinent labelled restriction fragments were purified by gel fractionation. These restriction fragments were further digested with *Fok*I (Figure 1) yielding templates labelled at the *Hin*dIII site, that included only one copy of the nonamer. This strategy yielded labelled templates including the nonamer motifs centered at −303 and −173 (Figure 1). In similar fashion, pJL104 and pJL105 were linearized by digestion with *Bam*HI, 5′ end-labelled at that site, and re-digested with *Hin*dIII (Figure 1). These fragments were in turn digested with *Fok*I to yield restriction fragments, labelled at the *Bam*HI site. These *Bam*HI*−*Fok*I fragments included the nonamer motifs centered at −282 and −233.

Purification and labelling of restriction fragments for use in EMSA

Restriction digests were performed according to manufacturer specifications. Restriction fragments were fractionated using either agarose or polyacrylamide gels essentially as previously described (14). The appropriate restriction fragments were excised from gels and eluted using a GEL/X Gel Extractor Kit (GENEX Corp., Gaithersberg, MD).

Purified restriction fragments were 5′ end-labelled with reverse transcriptase. Each reaction (40 μl final volume) contained a purified restriction fragment (amounts varied), 1× reverse transcriptase buffer (50 mm Tris/HCl pH=8.3, 40 mM KCl, 10 mM $MgCl_2$, 0.4 mM DTT) , 0.1 mM dGTP, 0.1 mM dTTP, 0.1 mM dCTP, 35 μCi [α-^{32}P]-dATP (3000 Ci/mmol; New England Nuclear, Wilmington, MD), and 67 units of AMV reverse transcriptase (Stratagene, La Jolla, CA). The reaction was allowed to proceed for 20 min at 42°C and stopped by the addition of 2 μl of 0.25 M EDTA. Labelled restriction fragments were separated from the unincorporated [α-^{32}P]-dATP through a Nuctrap Push Column using a Push Column Beta Shield Device (Stratagene, La, Jolla, CA) as per manufacturer's specifications.

Synthetic oligonucleotides to be used as templates in EMSA were 5′ end-labelled by T4 polynucleotide kinase. Typically, 1 μg of oligonucleotide was labelled in a 20 μl reaction containing 1× linker-kinase buffer (70 mM Tris/HCl pH=7.6, 10 mM $MgCl_2$, 5 mM DTT; 18), 100 μCi of [γ-^{32}P]-ATP (6000 Ci/mmol; New England Nuclear, Wilmington, MD), and 20 units of T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN) at 37°C for 30 min. Oligonucleotides were annealed as described below.

Preparation of yeast whole cell extracts

Preparation of whole cell extracts from *S.cerevisiae* was performed essentially as previously described (15). Typically, an appropriate yeast strain was grown to mid-logarithmic phase (70−100 Klett units; $2-3\times10^7$ cells/ml) in 1 liter of synthetic complete media (3). Where appropriate, media was supplemented with inositol to 75 μM and choline to 1 mM final concentrations. Cells were harvested by centrifugation (3,330×g/5 min/4°C) and washed once with 10 ml of extraction buffer (200 mM Tris/HCl pH = 8.0, 40 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 7 mM β-mercaptoethanol, 1 mM PMSF, 2 μM pepstatin A). Cells were again harvested by centrifugation (2,310×g/5 min/4°C), resuspended in 1 ml of extraction buffer, transferred to 15 ml Corex tubes, and stored overnight at −70°C. Cells were thawed on ice in the presence of an equal volume of sterile glass beads (0.45 μm diameter), disrupted by vortexing at full speed 6×15 seconds, and stored on ice for an additional 30 min. Cellular debris were removed by centrifugation in polycarbonate tubes using a Beckman TLA-100.3 rotor (16,486 × g/15 min/4°C). Cell extracts were transferred to 1.5 ml microcentrifuge tubes and further clarified by centrifugation (14,067 × g/1 hr/4°C) using a Beckman TLA-100.3 rotor. Extracts were collected into another 1.5 ml microcentrifuge tube and a concentrated solution of 100% $(NH_4)_2SO_4$ in 20 mM HEPES pH = 8.0, 5 mM EDTA, was added to 40% final concentration. Protein was precipitated by gentle inversion at 4°C for 30 min and pelleted by centrifugation (14,067 × g/10 min/4°C) using a Beckman TLA-100.3 rotor. Protein pellets were carefully resuspended in 250 μl of protein buffer (20 mM HEPES pH = 8.0, 5 mM EDTA, 20% glycerol, 7 mM β-mercaptoethanol, 1 mM PMSF, 2 μM pepstatin A) and stored at −70°C. Protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Rockville Center, NY). Whole cell extracts were also prepared from *S.pombe* using the method described for *S.cerevisiae*.

