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Multiple Basic Helix-Loop-Helix Proteins Regulate Expression of the *ENO1* Gene of *Saccharomyces cerevisiae*[▽]

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The basic helix-loop-helix (bHLH) eukaryotic transcription factors have the ability to form multiple dimer combinations. This property, together with limited DNA-binding specificity for the E box (CANNTG), makes them ideally suited for combinatorial control of gene expression. We tested the ability of all nine *Saccharomyces cerevisiae* bHLH proteins to regulate the enolase-encoding gene *ENO1*. *ENO1* was known to be activated by the bHLH protein Sgc1p. Here we show that expression of an *ENO1-lacZ* reporter was also regulated by the other eight bHLH proteins, namely, Ino2p, Ino4p, Cbf1p, Rtg1p, Rtg3p, Pho4p, Hms1p, and Ygr290wp. *ENO1-lacZ* expression was also repressed by growth in inositol-choline-containing medium. Epistatic analysis and chromatin immunoprecipitation experiments showed that regulation by Sgc1p, Ino2p, Ino4p, and Cbf1p and repression by inositol-choline required three distal E boxes, E1, E2, and E3. The pattern of bHLH binding to the three E boxes and experiments with two dominant-negative mutant alleles of *INO4* and *INO2* support the model that bHLH dimer selection affects *ENO1-lacZ* expression. These results support the general model that bHLH proteins can coordinate different biological pathways via multiple mechanisms.

The basic helix-loop-helix (bHLH) proteins are a large family of transcriptional regulators. Over 500 bHLH proteins have been identified in organisms from *Saccharomyces cerevisiae* to mammals (3, 5, 26, 35, 44, 45, 53, 63, 65, 78). They regulate a diverse array of cellular processes, including cell differentiation, development, and cell proliferation (7, 29, 30, 53, 57, 63, 66, 77). Five different properties of bHLH proteins generate sufficient diversity to regulate a variety of different transcriptional programs (53, 63). First, the structure of bHLH proteins includes two amphipathic α-helices, separated by a variable loop, which present hydrophobic residues on one face of each helix. This structure allows for the formation of homodimers or heterodimerization with several different partners (51, 76). In mammals, regulation of genes involved in proliferation and differentiation is governed by Max dimerized with Myc, Max, Mad, and Mxi (29, 30, 57, 66). Similarly, the *S. cerevisiae* Ino4p bHLH protein is a hub that interacts with all yeast bHLH proteins tested thus far (62). Second, dimerization juxtaposes two helices containing basic charged residues that create a DNA-binding interface (51, 53, 63, 76). Therefore, dimerization is a prerequisite for DNA binding. However, bHLH proteins have relatively limited DNA-binding specificity, since most interact with a sequence known as the E box (5′-CANNTG-3′) (9, 19, 21, 51, 76, 80). Conserved amino acids within the DNA-binding region interact with invariant nucleotides, while other residues provide specificity by interacting with the central variant nucleotides or, in some cases, nucleotides that flank the core sequence (9, 19, 21, 51, 76, 80). For example, Pho4p and Cbf1p homodimers both bind the consensus CACGTG sequence, but specificity is dictated by a flanking T nucleotide that inhibits Pho4p binding but not Cbf1p binding (21). Third, some bHLH proteins, such as Ino2p, autoregulate their own expression. The Ino2p-Ino4p heterodimer is required for derepression of the yeast phospholipid biosynthetic genes in response to inositol deprivation (32, 33, 67). Expression of an *INO2-cat* reporter requires both Ino2p and Ino4p (1, 2, 17). Fourth, some family members lack the basic charged DNA-binding domain (HLH) and therefore can dimerize with other bHLH proteins but prevent their binding to DNA. The Id HLH protein acts as a dominant inhibitor by heterodimerizing with other bHLH proteins (E12 and E47) (31, 47, 59, 77). Dimerization with the Id protein prevents these other bHLH proteins from binding, either as homodimers or heterodimers with MyoD, to the muscle creatine kinase enhancer (31, 47, 59, 77). Yeast contains one potential HLH protein, encoded by YGR290w (a dubious open reading frame [ORF]). Lastly, some bHLH proteins are regulated by intracellular compartmentation. Pho4p, Rtg1p, and Rtg3p are present in the cytoplasm under repressing conditions and translocate to the nucleus under activating conditions (36, 37, 42, 74). Collectively, these features make the bHLH protein family particularly suited for combinatorial control of gene expression. *S. cerevisiae* has only nine predicted bHLH proteins and is therefore an excellent model system to examine how this family of transcription factors function in the coordination of gene expression.

Yeast bHLH proteins regulate several important metabolic pathways, including phosphate utilization, glycolysis, and phospholipid biosynthesis (63). Pho4p was the first bHLH protein identified in yeast (6). Pho4p forms a homodimer that activates expression of the *PHO* regulon in response to phosphate limitation (Fig. 1) (60). The activity of Pho4p is regulated by nuclear translocation via phosphorylation at multiple residues (36, 37, 42). Like the case with Pho4p, Rtg1p and Rtg3p activities are also regulated by phosphorylation and nuclear translocation (34, 74). Rtg1p and Rtg3p form a heterodimer that regulates nuclear genes, such as *CIT2*, in response to

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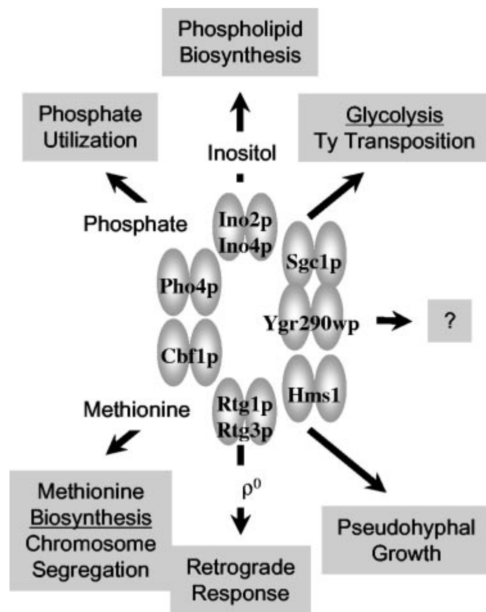

FIG. 1. Biological processes regulated by yeast bHLH proteins. Refer to the text for descriptions.

mitochondrial damage (ρ^0), a process known as retrograde regulation (Fig. 1) (11, 18). Ino2p and Ino4p form a heterodimer that regulates a large set of genes, including the phospholipid biosynthetic genes, in response to inositol deprivation (Fig. 1) (32, 33, 67). Cbf1p has a dual role in regulation of transcription and chromosome segregation (Fig. 1). Cbf1p binds the CACRTG element that is present in many *MET* gene promoters as well as in the centromere DNA element I (12, 39). Hms1p and Ygr290wp have similarity with the HLH family but are the least characterized of the yeast HLH proteins (Fig. 1) (50). Hms1p is required for pseudohyphal growth. Ygr290wp is listed as a dubious ORF (http://www.yeastgenome.org/) and retains some degree of sequence conservation with the HLH domain but lacks a basic charged DNA-binding region. Lastly, Sgc1p (Tye7p) forms a homodimer, activates the expression of glycolytic genes (i.e., *ENO1* and *ENO2*), and may also function in Ty1-mediated gene expression (Fig. 1) (48, 68).

