Cis and trans regulatory elements required for regulation of the *CHO1* gene of *Saccharomyces cerevisiae*

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ABSTRACT

A 34 base-pair (bp) fragment spanning sequences –154 to –120 of the promoter of the *CHO1* gene (structural gene for phosphatidylserine synthase) from the yeast *Saccharomyces cerevisiae* has been shown to place transcription of a promoter-less *Escherichia coli lacZ* gene under control of the phospholipid precursors inositol and choline. Furthermore, in deletion experiments the *CHO1* UAS_{INO} was localized to sequences between –151 and –123 of the *CHO1* promoter. A nine bp sequence was identified in the promoter region of the *CHO1* gene that shares an eight out of nine bp match with a sequence (consensus 5' ATGTGAAAT 3') that is repeated a total of 23 times upstream from several coregulated phospholipid biosynthetic genes. This sequence is contained within the –151 to –123 region to which the *CHO1* UAS has been localized. The nine bp repeated element is believed to be involved in the control of phospholipid biosynthetic gene transcription in response to changing levels of inositol and choline in the growth medium. This control has been shown to require activities encoded by the products of the three regulatory genes: *INO2*, *INO4*, and *OPI1*. A mutation in any of these regulatory genes results in aberrant *CHO1-lacZ* gene regulation, and affects regulation of the construct containing the 34 bp (–154 to –120) *CHO1* fragment demonstrating that the regulatory signal generated by these genes interacts with the 5' end of the *CHO1* gene.

INTRODUCTION

Phospholipid biosynthesis is a highly regulated process in the yeast *Saccharomyces cerevisiae*. One facet of this regulation involves the coordinated control of a number of phospholipid biosynthetic enzymes in response to changing levels of the soluble phospholipid precursors, inositol and choline in the growth media

(1, 2). Enzymes known to be subject to this control include the membrane-associated enzymes, CDP-diacylglycerol synthase (3), phosphatidylserine (PS) synthase (4) PS-decarboxylase (5), and the phospholipid N-methyltransferases (PMTs) responsible for the conversion of phosphatidylethanolamine (PE) into phosphatidylcholine (PC; 4, 6). Another coregulated enzyme is the cytoplasmic enzyme, inositol-1-phosphate synthase (IPS), that catalyzes the conversion of glucose-6-phosphate to inositol-1-phosphate, the first committed step in the synthesis of the inositol-containing phospholipids (7, 8). The coordinated regulation of these enzymes has been shown to involve *trans*-acting regulatory factors encoded by the *INO2*, *INO4*, and *OPI1* genes (2). Recessive mutations at the *INO2* and *INO4* loci lead to an inability of the cell to derepress any of the coregulated enzymes. Thus, *ino2* and *ino4* mutants express low (repressed) levels of all of the coordinately regulated enzymes (8, 9, 10). In contrast, *opi1* mutants constitutively express derepressed levels of the enzymes (3, 4, 8, 10, 11).

A number of structural genes encoding phospholipid biosynthetic enzymes have been cloned and sequenced (1). These genes include *INO1*, the structural gene for IPS (12, 13) and *CHO1*, the structural gene for PS synthase (14, 15). The regulation of the *CHO1* and *INO1* genes in response to changing levels of inositol and choline has been shown to occur at the level of mRNA abundance (8, 10). The *INO1* and *CHO1* genes are maximally derepressed in wild type cells grown in medium lacking inositol and choline, and maximally repressed when inositol and choline are both present. The addition of inositol to growth medium leads to an intermediate level of repression whereas the presence of choline alone has little effect. However, the repression ratio is substantially different for the products of each of these genes. For example, the abundance of the *INO1* transcript is only 10% of the fully derepressed level if cells are grown in medium containing inositol and 3% of the derepressed level if inositol and choline are both present in the medium (8). The transcript of the *CHO1* gene, in contrast, is expressed at 60% of its fully derepressed level if cells are grown in the

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presence of inositol alone, and it is expressed at 15–20% of its derepressed level if inositol and choline are both present in the growth medium (10). Thus, an important unanswered question is how the products of these two genes can be differentially regulated (i.e., have such different repression ratios) while being controlled by a single regulatory mechanism. Analysis of sequences in the 5' non-transcribed region of the *INO1* gene has revealed the presence of a nine bp repeated element (16) that is also present in the *CHO1* promoter region (1). The nine bp sequence does not, in isolation, serve as a UAS to activate transcription of a promoter-less reporter gene (16), but it does bind a specific protein found in extracts of all yeast strains including mutants defective in *INO1* regulation (17). This report describes the results of a molecular analysis of the 5' flanking sequences of the *CHO1* gene and their interaction with *trans*-acting regulatory genes.