EMSA

Standard binding reactions were carried out in a 20 μl final volume containing 4 mM Tris/HCl pH = 8.0, 4 mM $MgCl_2$, 4% glycerol, 250 mM KCl, 1 mM DTT, 50 pg/ml of pGEM1 (Promega, Madison, WI), DNA template (roughly 0.5 ng), and approximately 25 μg of cell extract. Complexes were allowed to form at room temperature for 10 to 15 min. After addition of 2 μl of dye solution (0.4% bromophenol blue, 0.4% xylene cyanol FF, 50% glycerol) complexes were resolved from free DNA by electrophoretic fractionation on a 4% acrylamide gel (30%:0.8% acrylamide:bis ratio) at 150V (20mA). Electrophoretic gels were dried onto sheets of 3MM Whatman paper and the reaction products were visualized by autoradiography. In some instances, products were excised from the dried gels and quantitated by liquid scintillation.

Synthetic oligonucleotides used in competition experiments

Six synthetic oligonucleotides, purchased from Operon Technologies, Inc. (Alameda, CA), were employed as competitors in mobility shift assays. JML-1 (5′-AGATCTATGTGAAATCTCAGA-3′) was annealed to JML-2 (5′-CTCGAGATTTCACATAGATCT-3′), JML-3 (5′-ATGTGAAAT-3′) was annealed to JML-4 (5′-ATTTCACAT-3′), and JML-5 (5′-CGCTTCGGCGGCTAAATGCGGC-3′) was annealed to JML-6 (5′-GCCGCATTTAGCCGCCGAAGCG-3′). Annealing reactions were carried out in 100 μl final volumes containing 1×TE 8.0 and each oligonucleotide at 10 μM final concentration by sequentially incubating at 85°C for 2 min, 65°C, 37°C, room temperature, and 4°C for 15 min each.

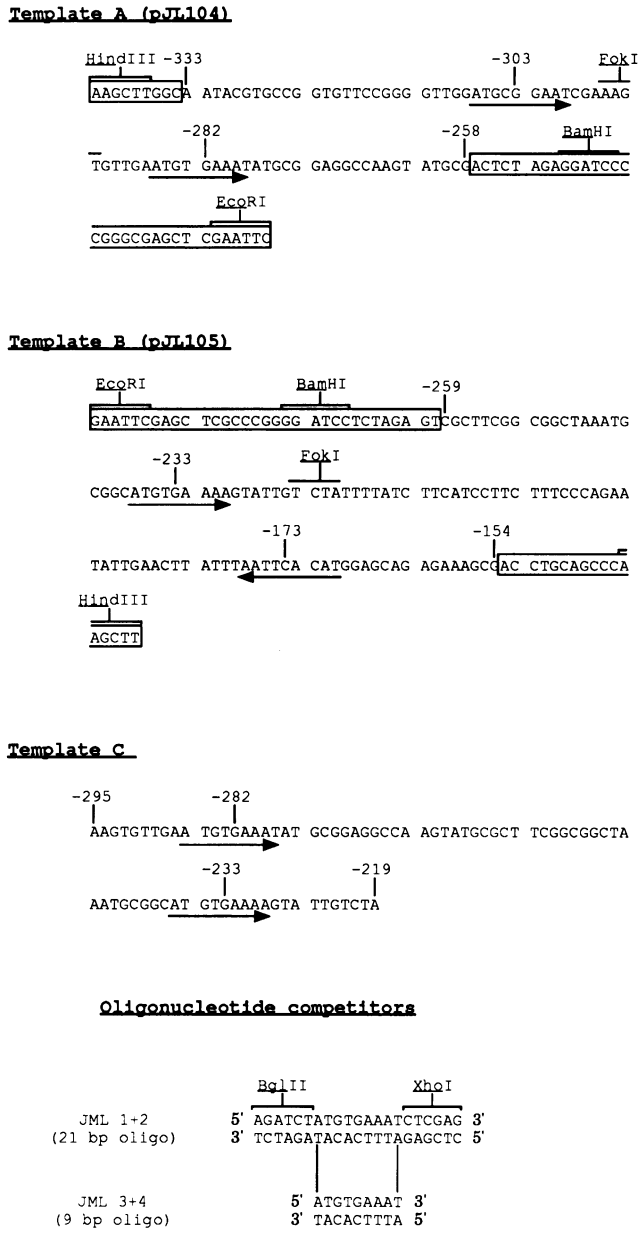

Figure 1. Sequences from the *INO1* promoter that were used as templates in binding reactions are denoted as templates A, B and C. The plasmid source of each template is indicated in parentheses. Each sequence is numbered relative to the *INO1* mRNA 5′ terminus (7). Boxed sequences represent flanking plasmid sequences from pGEM1 not related to the *INO1* gene. Several pertinent restriction sites are also indicated (discussed in text). Four copies of a conserved 9 bp element (discussed in text; 1) are denoted by an arrow which indicates orientation. Two synthetic oligonucleotides used as competitors and templates in binding reactions are also shown. The smaller 9 bp oligonucleotide is represented in the larger 21 bp oligonucleotide, flanked by two restriction sites.