Sgc1p was identified in a genetic selection for mutants that simultaneously restored growth on glucose and expression of an *ENO1-lacZ* reporter gene in a *gcr1* mutant strain (58). Grc1p is required for maximal expression of the enolase genes (*ENO1* and *ENO2*) and several other glycolytic genes (49). Sgc1p and Gcr1p function to stimulate expression of the *ENO1* and *ENO2* genes through parallel pathways, since a *gcr1 sgc1* double mutant strain is more defective in enolase gene expression than either of the single mutant strains (68). In this study, we found that in addition to Sgc1p, several other bHLH proteins affect the expression of the *ENO1* gene. This regulation requires that the bHLH proteins interact with three upstream activation sequence (UAS) elements that conform to the E box binding motif. Regulation through two of these UAS elements may be a recent evolutionary event, since these two elements are limited to the *S. cerevisiae* species. Epistasis analysis coupled with chromatin immunoprecipitation (ChIP) experiments suggests that novel bHLH combinations may interact with these UAS elements.

MATERIALS AND METHODS

Strains, media, and growth conditions. Plasmid-containing *Escherichia coli* DH5α cells (Invitrogen, Carlsbad, CA) were grown in LB-Amp medium (10% [wt/vol] Bacto tryptone, 5% [wt/vol] yeast extract, 10% [wt/vol] NaCl, and 50 μg/ml ampicillin) at 37°C. Plasmid-containing *E. coli* BL21(DE3)/pLysS cells (Novagen, Madison, WI) were grown at 37°C and 25°C in LB-Amp medium supplemented with 50 μg/ml chloramphenicol.

The *S. cerevisiae* strains used in this study were BY4742 (*MAT*α *his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0*), BY4741 (*MAT***a** *his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*), and isogenic strains containing *ino2*Δ, *ino4*Δ, *pho4*Δ, *cbf1*Δ, *sgc1*Δ, *rtg1*Δ, *rtg3*Δ, *hms1*Δ, and *ygr290w*Δ alleles (22, 81). Yeast cultures were grown at 30°C in a complete synthetic medium lacking inositol, choline, KH_2PO_4, and uracil (for reporter plasmid selection) (38). Where indicated, 75 μM inositol (I+) and/or 1 mM choline (C+) was added. Low-P_i medium contained 0.22 mM KH_2PO_4 and 20 mM KCl, and high-P_i medium contained 11 mM KH_2PO_4.

Plasmid construction. Plasmid YEp357R-*ENO1* contains 720 bp of the sequence upstream of the *ENO1* ORF and the first codon fused in frame to the *lacZ* reporter gene in YEp357R (56). This 720-bp region was previously shown to contain all of the regulatory elements necessary for *ENO1* expression (79). YEp357R is a multicopy episomal plasmid with a *URA3* selectable marker (56). This fusion plasmid was constructed by first amplifying 1,000 bp of the *ENO1* promoter from *S. cerevisiae* genomic DNA (Invitrogen, Carlsbad, CA), using primers ENO1 F and ENO1 R (Table 1). The 1,000-bp PCR product was cloned into pGEM-T (Promega, Madison, WI) and sequenced, and then the ORF-proximal 720-bp sequence was excised by digestion with EcoRI and inserted into YEp357R.

Plasmids that complemented the *cbf1*Δ, *sgc1*Δ, *ino2*Δ, and *ino4*Δ mutant alleles were constructed by cloning each ORF and promoter into pRS315. Plasmid pRS315-*CBFI* was constructed by amplifying a 1,556-bp fragment from *S. cerevisiae* genomic DNA (Invitrogen, Carlsbad, CA), using primers CBF1 F′ (position −500) and CBF1 R′ (position +1056) (Table 1). The 1,556-bp PCR product was cloned into pGEM-T (Promega, Madison, WI), sequenced, excised by digestion with NotI and XbaI, and ligated into pRS315. Likewise, pRS315-*SGC1* was made using primers SGC1 F′ (−1000) and SGC1 R′ (+1426) (Table 1), which amplified the *SGC1* ORF, 1,000 bp of upstream sequence, and 550 bp of downstream sequence. The 2,426-bp PCR product was cloned into pGEM-T, sequenced, excised by digestion with BamHI and HindIII, and inserted into pRS315. Plasmids pRS315-*INO2* and pRS315-*INO4* were constructed previously (K. R. Gardenour and J. M. Lopes, unpublished data). Briefly, pRS315-*INO2* was constructed by inserting a 2.4-kb SalI/ClaI fragment (containing 500 bp of promoter, the *INO2* ORF, and 400 bp of 3′-untranslated region [3′UTR]) from YCp50-*INO2* (17) into pRS200 (pRS200-*INO2*) and subsequently cloning a SalI/PstI fragment from pRS200-*INO2* into pRS315. Likewise, pRS315-*INO4* was constructed by inserting a 2.4-kb SacII/SalI fragment (containing 500 bp of promoter, the *INO4* ORF, and 400 bp of 3′UTR) from YCp50-*INO4*-496 (64) into pRS315.

Plasmids were made to contain dominant-negative mutants of *INO2* and *INO4*. The expressed mutants were capable of dimerization with other bHLH proteins but inhibited their binding to DNA because they either contained mutations in the DNA-binding basic charged domain (*ino2-R13L* and *ino4-R13L*) or completely lacked the basic charged domain (*ino4-BRD*). The *ino4-BRD* mutant was created by PCR. The region upstream of the basic charged domain (including 500 bp of the *INO4* promoter) was amplified using primers INO4F (containing a BamHI site) and INO′4R (Table 1) to yield a 650-bp product. The region downstream of the basic charged domain (including the *INO4* 3′UTR) was amplified using primers INO′4F and INO4R (containing a KpnI site) (Table 1) to yield an 800-bp product. The two PCR products were annealed (primers INO′4R and INO′ include a 30-bp overlap that deletes the basic charged region), extended, reamplified using primers INO4F and INO4R (to yield a 1,420-bp product), and cloned into pGEM-T. The insert was sequenced, excised by digestion with BamHI and KpnI, and inserted into pRS315 to yield pRS315-*INO4-BRD*. Plasmids pRS315-*INO2-R13L* and pRS315-*INO4-R13L* were constructed previously by site-directed mutagenesis (Gardenour and Lopes, unpublished data). Briefly, YCp50-*INO2* was mutagenized using a QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), using primers INO2 R13L 5′ SD and INO2 R13L 3′ SD (Table 1). The *INO2-R13L* mutant allele was sequentially cloned into pRS200 and pRS315 as described above. Plasmid pRS315-*INO4* was directly mutagenized using a QuikChange XL