MATERIALS AND METHODS

Strains, media and growth conditions

S. cerevisiae strains used in this study are described in Table 1. *E. coli* strains HB101 (18) and JM109 (19) were maintained on Luria-Bertani medium (LB; 20) and a medium lacking proline (19), respectively. Conditions and media used for growth and maintenance of *S. cerevisiae* strains has been described (8). A buffered medium containing X-gal (21) was used to score β -galactosidase activity in yeast. In all experiments, yeast cells were grown at 30°C.

Plasmid constructions and DNA preparation

A plasmid containing a 1.9 Kb *CHO1* subclone (pAB208) was cut with various restriction enzymes according to supplier specifications and the desired restriction fragments were recovered from electrophoretic gels. Plasmids pAB701, pAB702, and pAB703 were constructed by inserting a 1.0 Kb *XhoI/EcoRI* restriction fragment (Figure 1), bearing the 5' end of the *CHO1* gene, into plasmids YEp356R, YEp357R, and YEp358R, respectively (22). Plasmids YEp356R, YEp357R, and YEp358R (22) contain all but the first seven amino acids of the *E. coli lacZ* gene and the multiple cloning site (MCS) from pUC18. These elements are positioned to permit construction of gene fusions in all three reading frames. These plasmids also possess the *S. cerevisiae URA3* gene and the 2 μ autonomously replicating sequence (ARS) for selection and autonomous replication in yeast, respectively. Wild type yeast cells (W303-1A) that harbored the recombinant plasmids were scored for *CHO1-lacZ* gene expression on media containing X-gal. The pAB703 transformants were colorless. Cells transformed with pAB701 and pAB702 both produced the characteristic blue color indicative of β -galactosidase activity, but the pAB701 transformants produced much more color than the pAB702 transformants. Sequence analysis revealed that plasmid pAB701 contained an in-frame fusion with the first 13 codons of the putative *CHO1* ORF (starts at +13; Figure 2). Sequence analysis also revealed that the fusion in plasmid pAB702 places the first eight codons of an 18 codon ORF (starts at +26; Figure 2) in frame with the *lacZ* gene explaining the low level of β -galactosidase activity observed for transformants that harbor that fusion. The *lacZ* fusion plasmids, pAB707, pAB708, and pAB709 (Figure 3) were constructed in a similar fashion by ligating the appropriate purified *CHO1* restriction fragments with plasmids YEp356R, YEp357R,

and YEp358R cut at the proper restriction sites MCS. Plasmids pJL102 and pJL103 (Figure 3) were constructed using pAB709 as a source for *CHO1* sequences. Plasmids pAB751, pAB754, pTC102, and pTC103 were constructed by ligating the appropriate restriction fragments from pAB701, pAB709, pJL102, and pJL103 (respectively) into YIp356R (22); identical to YEp356R except that it lacks the 2 μ ARS. Plasmid pTL101 (Figure 3) was constructed by inserting a synthetic oligonucleotide with *XhoI* overhangs into the *XhoI* site of pJH304 (UAS-less *CYC1-lacI'*Z vector; 16). The double-stranded oligonucleotide consisted of two single-stranded oligonucleotides that included DNA sequences (Figure 2) from –154 to –120 of the *CHO1* promoter: LOPES-1 5' TCGAGAGCTTTTCACATGGACCCATCTAAAGATGAAGATC 3'; LOPES-2 5' TCGAGATCTTCATCTTTAGATGGGTCCATGTGAAAGCTC 3'. Plasmids pAB801 and pAB802 were constructed by ligating subclones of the *CHO1* and *INO1* genes (respectively) into the MCS of YEp351 (23) an *E. coli*/yeast shuttle vector. These derivatives also contain the 2 μ ARS and the yeast *LEU2* gene. Subclones of *CHO1* to be used for preparation of sequencing templates were constructed in M13mp18 and mp19 RF DNA.

Plasmid DNA was isolated from bacterial strain HB101 in mini-prep or larger quantities from cesium chloride gradients as previously described (24, 25). Single-stranded DNA, to be used as sequencing templates, was prepared from JM109 transformed with M13mp derivatives according to standard procedures (18).

Yeast transformation

Wild type and regulatory mutant (*ino2*, *ino4*, and *opi1*) yeast strains harboring a *ura3* mutation were transformed to uracil prototrophy with autonomously replicating plasmids (YEp versions), encoding the various *CHO1-lacZ* fusions, using a divalent cation method (26). Chromosomal integration at the *ura3* locus was accomplished by transforming yeast strains with integrating plasmids (YIp versions) linearized at a single *NcoI* site within the *URA3* gene. Integration at the *ura3* locus and copy number was assessed by Southern blot hybridization of genomic DNA isolated from transformants (16).