RESULTS

An *INO2/INO4*-dependent complex assembles with the *INO1* promoter between nucleotides −259 and −219

Several complexes were observed when a restriction fragment (template B; Figure 1) composed of nucleotides −259 to −154 from the *INO1* promoter, was incubated with a cellular extract prepared from a wild type *S.cerevisiae* strain (Figure 2A, lane 1; see also Ref. 13). Two of these complexes were absent from reactions carried out using extracts from strains harboring *ino2* or *ino4* point mutations (Figure 2A, lanes 2 and 3; denoted *INO2/INO4*), or a strain (isogenic to the wild type strain used in these studies, BRS1001) with an *ino4* null mutation (data not shown). The *INO2/INO4*-dependent complexes were observed when the extracts were derived from strains containing *opi1* mutations (Figure 2A, lanes 4 and 5). Extracts were also prepared

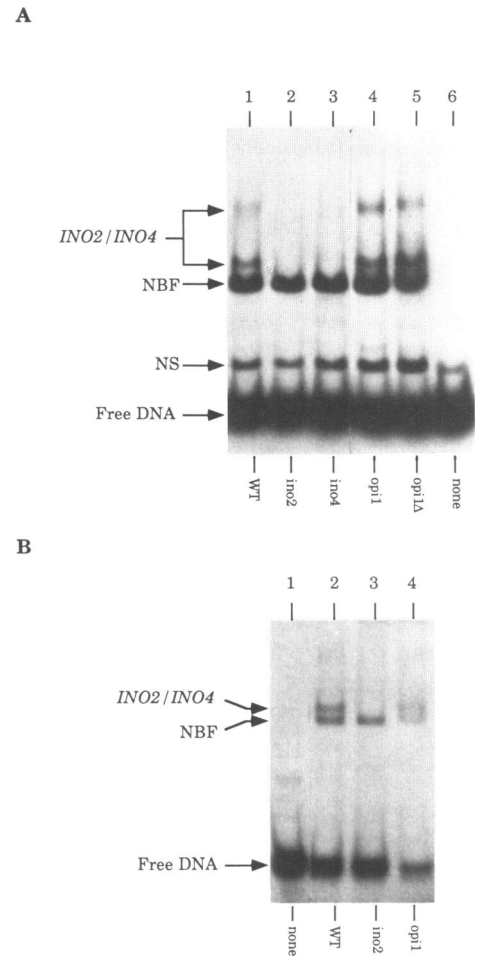

Figure 2. (A) EMSA identifying two *INO2/INO4*-dependent complexes that assemble with template B (−259 to −154). Also indicated is an NBF-specific complex. Relevant genotype of the strain used as source of extract is indicated below each lane. NS marks a non-specific free DNA band. (B) EMSA identifying a single *INO2/INO4*-dependent complex that assembles with template C (−259 to −219). Note that less template was inadvertantly added to lane 4.

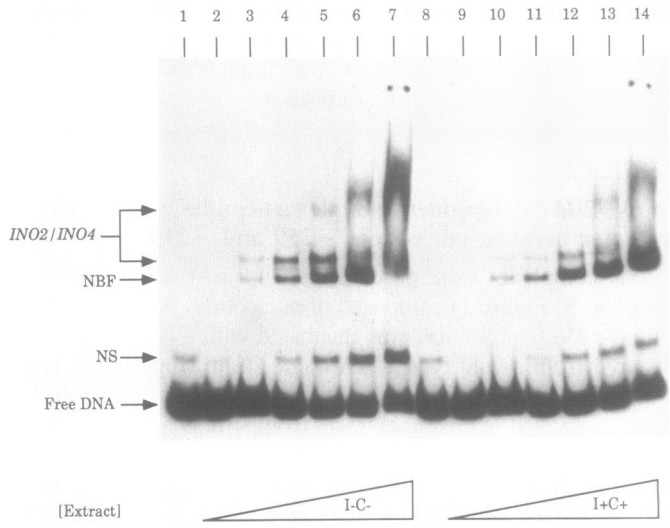

Figure 3. EMSA using template B (−259 to −154) and varying quantities of extracts prepared from cells grown under derepressing (absence of inositol and choline; I−C−; lanes 1−7) and repressing conditions (75 μM inositol and 1 mM choline; I+C+; lanes 8−14). Two *INO2/INO4*-dependent complexes, a single NBF-specific complex and a non-specific (NS) free DNA band are indicated. Lanes, from left to right, contain binding reactions carried out with increasing amounts of total cellular extracts: lanes 1 and 8, no extract; lanes 2 and 9, 1 μg of extract; lanes 3 and 10, 5 μg; lanes 4 and 11, 10 μg; lanes 5 and 12, 25 μg; lanes 6 and 13, 50 μg; and lanes 7 and 14, 100 μg. Bands that resolved the best (i.e. lanes 1−6 and 8−13) were excised and quantitated by liquid scintillation and the results are presented graphically in the next Figure (4).