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)
ENO1 F	AAGCTTTAGAAAGCATACTAT
ENO1 R	GAATTCGCCATTTTGATTTAG
CBF1 F′	GCGGCCGCTAATTCCCTCTTTTATGC
CBF1 R′	TCTAGATCAAGCCTCATGTGGATT
SGC1 F′	GGATCCTTCTATTATGCCAAAGC
SGC1 R′	AAGCTTAATAACGGGTTGTGAAT
INO4F	AAGCTTCACCTTCCAAGCTTT
INO′4R	AGCTCTTTCCAATTCTTGACCATCAGTCAA
INO′4F	TTGACTGATGGTCAAGAATTGGAAAGAGCT
INO4R	AGGCCTCCGGAGGAAAAAAAG
INO2 R13L 5′ SD	CACGTTCAAATGGAGAAGATACGATTAATAAACACCAAAGAAGCC
INO2 R13L 3′ SD	GGCTTCTTTGGTGTTTATTAATCGTATCTTCTCCATTTGAACGTG
INO4 R13L 5′ SD	GTTTCGTCTGAAAAAAAAAGGCTCGAGTTGGAAAGAGCTA
INO4 R13L 3′ SD	TAGCTCTTTCCAACTCGAGCCTTTTTTTTTCAGACGAAAC
CBF1A	GGGAGATCTCCCTCTTTTATGCTTTAGTATCGTCATATTC
CBF1-HA′	GCTTATTATTATTTGCCAGAGATCTCATCG
CBF1-HA 5′	ATGAGATCTCTGGCAAATAATAATAAGC
CBF1B	GCCCCAAAGTAGAAATAGGC
E-460 F	CCATCAGGATAGCACCCAAACTGCAGCATATTTGGACGACC
E-460 R	GGTCGTCCAAATATGCTGCAGTTTGGGTGCTATCCTGATGG
E-656 F	CGTCTATAAATGCCGGCCCGGGCGATCATCGTGGCGGGG
E-656 R	CCCCGCCACGATGATCGCCCGGGCCGGCATTTATAGACG
E-704 F	CGGTCATTGATGCATGCCATGGCCGTGAAGCGGGACAACC
E-704 R	GGTTGTCCCGCTTCACGGCGATGGCATGCATCAATGACCG
TCM1 chip F	GTAGGCAAAGGCAAACAAGA
TCM1 chip R	ATACGAGCGGCACTAACAGA
INO1 chip F	ATGCGGCATGTGAAAAGTAT
INO1 chip R	GAACCCGACAACAGAACAAG
ENO1-460 chip F	TCTACTGATCCGAGCTTCCA
ENO1-460 chip R	GAGAGGCGAAAGTGGTTTTT
ENO1-656 chip F	GGGACAACCAGAAAAGTCGT
ENO1-656 chip R	TGCGACAATTTGTGATATGC
ENO1-704 chip F	CAACCTGAATTCGGTCATTG
ENO1-704 chip R	AGACGACTTTTCTGGTTGTCC

site-directed mutagenesis kit and primers INO4 R13L 5′ SD and INO4 R13L 3′ SD (Table 1).

Hemagglutinin (HA)-tagged derivatives of *CBF1*, *SGC1*, *INO2*, and *INO4* to be used for ChIP assays were either generated or purchased. A YCp50-*CBF1-HA* construct was created by mutational PCR. PCR was used to replace the second and third codons of *CBF1* with a BglII site. To do this, a PCR using primers CBF1A and CBF1-HA 3′ (Table 1) yielded a 500-bp product containing the *CBF1* promoter and the new BglII site. A second PCR with the primers CBF1B and CBF1-HA 5′ (Table 1) yielded a 1,520-bp product containing the *CBF1* ORF and the new BglII site. The PCR products were digested with BglII and ligated to create a product which contained the *CBF1* promoter and ORF with the BglII site. The ligated fragment was used for another round of PCR with the primers CBF1A and CBF1B, resulting in a 2,056-bp product. This PCR fragment was cloned into pGEM-T. A 120-bp BglII fragment containing three tandem copies of the HA epitope was isolated from pSM492 (8) and inserted into the pGEM-T derivative partially digested with BglII. A SalI-BamHI fragment was isolated from the pGEM-T derivative and cloned into YCp50. The YCp50-*CBF1-HA* construct was confirmed by DNA sequencing. The YCp50-*INO2-HA* and YCp50-*INO4-HA* plasmids have been described previously (17, 64). A strain containing an HA-tagged *SGC1* gene was purchased from Open Biosystems (Huntsville, AL).

Three E boxes in the *ENO1* promoter (positioned at −460, −656, and −704) were mutagenized using a QuikChange XL site-directed mutagenesis kit and the pGEM-T-*ENO1* promoter derivative described above. The E primer set (Table 1) was used to create three single E box mutants. The single mutants were used to create the three possible combinations of double mutants and the triple mutant. The mutant *ENO1* promoters were cloned into YEp357R as described above.

Reporter enzyme assays. To assay β-galactosidase (β-Gal) activity, yeast strains were grown in 5 ml of appropriate medium to mid-log phase (60 to 80 Klett units) and pelleted by centrifugation at 5,000 × *g* for 10 min. The cell pellet was suspended in 200 μl of β-Gal assay buffer (20% glycerol, 0.1 M Tris-HCl [pH 8.0], 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride [PMSF]), transferred to a sterile 1.5-ml microcentrifuge tube, and stored at −80°C overnight. Cells were thawed on ice, and 100 μl of glass beads (0.45-mm diameter) was added. Cells were lysed by use of a vortex mixer six times for 15 s each. Cellular debris was removed by centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was transferred to another 1.5-ml microcentrifuge tube. To assay β-Gal activity, reaction mixtures were set up with 20 μl of cell extract and 80 μl of β-Gal assay buffer and incubated for 5 min at 28°C. The reaction was initiated by the addition of 40 μl of ONPG (*o*-nitrophenyl-β-D-galactopyranoside; 4 mg/ml). The absorbance of the reaction was measured by determining the optical density at 420 nm at 12-second intervals for a total of 30 min. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad, Rockville Center, NY). Both the β-Gal activity reactions and the protein concentration reactions were quantified using Soft_{max}Pro software and a Versa_{max} tunable microplate reader (Molecular Devices, Sunnyvale, CA). Units of β-Gal activity are given as A_{420}/min/mg total protein × 1,000.

ChIP assay. Yeast cell cultures (200 ml) were grown in I−C− medium at 30°C to mid-log phase (60 to 80 Klett units). Formaldehyde was added to a 1% final concentration, followed by a 30-min incubation at 30°C. Glycine was added to 125 mM, and the mixture was incubated for an additional 5 min. Cells were pelleted at 1,500 × *g* for 5 min, and pellets were washed twice with 700 ml of 1× phosphate-buffered saline (0.43 mM Na_2HPO_4, 0.14 mM KH_2PO_4, 13.7 mM NaCl, and 0.27 mM KCl) and once with 15 ml of bead-beater lysis buffer (50 mM HEPES-KOH, pH 7.5, 10 mM $MgCl_2$, 150 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM dithiothreitol, 1 mM sodium metabisulfite, 0.2 mM PMSF, 1 mM benzamidine, and 1 μg/ml pepstatin). The cell pellet was weighed and resuspended in 2.5× bead-beater lysis buffer. One milliliter of the resuspended cells was added to 1 ml of 0.45-mm glass beads. Cells were lysed in a mini-Beadbeater 8 with four 1-minute pulses at the highest setting (with cells being placed on ice for 2 min between pulses). The extract was recovered by pouring the bead-extract slurry into a 6-ml syringe fitted with a 25-gauge, 5/8-inch-long needle. The syringe was washed with 0.75 ml of bead-beater lysis buffer. The extract was sonicated three times for 30 seconds each, using a model 100 Sonic Dismembrator with a Branson 250 microtip sonicator (Fisher Scientific, Pittsburgh, PA) at 50% duty cycle with a power setting of 5, with cells being