Table 1. Yeast Strains (*S. cerevisiae*)

Strain	Genotype	Source	Reference
ade5	<i>MATa, ade5</i>	Lab strain	36
clu18	<i>MATa, cho1-3, leu2-3,112, his2, his3, ura3-52</i>	Lab strain	10
W303-1A	<i>MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1</i>	R. Rothstein	10
DKT307	<i>MATa, ino2-21, leu2-3,112, ade2-1, trp1-1, ura3-1, his3-11,15</i>	Lab strain	10
it8	<i>MATa, ino2-21, ura3-1, lys2</i>	Lab strain	This study
DKT308	<i>MATa, ino4-39, leu2-3,112, ura3-1</i>	Lab strain	10
it20	<i>MATa, ino4-40, leu2-3,112, his3, lys2, ura3-1</i>	Lab strain	This study
DKT313	<i>MATa, opi1-1, ura3-1, trp1-1, ade5, lys2</i>	Lab strain	10
A10	<i>MATa, ino2, ino4, ade5, lys2, trp1</i>	Lab strain	This study
A6	<i>MATa, ino2, opi1, ade1, lys1</i>	Lab strain	This study
A4	<i>MATa, ino4, opi1, ade5, lys1</i>	Lab strain	This study
BY101	<i>MATa, ade2-1, can1-100, trp1-1, his3-11,15, leu2-3,112, ura3::pAB751::URA3 (CHO1-lacZ)</i>	Lab strain	This study
BY121	<i>MATa, ade2-1, can1-100, trp1-1, his3-11,15, leu2-3,112, ura3::pAB754::URA3 (CHO1-lacZ)</i>	Lab strain	This study

RNA isolation and analysis: S1 and slot blot

Total cellular RNA was collected from yeast cells by a procedure previously described (27). Slot blots were hybridized to cRNA probes prepared from pAB306 (*CHO1*-specific) and pAB309 (*T-CMI*-specific) and quantitated by densitometric analysis of autoradiograms (10).

The 5' terminus of *CHO1* mRNA and mRNA transcribed from gene fusion constructs was determined by S1 analysis of total cellular RNA from yeast (18). Restriction fragments were prepared by electrophoretic fractionation and end-labelled with [γ - 32 P]ATP (Amersham Corp.) using T4 polynucleotide kinase (Boehringer-Mannheim, Inc.). Single-stranded probes were generated by digesting labelled restriction fragments with a second enzyme that cuts between the labelled sites and denaturing at 80°C in the presence of 10 μ g of total yeast RNA. RNA/DNA hybrids were formed at 42°C for 3 hr in S1 reaction buffer (0.28 M NaCl, 50 mM NaAcetate pH 4.5, 4.5 mM ZnSO₄) and exposed to 0, 10, or 20 units of S1 nuclease (Boehringer-Mannheim, Inc.) at 37°C for 30 min. S1-resistant products were resolved by fractionation on 8% acrylamide, 8 M urea, 0.5 $\times$ TBE gels alongside a sequencing ladder generated from the cloned *CHO1* gene (32), and visualized by autoradiography.

β -galactosidase activity assay

Assays for β -galactosidase activity were performed as previously described (16). Briefly, 330 μ l of cell extract were added to 1.32 ml of β -gal assay buffer (20% glycerol, 0.1 M Tris/HCl pH=8.0, 1 mM PMSF, 2 μ M pepstatin A, 1 mM DTT) and incubated at 28°C for five minutes. The reaction was initiated by adding 660 μ l of 4 mg/ml ONPG (in distilled water) and aliquots were removed at several time intervals and added to 500 μ l of 1M Na₂CO₃ (stop solution). The optical density of the final solution was measured at 420 nm. The total protein concentration of each

lysate was determined using a BioRad assay kit (28). Units of β -galactosidase activity are defined as (optical density @ 420 nm/min/mg of total protein) $\times$ 1000.

DNA sequencing

DNA sequencing was performed as previously described (29) using materials supplied with a BRL sequencing kit. Single-stranded DNA templates were prepared as described above.

RESULTS

Localizing the *cis*-acting elements required for *CHO1* gene regulation

A 2.8 Kb restriction fragment of DNA bearing the *CHO1* gene was previously shown to complement the choline/ethanolamine auxotrophy of *cho1* mutants (pAB206; 10). Expression of *CHO1* mRNA from this subclone exhibits full regulation in response to inositol and choline, demonstrating that this 2.8 Kb *CHO1* clone possesses all of the *cis*-acting sequences required for the regulation of *CHO1* gene expression in response to changing levels of the phospholipid precursors inositol and choline. Subsequently, plasmid pAB208 was constructed by inserting a 1.9 Kb *XhoI*-*PvuII* restriction fragment from pAB206 into the multicopy plasmid YEp351 (23; Figure 1). The 1.9 Kb *CHO1* subclone in pAB208 lacks 0.9 Kb of DNA from one end of the

Figure 1. Schematic representation of several plasmids (YEep-based) used in this study. Shown for reference are the relative positions of the *CHO1* and *INO1* ORFs. Each of the restriction fragments noted were inserted into YEp351 (23). Abbreviations: X, *XhoI*; A, *AccI*; S, *SspI*; V, *EcoRV*; L, *AluI*; Z, *XhoII*; E, *EcoRI*; N, *NdeI*; C, *NcoI*; P, *PvuII*; T, *PstI*; Q, *SstI*; B, *BglII*; H, *SphI*.