from an *ino2* mutant strain transformed with a multicopy plasmid that includes the *INO2* gene (D.M.Nikoloff personal communication). This plasmid restores formation of the *INO2/INO4*-dependent complex with template B (data not shown). To delineate the region within the *INO1* promoter that interacts with the *INO2/INO4*-dependent factor, a second template (C; −295 to −219; Figure 1) was used that overlapped template B by 41 nucleotides. A single *INO2/INO4*-dependent complex formed with template C (Figure 2B, lanes 2 and 4). The template C complex co-migrated with the faster-migrating *INO2/INO4*-dependent complex that formed on template B when fractionated on the same gel (data not shown) even though the sizes of the templates are different (i.e. template B is 105 bp and template C is 77 bp). Therefore, it is not clear whether the two complexes are identical but they are both *INO2/INO4*-dependent.

Extracts prepared from cells grown under derepressed conditions form more of the *INO2/INO4*-dependent complex

Repeated, but qualitative, examination of EMSA suggested that a greater amount of *INO2/INO4*-dependent complex formed when extracts were prepared from cells grown under derepressing conditions (i.e. absence of inositol and choline) as compared to extracts derived from cells grown under repressing conditions (i.e. presence of inositol and choline). To quantitate formation of this complex, aliquots containing between 1 μg and 100 μg of total protein prepared from wild type cells grown under derepressing and repressing conditions were allowed to react with template B (Figures 3 and 4). As previously noted, template B

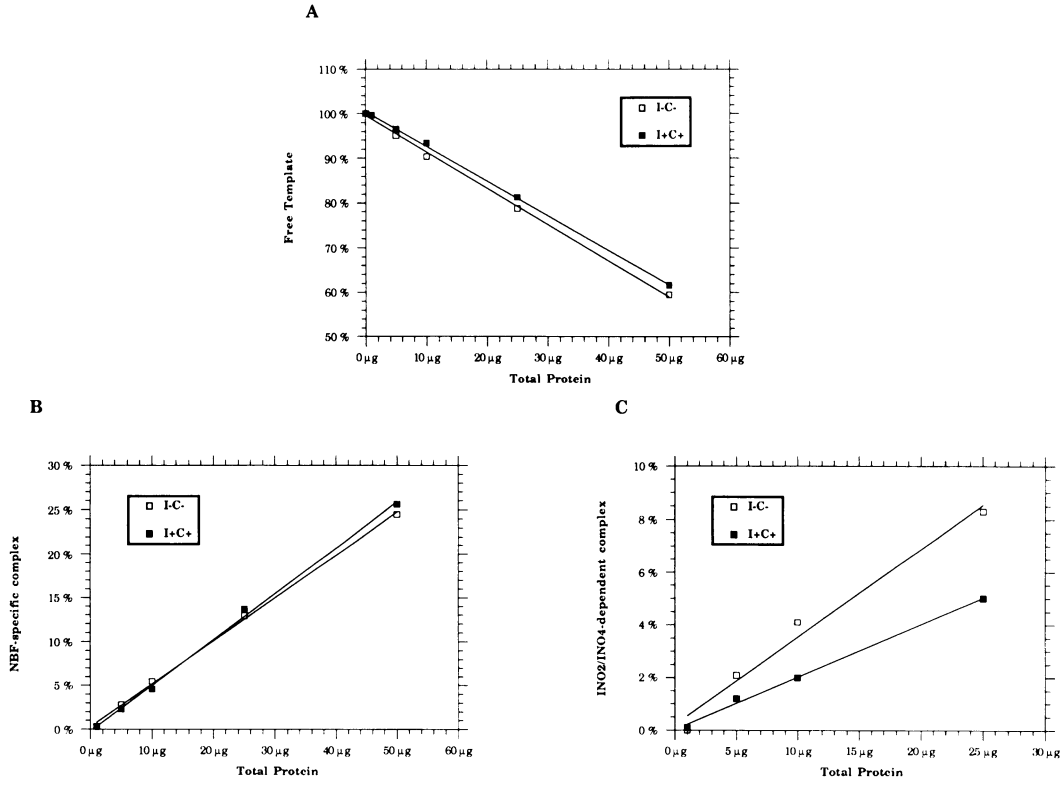

Figure 4. Bands were excised from the gel depicted in Figure 3 and counted by liquid scintillation counting. Note, only those bands that resolved clearly were used in this analysis. Reactions were carried out with template B (−259 to −154) and extracts prepared from cells grown under derepressing (absence of inositol and choline; I−C−) and repressing conditions (75 μM inositol and 1 mM choline; I+C+). (A) Graph showing the disappearance of free DNA (template B; represented as a percentage of total cpm in each lane) in an EMSA as a function of increased amount of extract. (B), and (C) Graphic representations of the appearance of: (B) an NBF-specific complex as a function of increased extract concentration, and (C) the faster-migrating *INO2/INO4*-dependent complex.