placed on ice for 2 min between pulses. The extract was cleared of debris twice in a microcentrifuge at full speed at 4°C for 5 min. Samples were fractionated in an agarose gel to ensure that DNA was sheared to a size range from 500 to 2,000 bp. The protein concentration was determined using the Bio-Rad Protein Assay, and 750 ng of extract was diluted with IP buffer (25 mM HEPES-KOH, pH 7.5, 150 mM KCl, 1 mM EDTA, 12.5 mM $MgCl_2$, 0.1% NP-40, 1 mM sodium metabisulfite, 0.2 mM PMSF, 1 mM benzamidine, and 1 μg/ml pepstatin) to a final reaction volume of 500 μl. Mouse anti-HA (clone 12CA5; Boehringer Mannheim) was added (to 2.5 ng/μl) and incubated overnight at 4°C. The antibody-protein-DNA complexes were recovered with protein G beads (equilibrated in IP buffer) by incubation at 4°C for 1 to 2 h. The beads were washed four times for 15 min each with 1 ml of IP buffer at 4°C, 100 μl of IP elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate) was added, and the samples were incubated at 65°C for 30 min. Samples were fractionated by centrifugation, and 80 μl of the supernatant was recovered. The elution step was repeated with 50 μl of IP elution buffer, and 50 μl of the supernatant was recovered and combined with the first eluate. Seventy microliters of each eluate was incubated overnight at 65°C to reverse cross-linking. Seventy microliters of Tris-EDTA (TE), pH 7.4, 1 μl of 20-mg/ml glycogen, and proteinase K (final concentration, 100 μg/ml) were added and incubated at 37°C for 2 hours. Samples were extracted with phenol-chloroform, and the organic phase was reextracted with 100 μl of TE. Sodium acetate was added to 0.3 M, and 2 volumes of 100% ethanol was added. DNAs were precipitated at −20°C for 1 h, collected by centrifugation, washed with 70% ethanol, and dried. DNAs were resuspended in 25 μl of TE with 100 μg/ml RNase A and incubated at 37°C for 30 min.

Immunoprecipitated DNA and input DNA were analyzed by real-time quantitative PCR using an Mx3000P QPCR thermocycler and MxProQPCR software (Stratagene, La Jolla, CA). Specific primers (Table 1) flanking 60 to 90 base pairs of each *ENO1* E box, the *INO1* promoter, and the *TCM1* promoter were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer and template DNA concentrations were optimized, and amplification reactions with SYBR green were carried out for 1 cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 55°C for 1 min. The data were calibrated using the *TCM1* ChIP signal and normalized to the input DNA.

RESULTS

***ENO1-lacZ* is regulated by multiple bHLH proteins.** It was known that *ENO1* expression is regulated by the Sgc1p bHLH protein and Gcr1p (49, 58, 68). Here we tested if other bHLH proteins also regulate *ENO1* expression by using an *ENO1-lacZ* reporter. The *ENO1-lacZ* reporter was assayed in a wild-type and nine isogenic bHLH knockout strains. Since inositol, choline, and phosphate concentrations affect the functions of different bHLH proteins (Ino2p:Ino4p and Pho4p, respectively), *ENO1-lacZ* expression was tested under different growth conditions. We used the following four growth conditions: I−C− low-P_i medium, I−C− high-P_i medium, I+C+ low-P_i medium, and I+C+ high-P_i medium. In the case of the *ino2*Δ and *ino4*Δ strains, the I−C− medium contained 10 μM inositol, which is required for growth of these mutant strains but still yields derepressed expression of their target genes (1, 17, 24).

As expected, the *SGC1* gene was required for maximal expression of *ENO1-lacZ* under all four growth conditions (Fig. 2A) (58, 68). In addition, the data showed that *ENO1-lacZ* expression in the wild-type strain was repressed in the presence of inositol-choline regardless of the phosphate concentration (Fig. 2A). *ENO1-lacZ* expression was reduced 51% and 21% by inositol-choline in low- and high-phosphate media, respectively (Fig. 2A). While these levels of repression are relatively modest, they are within the range observed for several well-characterized inositol-choline-responsive genes (14, 55, 69, 71). However, inositol-choline repression was not observed in

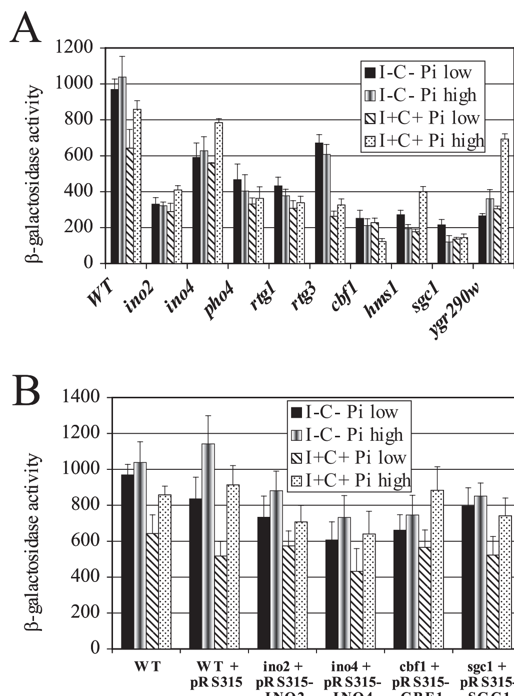

FIG. 2. *ENO1-lacZ* expression is regulated by all bHLH proteins of *S. cerevisiae*. (A) An *ENO1* promoter-*lacZ* reporter gene was transformed into a wild-type strain (WT) and nine bHLH knockout strains. Transformants were grown in the following four different media: I−C−, medium lacking inositol and choline (filled bars); I+C+, medium containing inositol and choline (bars with single horizontal stripe); Pi low, low-phosphate medium (hatched bars); and Pi high, high-phosphate medium (dotted bars). In the case of the *ino4*Δ and *ino2*Δ strains, the I−C− medium contained 10 μM inositol to allow for growth of these inositol auxotrophs. Cells were harvested in mid-log phase, and β-galactosidase activity was quantified. The data represents the means ± standard errors of the means for at least three experiments. (B) Complementation of *ENO1-lacZ* expression in the wild-type, *ino2*Δ, *ino4*Δ, *cbf1*Δ, and *sgc1*Δ strains. As a control, the *ENO1-lacZ* plasmid and empty pRS315 plasmid were cotransformed into the wild-type strain. The *ENO1-lacZ* plasmid and a pRS315-bHLH plasmid were cotransformed into the relevant bHLH knockout strains.

most other bHLH knockout strains (Fig. 2A). While this would be expected for the *ino2*Δ and *ino4*Δ strains, it was unexpected for the other bHLH knockout strains (Fig. 2A).

In addition, this experiment revealed that every yeast bHLH protein was required for maximal *ENO1-lacZ* expression, although the *ino2*Δ, *pho4*Δ, *rtg1*Δ, *cbf1*Δ, and *hms1*Δ strains showed the most dramatic effects, which resembled the effect of an *sgc1*Δ mutant. In this study, we focus on the Ino2p, Ino4p, Cbf1p, and Sgc1p bHLH proteins because the first three are associated with the response to inositol-choline (33, 67; Y. He, A. Shetty, and J. M. Lopes, unpublished results) and Sgc1p is well established as being required for maximal expression of *ENO1* (58, 68).