Figure 2. Sequence of regulatory sequences and amino terminus of the *CHO1* gene. Sequence data presented here confirms that of others (15, 35). Consensus binding sites for ABF1 and the nine bp element (discussed in the text) are denoted with arrows indicating orientation. The consensus TATA (30, 31) sequence is boxed and several transcription initiation start sites are noted with arrows (32). Sequences are numbered relative to the major transcription start site (+1). Marked for reference are the *EcoRI* restriction site (used as the 3' boundary for *CHO1-lacZ* fusions) and two ORFs (discussed in text). The -123 and -151 sites were used as the 5' boundaries for the construction of gene fusions described in the text.

2.8 Kb *CHO1* clone. When transformed into *cho1* mutant strains, plasmid pAB208 complements the choline/ethanolamine auxotrophy and restores PS biosynthesis (data not shown).

The DNA sequence of the 475 bp *EcoRV* restriction fragment from pAB208 is shown in Figure 2. This fragment includes the entire 5' region of the *CHO1* gene used to make the *lacZ* fusion in pAB709 (Figure 3) and includes 150 bp of additional *CHO1* coding sequence downstream of the fusion site (Figure 2). Two open reading frames (ORFs) are contained within the sequence shown in Figure 2. The shorter ORF starts at position +26 on the sequence and ends at position +79 and encodes 18 amino acids (Figure 2). The second ORF, which runs from the first ATG (position +13) to the end of the fragment, following the TATA box sequence (30, 31) to the end of the fragment, is the coding sequence for the *CHO1* gene product, PS-synthase (15). The 5' end of the predominant *CHO1* mRNA species has been mapped to the A residue at +1 and additional minor transcripts were mapped to +5, +7 and +12 (32; Figure 2). The 5' ends of the *CHO1-lacZ* fusion transcripts from plasmids pAB701 were mapped (data not shown) to positions in the *CHO1* region of the fusions identical to those previously reported for the intact *CHO1* gene (32) and illustrated in Figure 2. An inverted copy of a nine bp element (5' ATGTGAAAT 3') that is found repeated 23 times upstream of several phospholipid biosynthetic genes (1), is centered at position -147 (Figure 2). In addition, a consensus ABF1 binding site (5' RKCDYNNNNNACG 3'; 33) is located at position -185.

To facilitate analysis of the *cis*-acting elements required for *CHO1* gene regulation, the 5' end of the 1.9 Kb *CHO1* subclone from plasmid pAB208, encoding the amino-terminus of PS synthase, was fused to the *E. coli lacZ* gene to create plasmid pAB701 as described in the Methods. Crude extracts of pAB701-transformed wild-type cells grown in varying conditions of inositol and/or choline supplementation were assayed for β -galactosidase (Table 2). Addition of inositol to the growth medium resulted in a reduction of the level of fusion protein activity by approximately one-third compared to the level found in transformants grown in unsupplemented medium. Addition of choline and inositol to the growth medium led to a further reduction in the fusion protein activity level to approximately 30% of the level found in transformants grown in the unsupplemented

medium. Addition of choline (in the absence of inositol) to the growth medium resulted in only slight repression of the fusion protein activity. The relative levels of *CHO1-lacZ* fusion activity in the transformed wild-type cells closely resembled the relative levels of PS synthase and *CHO1* mRNA found in wild-type cells grown under equivalent conditions (10). These results suggest that the 1.0 Kb restriction fragment of the *CHO1* gene used to make the *CHO1-lacZ* fusion in the plasmid in pAB701 contains all of the *cis*-acting elements necessary for fully-regulated expression of the fusion gene. Expression of β -galactosidase from the gene fusion contained in pAB701 was also explored in transformants containing a single copy of the fusion chromosomally integrated. Yeast strain BY101 (Table 1) contains this *CHO1-lacZ* fusion integrated at the *URA3* locus. The *CHO1-lacZ* gene fusion contained in BY101 is regulated in a fashion identical to the regulation observed for the *CHO1-lacZ* gene fusion contained on the high copy plasmid (Tables 2 and 3). Strain BY101 is wild type for all known phospholipid biosynthetic functions.