forms two *INO2/INO4*-dependent complexes (Figure 3, lanes 4−7 and 12−14) . Approximately 40% less of the faster-migrating *INO2/INO4*-dependent complex was formed when the extract was prepared from cells grown under repressing conditions (Figure 4C). Furthermore, as increasing amounts of extract were added to the reaction, the slower- migrating *INO2/INO4*-dependent species formed exclusively (Figure 3, lanes 7 and 14). These results suggest that the slower- migrating species may be a multicomponent complex derived at the expense of the faster-migrating *INO2/INO4*-dependent complex. It is interesting that the non-specific (NS) complex also increased as a function of increased protein concentration. This was unexpected, since the NS band was present in the absence of extracts (Figure 3, lanes 1 and 8). It is possible that the NS band reflects probe in a secondary structure that is enhanced by some component in the extract.

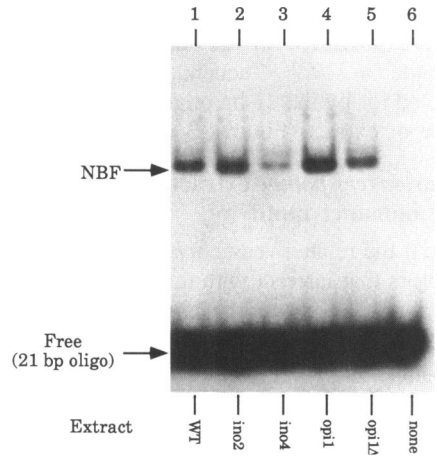

Figure 5. EMSA using a 21 bp oligonucleotide, that includes the 9 bp conserved element (Figure 1), and extracts from wild type and mutant strains. Marked for reference is an NBF-specific complex. Relevant genotype of the strain used as source of extract is indicated below each lane. The apparent relative differences in the formation of the NBF-specific complex are due only to variation in amount of cellular extract used in each reaction.

A factor (NBF) present in *S.cerevisiae* cell extracts binds specifically to the nonamer motifs in the *INO1* promoter

To identify complexes that assemble on 4 of the 7 copies of the nonamer motif present in the *INO1* promoter, competition assays were carried out using a synthetic oligonucleotide containing the nonamer. Three synthetic oligonucleotides were used: one included solely the nonamer (9 bp oligonucleotide; Figure 1); the second contained the nonamer embedded in 12 bp of flanking DNA sequences unrelated to the *INO1* promoter (21 bp oligonucleotide; Figure 1); the third included sequences spanning nucleotides −259 to −238 of the *INO1* promoter and does not contain a copy of the nonamer (22 bp oligonucleotide) The 9 bp and 22 bp oligonucleotides failed to compete away any of the observed complexes (data not shown) whereas the 21 bp oligonucleotide effectively competed for the formation of several complexes (discussed below). In further support of the specificity of NBF for the nonamer motif, the synthetic 21 bp oligonucleotide was shown to form a single complex (Figure 5). Consistent with the results obtained in competition experiments, the synthetic oligonucleotide containing exclusively the nonamer (Figure 1) does not form any complexes (data not shown). Since both the 9 bp and 21 bp oligonucleotide templates include the nonamer, these experiments suggest that binding of NBF requires sequences flanking the nonamer motif. It seems unlikely that there is any specificity for sequences other than the nonamer since previous computer analysis (1) failed to detect any sequence conservation