A complementation test was performed to confirm that the decreased *ENO1-lacZ* expression (Fig. 2A) was due to the bHLH knockout alleles. The mutant strains were transformed with pRS315-based plasmids carrying the *INO2*, *INO4*, *CBF1*, or *SGC1* gene under the control of its own promoter. The plasmids carrying the *INO2*, *CBF1*, and *SGC1* genes partially restored *ENO1-lacZ* expression to the wild-type levels of ex-

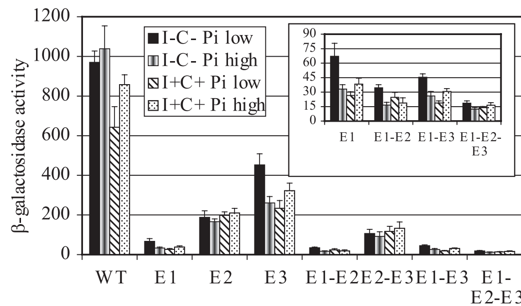

FIG. 3. Three E boxes (CANNTG) in the *ENO1* promoter are required for expression. Each of three *ENO1* promoter E boxes was mutated singly and in every possible combination. The wild-type (WT) and mutant promoters were assayed in a wild-type strain grown under the four conditions described in the legend to Fig. 2. The data represent the means ± standard errors of the means for at least three experiments. (Inset) Mutants that yielded low levels of activity, using a different scale.

pression (Fig. 2B). It is not unusual that these genes did not yield completely restored expression. However, the *INO4* plasmid did not appear to complement the defect in the *ino4*Δ strain, even though it did complement the inositol auxotrophy and defective *INO1-lacZ* expression in an *ino4*Δ strain (He et al., unpublished data). The reasons for this are not clear.

Three E boxes are required for *ENO1-lacZ* expression. The bHLH proteins regulate transcription by forming protein dimers, most of which recognize DNA sequences called E boxes (CANNTG) (53, 63). There are five potential E boxes in the *ENO1* promoter region present in the *ENO1-lacZ* construct used in this study. It was previously reported that sequences required for *ENO1* expression localize to the −724-to-−353 region, thereby eliminating two ORF-proximal E boxes (79). Therefore, we focused on the three ORF-distal E boxes (E-460 [E1], E-656 [E2], and E-704 [E3]; distances are relative to the start codon). We mutated each E box by site-directed mutagenesis and assayed the strains for β-galactosidase activity (Fig. 3). All three E boxes were found to play a role in *ENO1-lacZ* expression. Mutating the E1 box had the biggest impact, reducing expression 21.2-fold (average for the four growth conditions), while mutating the E2 and E3 boxes reduced expression 4.6- and 2.8-fold, respectively (Fig. 3). Deleting the E2 element, alone or in combination with E1 and E3, completely eliminated repression in response to inositol and choline (Fig. 3). However, deletion of E1 and/or E3 eliminated repression in response to inositol and choline only in high-P_i medium (Fig. 3).

To determine if there is any synergy between the E boxes, every double mutant and triple mutant combination was created, and the effect of each pairwise combination of elements was determined. The triple mutant was almost completely devoid of UAS activity, suggesting that the three E boxes constitute all of the required positive regulatory elements (Fig. 3). To define the contribution of each E box to activation, double E box mutants were compared to the triple E box mutant. For example, to calculate the E1 contribution in I−C− low-P_i medium, the activity in the E2+E3 mutant was divided by the activity in the E1+E2+E3 triple mutant (5.7-fold activation) (Table 2). To calculate the combined activation from the E1 and E2 elements, the activity in the E3 mutant was divided by the activity in the E1+E2+E3 triple mutant (24.3-fold activation) (Table 2). The ratio of the observed activation from two elements to the product of the activation levels from the individual elements represents the synergy factor. A synergy factor of 1.0 indicates additive activation (i.e., no synergy) (41). The data showed that most E box relationships yielded synergy factors in the range of 0.8 to 1.8 under all growth conditions, showing the absence of synergy between the three E boxes.

Ino2p, Ino4p, Cbf1p, and Sgc1p regulate *ENO1-lacZ* expression through three E boxes. To define the bHLH protein–E-box interactions, we used two approaches, namely, an epistatic analysis and ChIP. The dual approach was expected to provide corroborating evidence, and the epistatic analysis could additionally provide information about indirect regulation. For the epistatic analysis, the three E box mutant promoters were transformed into a wild-type strain and the nine bHLH knockout strains. Cells were grown in I−C− low-P_i and I+C+ low-P_i media.

The results suggest that Sgc1p functions exclusively through the E1 element, since deleting the *SGC1* gene had no effect on expression from the E1 mutant promoter but affected expression from the E2 and E3 mutants (Fig. 4). Likewise, Cbf1p functions through the E2 element (Fig. 4). The data suggest that Ino2p functions through the E3 element because deleting the *INO2* gene had virtually no effect on expression from the E3 mutant promoter. The data also suggest that Ino4p functions through the E1 and E3 boxes (Fig. 4). While deleting the *INO4* gene affected expression from all three E box mutants,

TABLE 2. Activation of *ENO1-lacZ* expression

ENO1-lacZ mutant promoter	Fold activation (synergy factor)[a]											
	I−C−, low P_i			I−C−, high P_i			I+C+, low P_i			I+C+, high P_i		
	Interaction between E1 and E2	Interaction between E1 and E3	Interaction between E2 and E3	Interaction between E1 and E2	Interaction between E1 and E3	Interaction between E2 and E3	Interaction between E1 and E2	Interaction between E1 and E3	Interaction between E2 and E3	Interaction between E1 and E2	Interaction between E1 and E3	Interaction between E2 and E3
E1			3.6 (0.8)			2.7 (0.9)			1.9 (0.8)			2.3 (1.1)
E2		10.1 (1.0)			13.6 (1.3)			14.3 (0.9)			12.8 (1.4)	
E3	24.3 (1.8)			21.4 (1.4)			16.9 (1.6)			19.7 (1.3)		
E1+E2		1.8	1.8		1.4	1.4		1.8	1.8		1.1	1.1
E2+E3	5.7[b]	5.7		7.5	7.5		8.4	8.4		8.1	8.1	
E1+E3	2.4		2.4	2.1		2.1	1.3		1.3	1.9		1.9

[a] The amount of activation and synergy factor were calculated as described in the text.

[b] The amount of activation was determined relative to the activities in the triple mutant, which were as follows: for I−C−, low-P_i medium, 18.6 units; for I−C−, high-P_i medium, 12.2 units; for I+C+, low-P_i medium, 13.9 units; and for I+C+, high-P_i medium, 12.4 units.

