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.

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

suggests that novel bHLH combinations may interact with these UAS elements.

MATERIALS AND METHODS

Strains, media, and growth conditions. Plasmid-containing *E. 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)/pLYS 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 (MATa his3-D1 leu2-D0 lys2-D0 ura3-D0), BY4741 (MATa his3-D1 leu2-D0 met15-D0 ura3-D0), and isogenic strains containing *ino2Δ, ino4Δ, pho8Δ, cbf1Δ, sgc1Δ, rgl1Δ, rgl2A, hmsΔ*, and *ygr290wΔ* alleles (22, 81). Yeast cultures were grown at 30°C in a complete synthetic medium lacking inositol, choline, KH2PO4, and uracil (for reporter plasmid selection) (38). Where indicated, 75 μM inositol (I−) and/or 1 mM choline (C+) was added. Low-P medium contained 0.22 mM KH2PO4 and 20 mM KCl, and high-P medium contained 11 mM KH2PO4.

Plasmid construction. Plasmid YEp357-ENO1 contains 720 bp of the sequence upstream of the ENO1 ORF and the first coding exon in frame to the lacZ reporter gene in YEplac332 (56). This 720-bp region was previously shown to contain all of the regulatory elements necessary for ENO1 expression (79). YEplac332 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 ORFproximal 720-bp sequence was excised by digestion with EcoRI and inserted into YEplac332.

Plasmids that complemented the *cbf1Δ, sgc1Δ, ino2Δ*, and *ino4Δ* mutants were constructed by cloning each ORF and promoter into pRS315. Plasmids that complemented the *INO4*, *INO2*, and *INO1* ORFs were constructed previously by site-directed mutagenesis (Gardenour and Lopes, unpublished data). Briefly, YCp50-INO4 was mutagenized using a QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), using primers INO4 F and INO4 R (Table 1) to yield a 1,420-bp product, and cloned into pGEM-T. The insert was sequenced, excised by digestion with BamHI and HindIII, and inserted into pRS315-M into pGEM-T (Promega, Madison, WI) and sequenced, and then the ORFproximal 720-bp sequence was excised by digestion with EcoRI and inserted into YEplac332.

Plasmids for the *INO4*, *INO2*, and *INO1* ORFs 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 RBS, and 400 bp of a 3′-untranslated region [3′UTR]) from YCp50-INO2 (17) into pRS200 (pRS200-INO2) and subsequently cloning a SacI/XbaI fragment from pRS504-INO2 into pRS315. Likewise, pRS315-INO1 was constructed using a 2.4-kb SacI/Sall fragment (containing 500 bp of promoter, the INO1 RBS, and 400 bp of 3′UTR) from YCp50-INO4-496 (64) into pRS315.

Plasmids were constructed 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-Brad*). The ino4Brad 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 INO4′R (Table 1) to yield an 800-bp product. The two PCR products were annealed (primers INO4′R and INO4 include a 30-base overlap that deletes the basic charged region), extended, reamplified using primers INO4F and INO4M (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-INO4-4Brad. 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.

mitochondrial damage (p0), 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 *S. cerevisiae* HLH family but lacks a basic charged DNA-binding region. Lastly, Sgc1p (Tyr7p) 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...
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 were used for ChIP assays were either generated or purchased. A pGEM-T-ENO1 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 CBF1 F and CBF1-HA 5′ (Table 1) yielded a 500-bp product containing the CBF1 promoter and ORF with the BglII site. The PCR fragment was cloned into pGEM-T. A 120-bp BglII fragment containing three tandem copies of the HA epitope was isolated from pSM902 (6) 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 pGEM5. The YCp50-INO4-INO2-HA construct was confirmed by DNA sequencing. The YCp50-INO2 R13L 3

Reporter enzyme assays. To assay β-galactosidase (β-Gal) activity, yeast 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. Glycerol 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 (0.13 mM Na2HPO4, 0.14 mM KH2PO4, 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 MgCl2, 150 mM KCl, 0.1 mM EDTA, 10% glycerol, 1.0% NP-40, 1.0 mM diethiothreitol, 0.1 mM sodium metabisulfite, 0.2 mM PMSF, 1 mM benzamidine, and 1 μM pepstatin). The cell pellet was weighed and resuspended in 25× β-bead-bacter lysis buffer. One milliliter of the resuspended cells was added to 1 ml of 0.45-mm glass beads. Cells were lysed by pouring the bead-extract slurry into a 3-ml syringe filled with a 25-gauge, 58-inch-long needle. The syringe was washed with 0.75 ml of β-bead-bacter lysis buffer. 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-b-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, MD). Both the β-Gal activity reactions and the protein concentration reactions were quantified using Softmax Pro software and a Versa max tunable microplate reader (Molecular Devices, Sunnyvale, CA). Units of β-Gal activity are given as A420/min/mg total protein × 1,000.