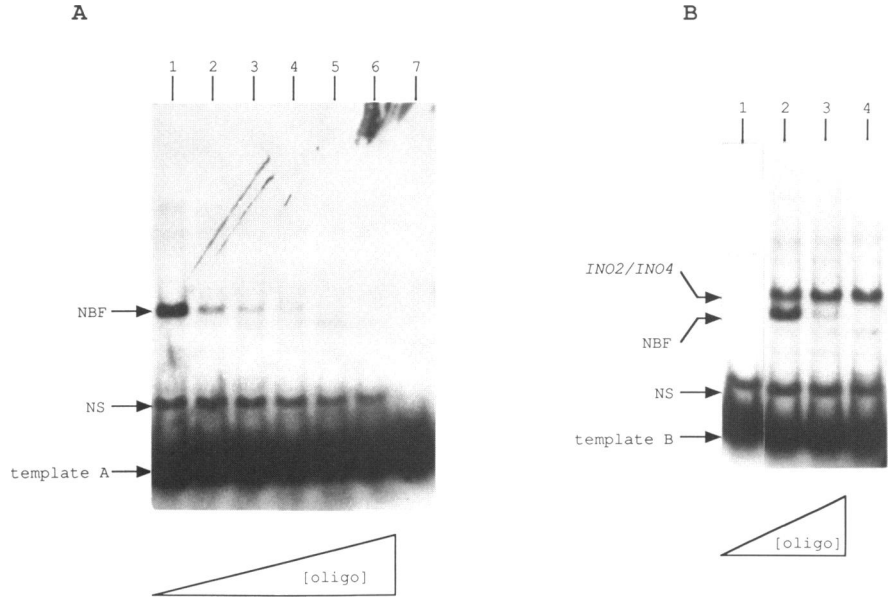

Figure 6. (A) EMSA carried out using extract from a wild type strain and template A (−333 to −258). Reactions, from left to right, contained increasing quantities of a 21 bp oligonucleotide that includes the conserved nonamer (discussed in text): lane 1, 125 nM; lane 2, 625 nM; lane 3, 1.25 μM; lane 4, 2.5 μM ; lane 5, 12.5 μM; lane 6, 25 μM; and lane 7, no extract or oligonucleotide added. The NBF-specific complex is indicated for reference. NS denotes a non-specific free DNA band. (B) EMSA carried out using extract from a wild type strain and template B (−259 to −154). Reactions, from left to right, contained increasing quantities of a 21 bp oligonucleotide that includes the nonamer motif: lane 1, no extract or oligonucleotide added; lane 2, 125 nM of oligonucleotide; lane 3, 1.25 μM; and lane 4, 12.5 μM. The *INO2/INO4*-dependent and NBF-specific complexes are indicated for reference.

on either side of this element among the 23 copies found in the phospholipid biosynthetic genes. Moreover, the 22 bp oligonucleotide containing sequences 5′ to the nonamer motif centered at −233 (Figure 1) failed to compete for formation of any complexes (data not shown). As a further test for sequence conservation in the vicinity of the nonamer element, a matrix alignment analysis (16) was performed on DNA sequences comprising the 23 copies of the nonamer (1) and 30 nucleotides on either side of each copy. This analysis confirmed that there is no statistically significant sequence conservation upstream or downstream of the nonamer motif in the 23 copies present in the promoters of the phospholipid biosynthetic genes (data not shown). The protein that recognizes the nonamer motif, and is competed away by the 21 bp oligonucleotide, has been designated nonamer binding factor (NBF).

Template A (−333 to −258) includes two copies of the nonamer motif (Figure 1, denoted as arrows). This template forms two complexes that are competed away by the 21 bp oligonucleotide (Figure 6A). Based on competition assays, template B (−259 to −154) which also includes two copies of the nonamer motif formed only a single nonamer-dependent complex (Figure 6B). The single NBF-specific complex with template B was the fastest-migrating complex in those reactions, distinguishing it from the two *INO2/INO4*-dependent complexes previously discussed. Template C (−295 to −219) also forms a single complex that is competed away by the 21 bp oligonucleotide (data not shown).

Templates B and C each contain two copies of the nonamer but formed only a single NBF-specific complex. In both cases the complex formed co-migrates with the faster-migrating species detected in reactions with template A (i.e the complex noted as NBF-specific in Figure 6). Since templates B and C overlap (−259 to −219) it is possible that in each case the single complex that forms interacts with the nonamer shared by the two templates (Figure 1). To determine which of the nonamer copies formed complexes in these experiments, four templates (collectively spanning sequences −333 to −154 of the *INO1* promoter) were generated that contained a single copy of the nonamer motif. All four templates were used in binding reactions with extracts from a wild type strain. Interestingly, all four templates formed a single complex that was competed away by the 21 bp oligonucleotide that contains the nonamer (data not shown). This observation suggests that while all four sites are able to form complexes with NBF from *S.cerevisiae* extracts, binding appears to occur exclusively to one copy when two or more copies of the nonamer are present on the same template.

Unlike the *INO2/INO4*-dependent complex, formation of the NBF-specific complexes is not regulated in response to supplementation of the growth media. Levels of NBF complex formation were measured in the same fashion as previously described for the *INO2/INO4*-dependent complex. The complex that is competed for by the 21 bp oligonucleotide is not affected by the source of extract (Figures 3 and 4).

Schizosaccharomyces pombe extracts contain a factor(s) that binds to the nonamer motif

To determine if the related yeast, *Schizosaccharomyces pombe*, possesses factors that interact with the *INO1* promoter, binding reactions were carried out using templates A (−333 to −258) and B (−259 to −154) with extracts prepared from *S.pombe* cells. Two complexes assembled on both templates A and B (Figure 7, lanes 2 and 5). One of the *S.pombe* complexes on each template was specific for the nonamer in these templates as defined by oligonucleotide competition (Figure 7, lanes 3 and 6). In addition, the 21 bp oligonucleotide that contains the nonamer motif (Figure 1) yielded two closely migrating complexes when extracts from *S.pombe* were used, suggesting that there may be more than one protein present that recognizes the nonamer motif (data not shown).