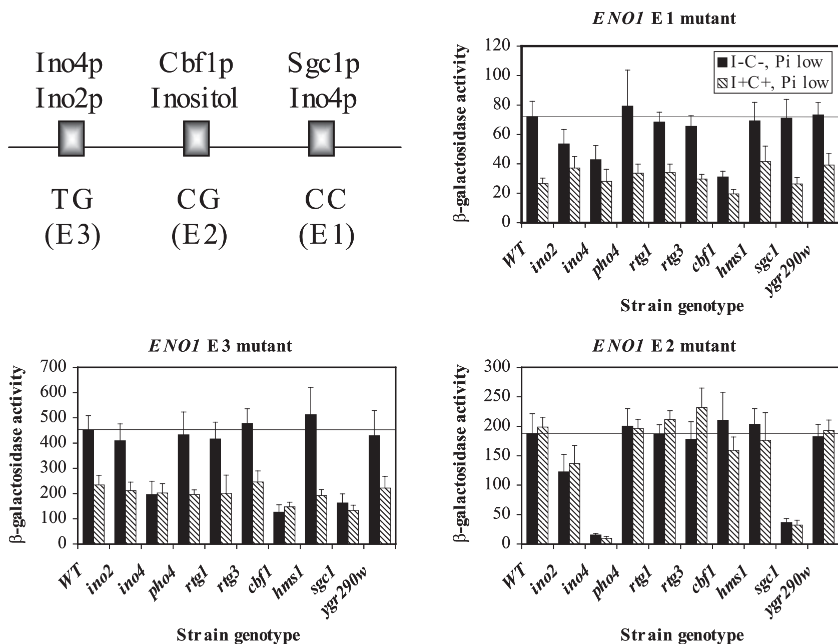

FIG. 4. Epistatic analysis of E box mutations in the *ENO1* promoter and bHLH knockout alleles reveals *cis*-element *trans*-factor relationships. Individual *ENO1* E box mutant promoters were assayed in wild-type (WT) and bHLH mutant strains grown in I−C−, low-P_i medium (filled bars) and I+C+, low-P_i medium (hatched bars). The data represent the means ± standard errors of the means for at least three experiments. Lines denoting the level of expression in the wild-type strain were drawn to facilitate comparisons between strains. A summary of the bHLH protein–E-box relationships (as well as the inositol–E-box relationship) is diagrammed along with the identities of the variable nucleotides in each E box (CANNTG).

the most dramatic effect was with the E2 mutant. Thus, while Ino4p may function through all three E boxes, it is more likely that it functions through the E1 and E3 elements. It is curious that the inositol-choline response is mediated through the E2 element (Fig. 3 and 4), which does not appear to bind the Ino2p-Ino4p dimer but instead interacts with Cbf1p. As for the other five bHLH proteins, it was not possible to assign them to specific E boxes since the bHLH knockout alleles did not affect expression from any of the E box mutants. The simplest explanation is that the other five bHLH proteins may function cooperatively through multiple sites.

ChIP was used to define the direct regulators of *ENO1* expression. The *TCM1* promoter was used as a negative control, and the *INO1* promoter served as a positive control for the binding of Ino2p and Ino4p. As expected, none of the bHLH proteins interacted with the *TCM1* promoter, whereas Ino2p and Ino4p interacted with the *INO1* promoter (Fig. 5). The data show that all four bHLH proteins bind the *ENO1* promoter directly in vivo via different E boxes. Moreover, the ChIP results are in complete agreement with the results of the epistatic study (compare Fig. 4 and 5). Ino2p and Ino4p interacted with the E3 box, but Ino4p also interacted with the E1 element (Fig. 5). This is interesting because Ino4p does not homodimerize and usually requires Ino2p for binding to DNA (72). However, Ino4p has been shown to regulate some genes in the absence of Ino2p (67). Furthermore, Sgc1p also interacted with the E1 box, and while Sgc1p binds a consensus E box as a homodimer (68), it may bind the *ENO1* E1 element as a heterodimer with Ino4p. Cbf1p was the only bHLH protein tested that interacted with the E2 box. This is curious since the

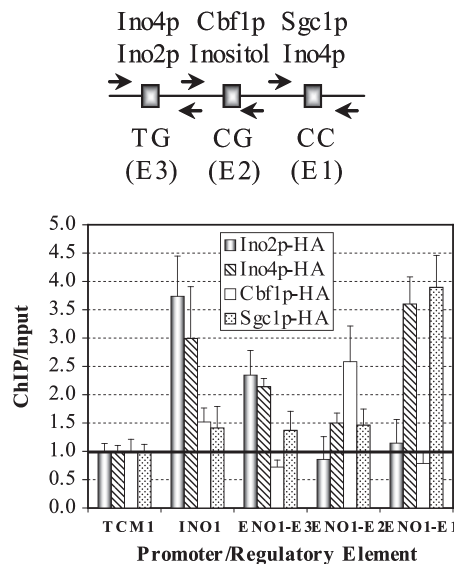

FIG. 5. ChIP analysis of bHLH protein binding to the *ENO1* promoter. ChIP and input DNAs were quantified by quantitative PCR and calibrated using the signal from *TCM1* ChIP. The bHLH protein ChIP levels were normalized to the input chromatin and are shown relative to that of the *TCM1* control (set at 1.0). The *TCM1* promoter served as a negative control, and the *INO1* promoter was a positive control for Ino2p and Ino4p binding. The data represent the means ± standard errors of the means for at least three experiments. A summary of the bHLH–E-box relationships (as well as the inositol–E-box relationship) is diagrammed along with the identities of the variable nucleotides in each E box (CANNTG). The relative locations of primers used in the PCR are also depicted (arrows).

response to inositol-choline was found to be mediated by the E2 box and inositol-choline regulation was typically believed to be effected by Ino2p-Ino4p (13, 23, 27, 63). However, a recent microarray study identified a set of genes that are repressed by inositol and do not bind the Ino2p-Ino4p dimer but are instead part of the unfolded-protein response regulon (33).

***INO4* and *INO2* dominant-negative mutants affect expression of *ENO1-lacZ*.** The ability to form multiple dimer combinations is a general property of bHLH proteins (29, 30, 57, 66). The results described above showed that Ino2p, Ino4p, Cbf1p, and Sgc1p regulate the expression of *ENO1-lacZ* and suggested that novel dimers (such as Ino4p-Sgc1p) may contribute to this regulation. We have also previously shown that Ino4p can dimerize with every bHLH protein in a yeast two-hybrid assay (62). To determine if partner selection plays a role in *ENO1-lacZ* expression in vivo, we used two *ino4* alleles and one *ino2* allele containing mutations in the basic region. In two of these mutants, *ino4-R13L* and *ino2-R13L* (Gardenour and Lopes, unpublished data), the amino acid in the 13th position of the basic region was changed from an R to an L. This position of the basic region is highly conserved throughout bHLH proteins and contacts a G nucleotide in the fourth position of the E box (CANNTG) (63). However, MyoD contains an L amino acid at the 13th position of the basic region and contacts a C nucleotide in the 4th position of the E box (9). Therefore, while the *ino4-R13L* and *ino2-R13L* mutants function as dominant-negative mutants with respect to *INO1* expression, they are able to bind to E boxes that contain a C nucleotide at the fourth position (Gardenour and Lopes, unpublished data). In the other mutant, *ino4-BRD*, we deleted the 13-amino-acid basic region.