When the *CHO1* gene was first isolated on a high copy plasmid, it was reported that strains bearing this construct expressed high, unregulated levels of PS synthase (14). Since a number of the constructs employed in this study involved high copy plasmids, this issue was explored by transforming strain BY101 (bearing a single integrated copy of the *CHO1-lacZ* fusion) with derivatives of the multicopy plasmid YEp351 (23) harboring the intact *CHO1* or *INO1* genes or fragments of those genes (Figure 1). Expression and regulation of the integrated *CHO1-lacZ* fusion gene was largely unaffected by the presence of high copy plasmids harboring the *CHO1* derivatives (Table 3). Likewise, plasmids including sequences from the *INO1* 5' or 3' regions had little effect on expression of the integrated *CHO1-lacZ* fusion. However, expression of the *CHO1-lacZ* fusion gene was affected when strain BY101 was transformed with the intact *INO1* gene (Table 3). In this case, expression of the gene fusion did not reach fully derepressed levels even when the transformant was grown in the absence of inositol and choline. The failure to achieve full derepression in this transformant grown under derepressing conditions is believed to be due to production of excess inositol by the intact *INO1* gene, a topic that will be taken up in the Discussion.

Table 2. Effect of Regulatory Mutations on *CHO1-lacZ* Levels

Plasmid/strain (relevant genotype)	Growth Conditions ^a			
	I+C+	I+C-	I-C+	I-C-
pAB701/W303-1A (wild type)	28.5 ^b	67.1	90.0	100.0
pAB701/it8 (<i>ino2</i>)	27.7	26.8	NP	NP
pAB701/DKT307 (<i>ino2</i>)	29.8	30.0	NP	NP
pAB701/it20 (<i>ino4</i>)	26.1	21.5	NP	NP
pAB701/DKT308 (<i>ino4</i>)	32.3	25.4	NP	NP
pAB701/DKT313 (<i>opi1</i>)	200.6	187.7	167.5	163.8

^a Wild type and regulatory mutant yeast strains transformed with the *CHO1-lacZ* fusion plasmid pAB701 (YEp-based plasmid) were grown in vitamin- and uracil-free media (I-C-) or media supplemented with 75 μ M inositol (I+C-), 1 mM choline (I-C+), or both 75 μ M inositol and 1 mM choline (I+C+). NP (not performed): transformants harboring *ino2* or *ino4* mutants could not be grown in the absence of inositol since they are inositol auxotrophs.

^b Transformed cell lysates were prepared and assayed for *CHO1-lacZ* fusion protein (β -galactosidase) activity. Each transformant was assayed at least twice and the average activity for each transformant was normalized relative to the pAB701/W303-1A transformant grown in unsupplemented media (I-C-).

Table 3. Effect of Plasmid Competition on *CHO1-lacZ* Expression

Plasmid/strain (plasmid description)	Growth conditions ^a			
	I+C+	I+C-	I-C+	I-C-
YEp351/BY101	23.3	52.5	98.8	100.0
pAB208/BY101 (<i>CHO1</i>)	29.4	52.5	110.2	141.4
pAB801/BY101 (<i>CHO1</i> 5' region)	30.2	78.2	92.4	121.1
pJH318/BY101 (<i>INO1</i>)	29.6	61.5	64.1	60.6
pAB802/BY101 (<i>INO1</i> 5' region)	28.5	36.6	66.9	87.4
pJH321/BY101 (<i>INO1</i> 3' region)	17.9	22.3	78.8	92.0

^a Wild type cells harboring genomic copies of the full-length *CHO1-lacZ* fusion gene (BY101 contains pAB751 [YIp-based version of pAB701] integrated at the *URA3* locus of W303-1A) were transformed with the multi-copy plasmid YEp351 (23) or with YEp351 derivatives bearing *INO1* or *CHO1* subclones. The transformants were grown in vitamin- and leucine-free media (I-C-) or media supplemented with 75 μ M inositol (I+C-), 1 mM choline (I-C+), or both 75 μ M inositol and 1 mM choline (I+C+).

^b Transformed cell lysates were prepared and assayed for *CHO1-lacZ* fusion protein (β -galactosidase) activity. Each transformant was assayed at least twice and the average activity for each transformant was normalized relative to the YEp351/BY101 transformant grown in unsupplemented media (I-C-).

In order to localize further the *cis*-acting elements responsible for the regulation of *CHO1-lacZ* fusion gene expression, several *CHO1* subclones were inserted into plasmid YEp356R (Figure 3). Each fusion shared the same 3' boundary as the *CHO1* clone in pAB701 (*EcoRI* site) but had different 5' boundaries. The resulting plasmids, pAB707 (–840), pAB708 (–500), pAB709 (–300), pJL102 (–151), and pJL103 (–123), were transformed into wild-type yeast cells and β -galactosidase was assayed in transformants grown under repressing and derepressing conditions (Figure 3). The relative levels of β -galactosidase activity produced by each of the gene fusion constructs corresponded completely with the relative steady-state levels of *CHO1-lacZ* mRNA as determined by slot blot analysis (data not shown). Moreover, the same relative levels of expression were observed in strains that harbored each of the plasmids integrated at the *URA3* locus in single or multiple copies (data not shown). Growth of the transformant strains harboring pAB701, pAB707, pAB708, pAB709, and pJL102 in medium containing inositol led to partial reduction in fusion protein activity while growth in the presence of inositol and choline led to maximal repression