An *OPI1*-dependent complex assembles between nucleotides −333 and −295

Extracts were prepared from cells harboring mutations at the *OPI1* locus and used in EMSA with templates A, B, and C. These

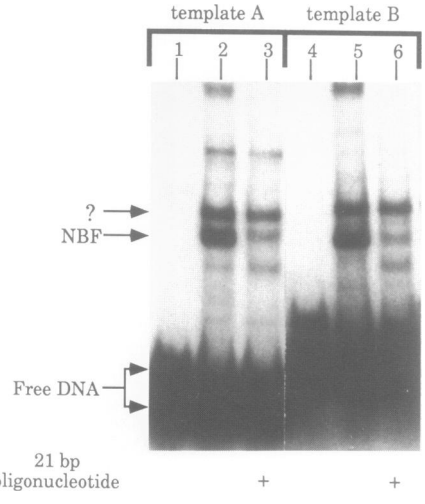

Figure 7. Binding reactions carried out using extracts from *S.pombe* and templates A (−333 to −258; lanes 1−3) and B (−259 to −154; lanes 4−6). Marked for reference are a nonamer-specific complex and an unidentified complex. The nonamer-specific complex is classified on the basis of competition by the 21 bp oligonucleotide (lanes 3 and 6). Lanes 1 and 4 contain reactions conducted in the absence of extract.

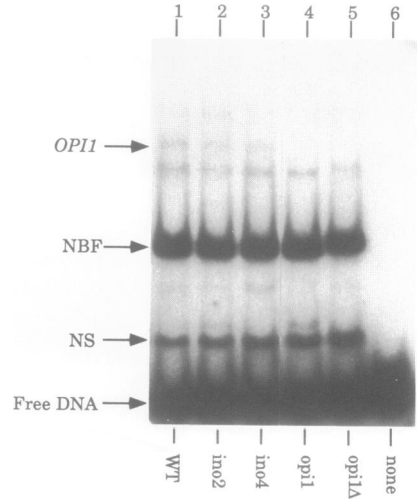

Figure 8. EMSA identifying an *OPI1*-dependent complex that assembles with template A (−333 to −258). Also indicated is an NBF-specific complex. Relevant genotype of the strain used as source of extract is indicated below each lane. NS marks a non-specific free DNA band.

experiments identified a single weak *OPI1*-dependent complex with template A (−333 to −258; Figure 8, lanes 1−3) but no such complexes were observed with either template B (−259 to −154) or C (−295 to −219; data not shown) Since no *OPI1*-dependent complex formed on template C (−295 to −219), the region required for its formation appears to be limited to sequences between −333 and −295 of the *INO1* promoter. The dependency of the complex on a wild type copy of the *OPI1* gene was confirmed by several experiments using extracts derived from mutants as compared to wild type, *ino2*, and *ino4* mutant strains (data not shown).

DISCUSSION

A number of protein:DNA complexes that form on the *INO1* promoter have been identified. Several of these complexes fail to form when extracts are prepared from strains carrying previously characterized mutations in regulatory genes known to control expression of phospholipid biosynthetic structural enzymes (2). In particular, several complexes failed to form when extracts were prepared from strains harboring *ino2*, *ino4*, or *opi1* mutations. Thus, formation of some of the observed protein:DNA complexes must, in some fashion, be dependent on components of the regulatory machinery previously defined by classical genetic analysis. However, some of these complexes were observed using extracts of all strains including those with lesions in the regulatory loci. In particular, a factor (NBF) present in all yeast strains tested was shown to interact specifically with the nonamer motif found in the promoters of a number of genes encoding phospholipid biosynthetic enzymes.

Two protein:DNA complexes formed only when extracts were prepared from strains that are wild type for the *INO2* and *INO4* regulatory genes and are thus designated '*INO2/INO4*-dependent' complexes. However the data presented here do not categorically establish that the products of these two genes interact directly with the *INO1* promoter or each other. There are several possible explanations for the observations reported here: (1) the *INO2* and *INO4* gene products may both be present in the complex and may interact to form a heteromeric protein; (2) one of the two gene products may be present in the complex but must be activated by or its expression may be regulated (directly or indirectly) by the product of the second gene; (3) neither gene product may be present in the complex but both may be required to activate or regulate the expression of another protein(s). To test the first possibility, extracts from *ino2* and *ino4* mutant strains were mixed and used in a binding reaction with template B. This experiment failed to restore either of the two *INO2/INO4*-dependent complexes (data not shown). However, this experiment does not rule out the possibility that the products of these two genes are physically present in the complexes because they may have to be co-synthesized in order to form a heteromeric protein. Experiments designed to determine if the products of the *INO2* and *INO4* genes are actually physically present in the *INO2/INO4*-dependent protein:DNA complexes are in progress.