The *ino4* and *ino2* mutants (in pRS315) were transformed into strains harboring the *ENO1-lacZ* reporter, grown under the four conditions described above, and assayed for β-galactosidase activity. The data clearly show that the presence of the R13L mutant bHLH proteins affects the expression of *ENO1-lacZ* in several strains (compare Fig. 6 and 2A). The *ino4-R13L* and *ino4-BRD* mutants reduced expression in the wild-type strain, mostly under I−C− conditions, and significantly reduced *ENO1-lacZ* expression in the *ino2*Δ and *cbf1*Δ strains (compare Fig. 6A and 2A; data not shown). This suggests that in the *ino2*Δ and *cbf1*Δ strains, expression is dependent on a bHLH protein(s) (likely Sgc1p) that is inhibited from binding by the Ino4p-R13L (and Ino4p-BRD) mutant. However, in the *ino4*Δ and *sgc1*Δ strains, expression was either unchanged or increased slightly (compare Fig. 6A and 2A; data not shown). Likewise, the presence of the *ino2-R13L* mutant allele either had no effect on expression (*cbf1*Δ and *sgc1*Δ strains) or significantly increased expression (wild-type, *ino2*Δ, and *ino4*Δ strains) (compare Fig. 6B and 2A). Because of the number of bHLH proteins that affect *ENO1-lacZ* expression (Fig. 2A), there are numerous possibilities that could account for the increase in expression in the presence of the *ino4-R13L* and *ino2R-13L* mutants. For example, the R13L mutation could inhibit binding of a repressor protein or could recruit a different bHLH protein to the E1 site, which has a C nucleotide at the fourth position. Regardless of the explanation for the increased expression, the data support the conclusion that bHLH dimer formation affects *ENO1-lacZ* gene expression in vivo.

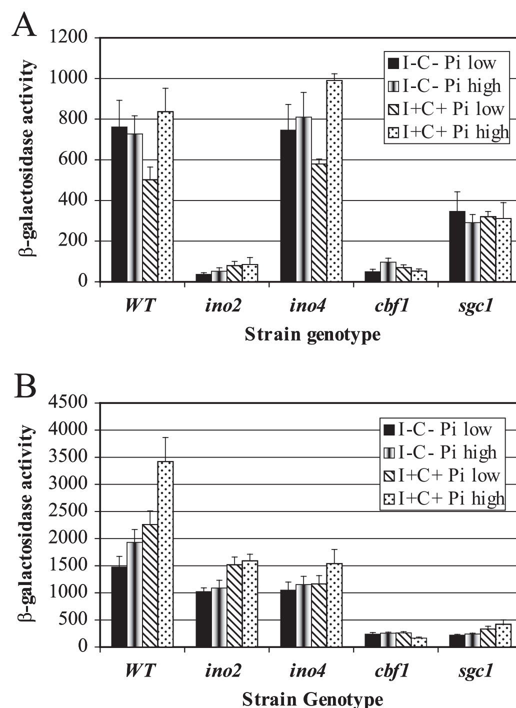

FIG. 6. Expression of *ENO1-lacZ* is altered in *ino4-R13L* (A) and *ino2-R13L* (B) dominant-negative mutants. The *ENO1-lacZ* reporter was assayed in wild-type (WT), *ino2*Δ, *ino4*Δ, *cbf1*Δ, and *sgc1*Δ strains grown under the four conditions described in the legend to Fig. 2. *ENO1-lacZ* transformants contained pRS315 plasmids bearing either the *ino4-R13L* or *ino2-R13L* mutant allele. The data represent the means ± standard errors of the means for at least three experiments.

DISCUSSION

The bHLH proteins have been studied extensively in higher eukaryotic cells. The bHLH family is a large and versatile family of transcription regulators (7, 30, 40, 43, 46, 77, 82). Most attention has been focused on their ability to form multiple dimer combinations and, to a lesser extent, on their limited DNA-binding specificity (4, 9, 10, 19, 21, 51, 57, 70, 75, 76, 80). Consistent with this, we have previously reported that Ino4p forms multiple dimers with other bHLH proteins via the yeast two-hybrid assay and biochemical copurification (62). This suggests that different bHLH proteins might also be involved in the coordination of different biological pathways through Ino4p. However, it has become evident that autoregulation and cross-regulation of bHLH-encoding genes, interorganellar transport, and inhibition of binding to promoters are also major contributors to how these proteins regulate gene expression (1, 59, 74). Naturally, in higher eukaryotes there are additional layers of complexity dictated by tissue-specific and development-specific distributions of bHLH proteins. Yeast has been a particularly fruitful system for studying this family of proteins with respect to how each protein or dimer functions in regulating a specific biological process (63) (Fig. 1). There is a relatively small number of bHLH proteins in yeast compared to those in *Drosophila*, *Caenorhabditis elegans*, and mammals (3, 5, 26, 35, 44, 45, 53, 65, 78). Therefore, yeast is ideally suited for the study of how the various

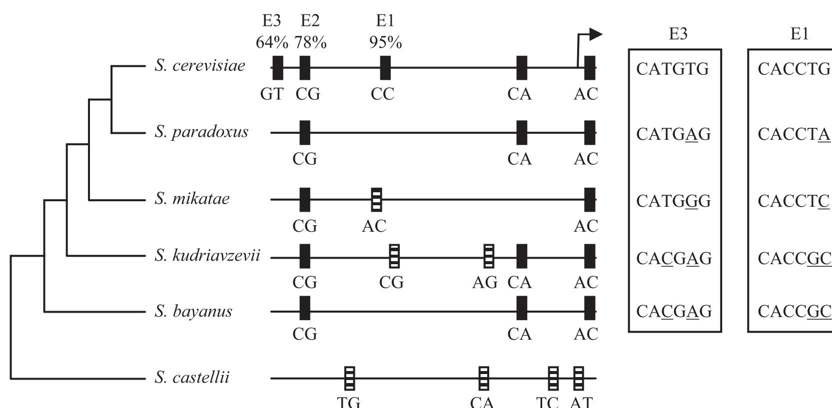

FIG. 7. Evolution of the E1, E2, and E3 E boxes in the *ENO1* promoter. The relevant sequences of the *ENO1* promoters from six *Saccharomyces* species were compared. The *Saccharomyces* phylogenetic tree was produced with data from published reports (15, 16, 20, 28). The relative position of the transcription start site is depicted as an arrow. *S. cerevisiae* E boxes that are found in other *Saccharomyces* species are shown as filled boxes, while putative E boxes not found in *S. cerevisiae* are shown as horizontally striped boxes. The identities of the variant nucleotides in each E box element are noted below each E box. The sequences of the E1 and E3 boxes are shown next to the relevant species. The numbers by each E box at the top of the figure represent the percent decrease in *ENO1-lacZ* expression when each E box is deleted.

mechanisms described above contribute to the coordination of different biological processes on the genomic scale.

The results presented here are striking because they show for the first time that multiple bHLH proteins, which are known to regulate different biological processes, also regulate a single gene in yeast. In the case of *ENO1*, all nine bHLH proteins were required to activate its expression (Fig. 2). Inositol-choline also repressed *ENO1-lacZ* expression, and therefore *ENO1* expression is coordinated with phospholipid biosynthesis. The phospholipid biosynthetic genes are induced in the absence of inositol-choline via the Ino2p-Ino4p dimer. *ENO1* did not emerge in genome-wide expression studies that identified inositol-choline- and Ino2p-Ino4p-regulated genes (33, 67). Furthermore, ChIP-chip analyses also did not identify Ino2p-Ino4p binding upstream of the *ENO1* ORF (25, 52, 61). This is due in part to the stringent cutoffs used in the genome-wide studies but also may be due to the growth conditions we employed.