(Figure 3). Plasmids pAB709 (–300) and pJL102 (–151) produced slightly higher levels of fusion protein activity under all growth conditions than did the other plasmids. However, in each case a similar repression ratio was observed (Figure 3). The DNA fragment from –300 to –151 contains an ABF1 binding site (33). Since plasmids pAB709 (–300) and pJL102 (–151) support expression of virtually identical levels of β -galactosidase under all growth conditions, it appears that the ABF1 element has no effect on *CHO1* expression. Transformants harboring pJL103 (–123) expressed levels of fusion protein activity identical to the repressed levels observed for pAB709 (–300) and pJL102 (–151) under all growth conditions (Figure 3). Thus, sequences required for fully regulated expression of the *CHO1-lacZ* fusion gene must be located between –151 and –123 while sequences controlling basal *CHO1* expression must be contained within the sequence remaining within the –123 construct.

To test if the *CHO1* upstream activation sequences that respond to growth in the presence of inositol and choline (UAS_{INO}) are located between –151 (pJL102) and –123 (pJL103), a synthetic

Figure 3. Schematic representation of *CHO1-lacZ* fusions and a *CHO1 UAS*_{INO} fusion to *CYC1-lacI'Z* (all the plasmids described are YEp-based). Noted are the relative positions of the *CHO1* and *CYC1* TATA sequences, the nine bp sequence (black box), the ABF1 binding site (open box) and the *CYC1* start site of transcription (+1). Each of the *CHO1-lacZ* fusions were generated by inserting the relevant restriction fragments into YEp356R (22) using the *EcoRI* site as a fusion junction. The *CHO1* promoter 5'-terminal nucleotide for each construct is indicated in parentheses and the pertinent 5' restriction site is noted next to each plasmid. Plasmid pTL101 was constructed by inserting a synthetic oligonucleotide that included *CHO1* promoter sequences from –151 to –123 (flanked by *XhoI* sites) into the *XhoI* site of pJH304 (16). β -galactosidase activity produced by the *CHO1-lacZ* fusion plasmids is shown normalized to the level generated by pAB701 when grown under fully derepressing conditions (I-C-). β -galactosidase units produced by the *CHO1 UAS-lacI'Z* are equal to 1000 $\times$ (optical density at 420 nm/min/mg of total protein). For each plasmid the values presented reflect the average of at least 3 independent experiments. Abbreviations: I-C-, unsupplemented medium; I-C+, medium containing 1 mM choline; I+C-, medium containing 75 μ M inositol; I+C+, medium containing 75 μ M inositol and 1 mM choline; I+(10)C+, medium containing 10 μ M inositol and 1 mM choline.

oligonucleotide (including sequences –154 to –120) was placed upstream of a heterologous UAS-less reporter gene (*CYCI-lacI'Z*) on plasmid pJH304 (16). Expression of the reporter gene was found to be activated and regulated in response to growth in the presence of inositol and choline when the UAS_{INO} containing oligonucleotide was inserted upstream (pTL101 in Figure 3). However, the relative degree of repression of this construct was far greater than the 3- to 4-fold repression observed for any of the *CHO1-lacZ* fusions or for the native *CHO1* transcript (10). Furthermore, this construct in contrast to the native *CHO1* gene and all of the *CHO1-lacZ* fusion constructs exhibited no detectable basal expression (Figure 3).

Trans-acting factors involved in the control of CHO1 gene expression

The *INO2*, *INO4*, and *OP1* regulatory genes have been shown to affect *CHO1* gene expression (10). *CHO1* mRNA levels are low and constitutive in *ino2* and *ino4* mutant cells (approximately equal to the wild-type strain [*ade5*] fully repressed level; Figure 4; 10). In *op1* mutant cells the *CHO1* mRNA level is high and constitutive (approximately equal to the fully derepressed level observed in wild-type cells; Figure 5; 10). β -galactosidase activity was measured in *ino2*, *ino4*, and *op1* single mutant strains transformed with either the *CHO1-lacZ* fusion plasmid pAB701 (Table 2) or the *CHO1-CYC1-lacI'*Z plasmid pTL101 (Figure 3). The activity of β -galactosidase expressed from the *CHO1-lacZ* plasmid (pAB701) was constitutive in *ino2* and *ino4* strains at a level approximating that observed in wild-type strains grown under repressing conditions (Table 2). β -galactosidase activity levels in *op1* mutant cells transformed with plasmid pAB701 were also constitutive; in this case at a level roughly twice that found in wild-type cells transformed with pAB701 and grown under fully derepressing conditions (Table 2). Expression of *CYC1-lacI'*Z from pTL101 was completely absent in the *ino2* mutant background and *ino4* mutant strain (Figure 3) grown under repressing conditions (I+ C+; Figure 3). Both of these