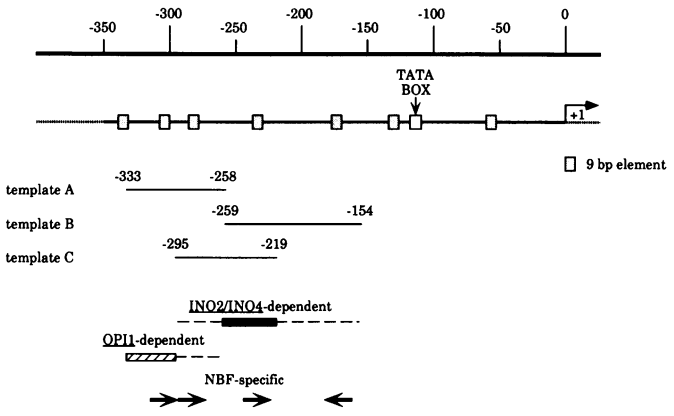

Figure 9. Schematic representation of the mapped binding sites for the four types of complexes that assemble with the *INO1* promoter: *INO2/INO4*-dependent complex (black box and dashed line); *OPI1*-dependent complex (thin cross-hatched box); and four NBF-specific complexes (arrows which indicate direction of binding site). Represented for reference are the *INO1* 5′ region numbered relative to the 5′ end of the *INO1* mRNA (7) and the three primary templates (A, B, and C) used in this study. Also noted are the position of the TATA box (open box) and seven copies of the nonamer motif (speckled boxes; 1; see also text).

The predicted amino acid sequences of the *INO2* and *INO4* genes (10; D.M.Nikoloff unpublished observations) revealed domains similar to those that have previously been observed in DNA-binding proteins. Specifically, both of the predicted gene products contain homology to the 'helix-loop-helix' motif identified in the myc family of DNA binding proteins (D.M.Nikoloff unpublished observations). This motif is postulated to be involved in heterodimer formation (17). In reproducible DNA binding experiments, approximately 40% less *INO2/INO4*-dependent complex is formed when the extracts were generated from cells grown under repressing conditions (Figure 4). Significantly, no other complex (including the NBF-specific complex) is affected in this fashion. This observation is consistent with the predicted role for the products of these two genes as transcriptional activators (10; D.M.Nikoloff unpublished observations).

Formation of one of the *INO2/INO4*-dependent complexes appears to require sequences present in the region spanning the 40 nucleotides from −259 to −219 within the *INO1* promoter (Figure 9). Identification of this region as a potential binding site was based on the observation that *INO2/INO4*-dependent complexes formed on two templates (B and C) that overlapped by nucleotides −259 to −219 (Figure 9) but not on template A (−333 to −259). Interestingly, template B (−259 to −154) confers full *INO1*-specific regulation to a heterologous gene fusion (*CYC1-lacI'Z*; 7).

The protein component(s) of the complex that is dependent on the nonamer is not yet genetically defined. Seven copies of the repeated nonamer are present in the *INO1* promoter (Figure 9) and in the present study four of these were found to be capable of forming a complex with a factor, designated NBF. This factor was shown to be specific in its interaction with the nonamer by competition experiments with the 21 bp oligonucleotide that includes the nonamer motif (Figure 6). Templates A, B, and C (Figure 1; −333 to −154) each contain 2 of these nonamer elements. However, when each of these templates were used in EMSA only a single NBF-specific complex was observed (Figures 2 and 8). Each of these templates includes two copies of the nonamer (Figures 1 and 9) and all four copies of the nonamer are able to form an NBF-specific complex when isolated on a smaller fragment (data not shown) Thus, the inability of templates A, B, and C to form doubly complexed species suggests that NBF binding to one copy of the nonamer site on any single template may preclude binding at the other sites.

Extracts from *S.pombe* appeared to contain a protein that recognizes the binding site represented by the nonamer. A

synthetic 21 bp oligonucleotide that includes the nonamer could compete away formation of a specific complex (Figure 7) formed by extracts prepared from *S.pombe*. The sequence to which these factors bind (5′-ATGTGAAAT-3′) is similar to the octamer sequence (5′-ATGCAAAT-3′) found in the promoters of the mammalian immunoglobulin genes (5, 6). However, the experiments reported here do not establish that the factors present in these two organisms are homologs of the octamer-binding proteins or that they are analogous to each other.

Another type of complex was defined as *OPI1*-dependent. Binding reactions carried out using extracts from strains harboring either a point mutation or a null mutation at the *OPI1* gene were shown to lack a high molecular weight complex (with template A; Figure 8). The *OPI1* gene has been cloned and sequenced and the predicted amino acid sequence identified several motifs that have previously been observed in other DNA-binding proteins (12).

The experiments described here have identified several complexes that assemble on the *INO1* promoter of *S.cerevisiae*. While these experiments do not formally prove that the products of the *INO2*, *INO4* and *OPI1* genes are directly involved in complex formation with the *INO1* promoter, this is a reasonable working hypothesis. Formal proof will require biochemical characterization of the factors present in these complexes. Experiments are in progress to address this issue. In addition, a complex has been identified that is, as yet, genetically undefined: the NBF-specific complex. Biochemical purification of the factor(s) present in this complex and cloning of the gene(s) that encodes it will greatly facilitate defining its function(s).

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