As described above, there are several possible mechanisms whereby bHLH proteins regulate *ENO1* expression. They might regulate it by directly binding to the *ENO1* promoter as homodimers or heterodimers. In this case, multiple dimers might bind multiple sites or compete for binding to the same site in the *ENO1* promoter. The *ENO1* promoter contains five potential E boxes, three of which were investigated here because published promoter deletion studies suggest that the two ORF-proximal elements are not required. The three distal E boxes were mutated, and the triple mutant virtually eliminated expression (<2% of wild-type promoter activity), supporting the conclusion that these elements are required for *ENO1* expression (Fig. 3). The epistatic analysis showed that Ino2p-Ino4p binds to the most distal element (E3), Cbf1p binds to the E2 element, and Sgc1p and Ino4p bind the E1 element to regulate *ENO1-lacZ* expression (Fig. 4). In support of these results, the ChIP experiments showed that these bHLH protein–E-box genetic interactions correlate with direct binding by the bHLH proteins (Fig. 5). Curiously, repression by inositol-choline appeared to occur through the E2 element which bound Cbf1p (Fig. 3 to 5). This was surprising since this response is most frequently associated with Ino2p-Ino4p, which bound the E3 element. However, inositol-choline also affected expression through the E3 and E1 elements in high-P_i medium. Nevertheless, the E2 response could in fact be due to Cbf1p since we recently found that Cbf1p also regulates another inositol-choline-regulated gene (He et al., unpublished data).

An important question to address is whether these elements and the cognate bHLH factors play an important role in *ENO1* expression or are minor contributors. To address this issue, we compared the *ENO1* promoter sequences for several species of *Saccharomyces* (Fig. 7). It is obvious that the E2 element evolved fairly early, as it appears in *Saccharomyces bayanus*. Thus, it appears that regulation in response to inositol-choline is an early event and must be important for several members of the *Saccharomyces* genus (Fig. 7). The response to inositol-choline is modest, which likely explains why it was not identified in the genome-wide expression studies (33, 67). However, the repression level of *ENO1* is certainly comparable to that of several well-characterized inositol-choline-regulated yeast genes involved in fatty acid synthesis (*FAS1*, *FAS2*, and *ACC1*) as well as the Kennedy pathway for phospholipid synthesis (*CPT1*) (14, 55, 69, 71). The E3 and E1 elements, however, appeared late and are restricted to *S. cerevisiae*, suggesting that they play a specialized role in this species (Fig. 7). Collectively, these observations suggest that these elements may have evolved for different reasons in the *Saccharomyces* genus. Another important consideration from these studies is that yeast promoter databases (e.g., see http://fraenkel.mit.edu/yeast_map_2006/) that list binding sites for transcription factors typically cross-list the ChIP-chip studies and conservation of DNA sequence elements. However, these three *ENO1* promoter elements do not satisfy the minimum cutoffs imposed in databases (15, 16, 20, 25, 52).

It was already known that expression of *ENO1* is regulated by Sgc1p (58, 68). Here we found that Sgc1p interacted with the E1 element, either as a homodimer or as a heterodimer with Ino4p (Fig. 4 and 5). Previous studies using electrophoretic mobility shift assays and DNase I footprinting experiments showed that recombinant Sgc1p binds one of the two

ORF-proximal E boxes (not analyzed in this study) (68). The difference in these studies can be explained if binding to the E1 box occurs as an Sgc1p-Ino4p heterodimer, which was not tested in the published studies (68). Alternatively, the electrophoretic mobility shift assay experiments did reveal additional bands at high Sgc1p concentrations that could reflect binding to the E1 element. Regardless of the explanation, the results we present here are corroborated by two distinct approaches, i.e., epistatic analysis and ChIP.

We and others have previously reported that *INO2* expression is regulated by Ino2p and Ino4p (1, 2, 54, 73). We have found that *SGC1* is autoregulated and cross-regulated by Cbf1p and Ygr290wp (M. Chen and J. M. Lopes, unpublished data), suggesting that these bHLH proteins may regulate *ENO1* by regulating the *SGC1* gene. It will be interesting to determine if regulation of *SGC1* expression affects global gene expression patterns. To do this, it will be necessary to define and mutate the elements in the *SGC1* promoter that are required for regulation by Sgc1p, Cbf1p, and Ygr290wp. Examination of the *SGC1* promoter reveals four potential E boxes, and three of these are conserved among at least four of the *Saccharomyces* species. The *ygr290w* mutant yielded increased expression of the *SGC1-cat* gene, which is consistent with the observation that the YGR290w ORF is predicted to encode an HLH protein that lacks the basic region. Therefore, if this gene is in fact expressed, it could behave like the Id family, which inhibits dimerization with bHLH proteins and inhibits binding to DNA (59). However, YGR290w is listed as a dubious ORF based on available experimental and sequence comparisons (http://db.yeastgenome.org/cgi-bin/locus.pl?locus=YGR290w). This dubious ORF partially overlaps the *MAL11* gene, which encodes a high-affinity maltose transporter. Thus, there is a possibility that the phenotype we observe with *SGC1-cat* is due to deletion of the *MAL11* gene. Nevertheless, the *SGC1-cat* phenotype will make it possible to distinguish between these two possibilities.

The *Saccharomyces cerevisiae* bHLH protein interaction map showed that Ino4p is a hub for binding of other bHLH proteins (62). Consistent with this observation, we showed that the *ino4-R13L* (Fig. 6A) and *ino4-BRD* (data not shown) mutants completely alter expression from the *ENO1* promoter. This was especially evident in the *ino2*Δ and *cbf1*Δ mutant strains, where the expression of *ENO1* was almost completely eliminated. Similarly, the *ino2-R13L* mutant also affected *ENO1-lacZ* expression (Fig. 6B). Thus, we can conclude that dimerization selection does play a role in the expression of *ENO1-lacZ*. Consequently, our analysis of the *ENO1* promoter has identified that multiple bHLH proteins are required for expression through distinct mechanisms, including direct binding to different E boxes, formation of multiple dimers, and regulation by a putative HLH protein (Ygr290wp).

Why *ENO1* is regulated by all of these bHLH proteins is, of course, the most important question to be asked. We favor the model that *ENO1* is a particularly striking example of the various mechanisms whereby bHLH proteins regulate gene expression in yeast. However, it may be that some bHLH-mediated regulation is simply a reflection of noise in regulation. This may very well explain the effects of some but not all of the bHLH proteins. For example, it seems unlikely that the *ENO1* promoter would have evolved the E1 and E3 boxes in *S. cerevisiae* if noise were the only explanation. Another important consideration is whether or not *ENO1* is unusual in its response to bHLH proteins. We are currently analyzing four other well-studied promoters (*INO1*, *CIT2*, *MET16*, and *PHO5*) targeted by bHLH proteins. We find that all four promoters are regulated by several bHLH proteins, but none to the extent of *ENO1* (M. Chen, Y. He, A. Shetty, and J. M. Lopes, unpublished data). This suggests that bHLH proteins themselves are not a source of unusually high noise in gene regulation, but we cannot preclude that the *ENO1* promoter is not noisy in and of itself. To a great extent, answering this question will depend on studies that determine the effects of regulation of *ENO1* on yeast metabolism and fitness.

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