strains were also grown in media supplemented with 10 mM inositol which allows for growth but does not yield full repression in a wild type strain. This growth condition yields 20% repression of *CYC1-lacI'Z* expression from pTL101 in a wild type strain as compared to the 40% repression observed for the native *INO1* gene under similar growth conditions (8). The *ino2* strain transformed with pTL101 gave no expression of β -galactosidase when grown under these partially derepressing conditions, but *ino4* transformant expressed low but detectable (1.6 units) of activity (Figure 3). Expression from pTL101 in an *opi1* strain was constitutive at a level roughly two-fold higher than wild type derepressed expression.

The epistatic interactions of the *INO2*, *INO4*, and *OPI1* genes were explored by constructing double mutant strains. Levels of *CHO1* mRNA in the *ino2 opi1* and *ino2 ino4* double mutant strains were found to be roughly equivalent to levels of *CHO1* mRNA found in the *ino2* and *ino4* single mutant strains grown under equivalent conditions (Figure 4). The *ino4, opi1* double mutant strain expressed *CHO1* mRNA constitutively at a level intermediate between the levels found in the *ino4* and *opi1* single mutant strains.

DISCUSSION

The regulation of genes transcribed by RNA polymerase II in *S. cerevisiae* requires the presence of elements upstream of the transcription start site. These upstream activating sequences (UAS) interact with *trans*-acting regulatory factors (34). Previously, expression of the *CHO1* gene was shown to be regulated at the level of mRNA abundance in response to changing levels of the soluble phospholipid precursors inositol and choline (10). We have now shown that fusion of fragments from the 5' end of the *CHO1* gene to the *E. coli lacZ* reporter gene result in *CHO1-lacZ* gene expression that is regulated in response to changing levels of inositol and choline in wild type cells. Furthermore, expression of the fusion gene is sensitive to mutations in previously defined regulatory genes in a pattern analogous to that of native *CHO1* gene transcription (10).

Expression of the *CHO1-lacZ* fusion gene integrated at the *URA3* locus of a wild type yeast strain was sensitive to multiple copies of the *INO1* gene on an episomal plasmid (Table 3). Multiple copies of the intact *INO1* gene, but not multiple copies of the *INO1* 5' or 3' ends, resulted in an inability to fully derepress the single copy of the *CHO1-lacZ* gene fusion integrated at the *URA3* locus. The level of β -galactosidase expression achieved under fully derepressing conditions (I-C-; Table 3) was comparable to the relative expression of the *CHO1* native transcript observed in cells grown under partially repressing conditions (i.e., when inositol alone is present; 10). This result suggests that altered regulation of the *CHO1-lacZ* fusion gene in the *INO1* transformants results from high endogenous levels of inositol due to elevated expression of IPS enzymatic activity. Clearly, however, multiple copies of the 5' regions of *CHO1* and *INO1* genes had little or no effect upon expression of the gene fusions, indicating that the regulatory apparatus was not saturated by the excess copies of the *cis*-acting regulatory sequences.

Regulation of the steady-state level of the *CHO1* and *CHO1-lacZ* fusion gene transcripts in response to alterations in the level of inositol and choline supplementation requires the products of at least three regulatory genes, *INO2*, *INO4*, and

Figure 4. Slot-blot analysis of steady-state levels of *CHO1* mRNA from wild type, single and double regulatory mutant cells grown under various conditions of choline supplementation. Wild type (*ade5* strain) cells or cells with one (*ino2* [DKT307], *ino4* [DKT308], and *opil* [DKT313]), or two (*ino2/ino4* [A10], *ino2/opil* [A6], *ino4/opil* [A4]) regulatory gene mutations were grown in inositol-containing medium (75 μ M) with (–) or without (+) supplemental choline (1 mM). Total RNA was prepared and applied to a slot blot (10 μ g/lane) and slot blots were sequentially hybridized with *CHO1* (pAB306; 10) and *TCM1*-specific (pAB309; 10) cRNA probes. The resulting autoradiograms with signals that were within the linear range were quantitated by densitometry. Employing the amounts of *TCM1* mRNA (encodes a ribosomal protein) as an internal standard, the amounts of *CHO1* mRNA found in each strain and growth condition were expressed as the quotient of the [*CHO1* RNA]/[*TCM1* RNA] value for that strain and growth condition relative to the value determined for the wild type strain grown under fully derepressing conditions (no supplements).