Table 1. Oligonucleotides used in this study

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<thead>
<tr>
<th>Oligonucleotide</th>
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<tr>
<td>ENO1 F</td>
<td>AAGCTTGGAAAGCATATAAT</td>
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<tr>
<td>ENO1 R</td>
<td>GAAATTGCCATTTTGATTTAG</td>
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<tr>
<td>CBF1 F</td>
<td>GCGCCGCTAATTCCTTTTTATGC</td>
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<tr>
<td>CBF1 R</td>
<td>TCTGATAAGTCTGCATGGAATTCGAT</td>
</tr>
<tr>
<td>SGC1 F</td>
<td>CCGTCAAGTACCTGAAGCTT</td>
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<tr>
<td>SGC1 R</td>
<td>AAGCTTTAAACGCGGTGTTGAAT</td>
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<tr>
<td>INO4 F</td>
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<td>INO4 R</td>
<td>AGGCTTCACCTCAGCTGTTTCCTT</td>
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<td>INO2 R13L 3′ SD</td>
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<tr>
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<tr>
<td>CBF1-HA 5′</td>
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<td>CBF1-B</td>
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<tr>
<td>ENO1-704 chip R</td>
<td>AGACCAAAAGGCGGTTTCAG</td>
</tr>
</tbody>
</table>

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, MD). Both the β-Gal activity reactions and the protein concentration reactions were quantified using Softmax Pro software and a Versa max tunable microplate reader (Molecular Devices, Sunnyvale, CA). Units of β-Gal activity are given as A420/min/mg total protein × 1,000.
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−, 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 ino4Δ and ino2Δ 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, ino2Δ, ino4Δ, cbf1Δ, and sgc1Δ 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 (WT). The ino4Δ mutant showed the most dramatic effects, which resembled the effect of an sgc1Δ mutant. In this study, we focus on the ino2p, ino4p, Cbf1p, and Sgc1p bHLH proteins because the first three are most well-established as being required for maximal expression of ENO1 (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 most other bHLH knockout strains (Fig. 2A). While this would be expected for the ino2Δ and ino4Δ 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 ino2Δ, pho4Δ, rgl1Δ, cbf1Δ, and lms1Δ strains showed the most dramatic effects, which resembled the effect of an sgc1Δ 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-
The amount of activation was determined relative to the activities in the triple mutant, which were as follows: for I−C−, low-Pi medium, 13.9 units; and for I−C−, high-Pi I−C−/H11001, high Pi I−C−/H11001, C−/H11002, low Pi I−C−/H11002, C−/H11001, high Pi I−C−/H11001, C−/H11001, low-Pi medium, 12.2 units; for I−C+, low-Pi medium, 13.9 units; and for I−C+, high-Pi medium, 12.4 units.

### Table 2. Activation of ENO1-lacZ expression

<table>
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<tr>
<th>ENO1-lacZ mutant promoter</th>
<th>I−C−, low Pi</th>
<th>I−C−, high Pi</th>
<th>I−C+, low Pi</th>
<th>I−C+, high Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction between E1 and E2</td>
<td>2.7 (0.9)</td>
<td>16.9 (1.6)</td>
<td>8.4</td>
<td>13.8 (1.4)</td>
</tr>
<tr>
<td>Interaction between E1 and E3</td>
<td>13.6 (1.3)</td>
<td>19.7 (1.3)</td>
<td>8.1</td>
<td>19.2 (1.4)</td>
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<tr>
<td>Interaction between E2 and E3</td>
<td>2.3 (1.1)</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

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

![ChIP analysis of bHLH protein binding to the ENO1 promoter](http://ec.asm.org/)

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).
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 1-C- conditions, and significantly reduced ENO1-lacZ expression in the ino2Δ and cbf1Δ strains (compare Fig. 6A and 2A; data not shown). This suggests that in the ino2Δ and cbf1Δ 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 ino4Δ and sce1Δ 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 (cbf1Δ and sce1Δ strains) or significantly increased expression (wild-type, ino2Δ, and ino4Δ 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 ino2-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.

**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
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 did not identify inositol-choline-regulated genes (33, 67). Furthermore, ChIP-chip analyses also did not identify inositol-choline-regulated genes (33, 67). 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-Pi 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://frawkel.mit.edu/yeast_map2006/) 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 E101 promoter. This was especially evident in the ino2Δ and cbf1Δ mutant strains, where the expression of E101 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 E101 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 (Ygr290w).

Why E101 is regulated by all of these bHLH proteins is, of course, the most important question to be asked. We favor the model that E101 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 E101 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 E101 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 E101 (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 E101 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 E101 on yeast metabolism and fitness.

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