OPII. *CHO1* mRNA abundance in the *ino2 ino4* and *ino2 opi1* double mutant strains was constitutive at a level roughly equivalent to that found in wild type cells grown in fully repressing conditions (I+C+; Figure 4). The *CHO1* mRNA levels in the *ino4 opi1* double mutant strains were constitutive at a level slightly higher than wild type repressed levels, but still well below wild type derepressed or *opi1* levels. The *CHO1* mRNA expression phenotypes of the double mutant combinations suggest a hierarchy of gene action in *CHO1* gene expression similar to that observed for the *INO1* gene (8). Recent studies have suggested that the *OPII*, *INO4* and *INO2* genes all encode DNA binding proteins (37, 38, 17 and M. Nikoloff, personal communication). Thus, all of these regulatory gene products may be involved directly in transcriptional control of *CHO1*, *INO1* and other coregulated genes.

The *CHO1-lacZ* fusion present in plasmids pAB701, pAB707, pAB708, pAB709, and pJL102 was found to be regulated in response to changes in the levels of inositol and choline in the growth medium. The regulation of the gene fusion in both wild type and mutant strains was identical to the regulation previously reported for the abundance of the native *CHO1* gene (10). However, wild type cells transformed with plasmid pJL103 (which has its 5' boundary at -123) expressed low constitutive levels of the *CHO1-lacZ* fusion gene product. This result indicates that removal of sequences between -151 and -123 eliminates regulation but not basal expression of the *CHO1* gene. Levels of β -galactosidase in the pJL103 transformants grown under both repressing and derepressing conditions were equivalent to the levels observed in the pAB709 and pJL102 (-151) transformants grown under the repressing conditions (Figure 3). Thus, the *CHO1* UAS_{INO} is likely to reside between 151 and 123 bp 5' to the site of initiation of *CHO1* gene transcription. The results of the deletion analysis were supported by finding that a fragment corresponding to residues -154 to -120 conferred inositol- and choline-sensitive regulation to the heterologous UAS-less *CYC1-lacI'Z* gene (pTL101 construct; Figure 3). This construct, unlike the native *CHO1* gene and the *CHO1-lacZ* fusion constructs, failed to support any detectable basal expression consistent with the hypothesis that sequences controlling *CHO1* basal expression lie outside this region.

Expression of *CYC1-lacI'Z* from pTL101 was also sensitive to mutations in the *INO2*, *INO4*, and *OPI1* regulatory genes (Figure 3). Thus, the information required both for activation and repression of *CHO1* expression and for interaction with signals generated by the gene products of the *INO2*, *INO4* and *OPI1* genes, must lie within the fragment spanning -154 to -120 of the *CHO1* promoter. Interestingly, the *ino4* mutant strain was still able to direct expression from pTL101 under partially derepressing conditions at a reduced level (1.6 units of activity when grown in 10 mM inositol; Figure 3). This observation may also be related to the incomplete epistasis of *ino4* on *opi1* when *CHO1* expression is monitored (Figure 4). Both of these effects constitute differences between the effect of the *ino2*, as compared to the *ino4* mutation, on *CHO1* expression. The *ino2-21* and *ino4-39* mutant alleles employed are both nonsense mutants that result in relatively early chain termination of their respective gene products and are believed to be null mutants on a biochemical level (J. Ambroziak, M. Nikoloff; personal communication). Studies of the effects of these same mutations on the expression of the *INO1* gene had failed to reveal any significant difference between the effects of these same *ino2* and *ino4* mutations (8, 16, 17). However, the results of these studies on *CHO1*

expression suggest that the *INO2* and *INO4* gene products may play subtly different roles in the overall pattern of regulation.

The best candidate for the *CHO1* UAS_{INO} is the nine bp sequence located at position -147. This sequence is an 8 out of nine bp match with a consensus sequence (5' ATGTGAAAT 3') that is repeated a total of 23 times in association with the promoters of several coregulated phospholipid biosynthetic genes (1). This element binds a specific protein from yeast extracts (17). One of these *INO1* upstream elements is in the same inverted orientation and position relative to the initiator ATG codon as the single element found in the *CHO1* promoter. Since the repeated element is the only segment of sequence similarity (with the exception of the TATA sequence) shared by the coregulated *CHO1* and *INO1* promoters it seems likely that this element is involved in *CHO1* gene regulation. This nine bp sequence, however, failed to activate transcription when it was inserted in single or multiple copies into the same promoter-less plasmid used to make pTL101 (16). Thus, this sequence by itself cannot constitute the UAS_{INO}. However, at least one copy of this sequence is included in every promoter that conveys inositol- and choline-sensitive regulation to a heterologous reporter gene (16, T. Gill unpublished observations). Thus, mounting evidence suggests that this repeated sequence is a crucial *cis*-acting regulatory element in the phospholipid biosynthetic regulon of *S. cerevisiae*.

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