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# Yeast transcriptional activator *INO2* interacts as an Ino2p/Ino4p basic helix–loop–helix heteromeric complex with the inositol/choline-responsive element necessary for expression of phospholipid biosynthetic genes in *Saccharomyces cerevisiae*

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## ABSTRACT

**Coordinate transcriptional control of yeast genes involved in phospholipid biosynthesis is mediated by the inositol/choline-responsive element (ICRE) contained in the respective promoter regions. Regulatory genes *INO2* and *INO4*, both encoding basic helix–loop–helix (bHLH) proteins, are necessary for ICRE-dependent gene activation. By the use of size variants and by heterologous expression in *E.coli* we demonstrate that Ino2p and Ino4p are both necessary and sufficient for the formation of the previously described *FAS* binding factor 1, Fbf1, interacting with the ICRE. Formation of a heteromeric complex between Ino2p and Ino4p by means of the respective bHLH domains was demonstrated *in vivo* by the interaction of appropriate two-hybrid constructs and *in vitro* by Far-Western analyses. Neither Ino2p nor Ino4p binds to the ICRE as a homodimer. When fused to the DNA-binding domain of Gal4p, Ino2p but not Ino4p was able to activate a $UAS_{GAL}$-containing reporter gene even in the absence of the heterologous Fbf1 subunit. By deletion studies, two separate transcriptional activation domains were identified in the N-terminal part of Ino2p. Thus, the bHLH domains of Ino2p and Ino4p constitute the dimerization/DNA-binding module of Fbf1 mediating its interaction with the ICRE, while transcriptional activation is effected exclusively by Ino2p.**

## INTRODUCTION

The genetic control of membrane biogenesis is exerted by the coordinate expression of a large number of phospholipid biosynthetic genes (reviewed in 1). We have previously identified and characterized an upstream activation site ($UAS_{FAS}$) common to the unlinked fatty acid synthase genes *FAS1* and *FAS2* which turned out to be of general importance for the activation of phospholipid anabolism (2). Synthetic minimal promoters controlled by $UAS_{FAS}$ mediated a differential expression of a reporter gene dependent on the amount of phospholipid precursors inositol + choline. Thus, due to its regulatory phenotype, $UAS_{FAS}$ may be more adequately characterized as an inositol/choline-responsive element (ICRE; 2). This ICRE (consensus sequence TYTTCACATGY) contains the core sequence CANNTG, which is also known as an E box and which serves as a recognition site for DNA-binding proteins of the basic helix–loop–helix (bHLH) family (3). Members of the bHLH family comprise determinants of cellular differentiation and proliferation in mammalian and invertebrate systems such as the myogenic transcription factors MyoD, MRF4, myogenin and Myf-5 (4) as well as factors not restricted to specialized tissues (E12, E47, daughterless, c-Myc and Mad; 5–7). Proteins of the bHLH group may form either homodimers or heterodimers or both, dependent on the individual structure of the respective interaction surface provided by the HLH domain (8).

Since the positively acting regulatory genes *INO2* and *INO4* required for the activation of the ICRE-controlled inositol-1-phosphate synthase gene *INO1* (9) encode proteins with similarity to the bHLH family (10,11), we became interested in the possible function of Ino2p and Ino4p for ICRE-dependent gene expression. As we reported earlier, Ino4p turned out to be necessary for the formation of the ICRE-associated protein complex Fbf1 (*FAS* binding factor 1) and, thus, for ICRE-controlled reporter gene activation (12).

In this work we demonstrate that both regulatory genes *INO2* and *INO4* are necessary and sufficient for the formation of Fbf1 and its binding to the ICRE. Heterodimer formation and DNA-binding specificity is dictated by the respective bHLH

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domains. Unlike DNA-binding, however, transcriptional activation of target promoters requires only a functional *INO2* gene.

## MATERIALS AND METHODS

### Yeast strains and media

Yeast wild-type strain JS91.15-23 (MATα *ura3 leu2 his3 trp1 can1 MAL3 SUC3*) and its isogenic derivatives SS92.3-1 (Δ*ino2::LEU2*), RSH3 (Δ*ino4::LEU2*; 12) or SS93.8-1 (Δ*ino2::LEU2* Δ*ino4::LEU2*) were used as recipients for ICRE-controlled reporter constructs. Selective synthetic media (SCD) supplemented for growth of inositol-requiring mutants have been described (12). Strain SIRP3.Δino2 is identical to SS92.3-1, but contains an integrated ICRE–*CYC1–lacZ* reporter plasmid (pJS375; see below) targeted to the *ura3* locus. $GAL4_{DBD}$ gene fusions were transformed into strain YJOZ (MATa *ura3 leu2 his3 trp1 ade1 gal4Δ gal80Δ GAL1-lacZ*; 13) or the respective Δ*ino2* or Δ*ino4* disruptants. Transformants were grown under conditions of glucose derepression ($SCD_{0.2}Lac_2$ medium: 0.2% glucose + 2% lactate) selecting for plasmid markers.

### Plasmid constructions and gene isolation

Reporter plasmids pJS200, pJS203, pJS234N, pJS246N, pJS263N, pJS264N, pJS325 and pFT3N containing various *lacZ* fusion constructs have been described (2,12). Plasmid pJS375 (ICRE–*CYC1–lacZ URA3*) is an integrative derivative of pJS264N. In order to obtain a Δ*ino2* null mutation, the *INO2* gene was amplified by the polymerase chain reaction (PCR) using primers designed according to its published sequence (11) and subsequently cloned as a 1.8 kb fragment in pUC19 to give pSS13. Almost the entire reading frame (codons 15–286) containing the transcriptional activation domain as well as the bHLH region of *INO2* was deleted by *Bgl*II/*Eco*RV cleavage, followed by insertion of *LEU2* giving plasmid pSS20. The Δ*ino2::LEU2* mutant strain obtained by integrative transformation of a wild-type with the disruption construct present in pSS20 (SS92.3-1) was then used for the isolation of a larger *INO2* clone by complementation of its inositol auxotrophy. Plasmid YEp24-INO2.1 (10.4 kb insert of chromosomal DNA in YEp24) isolated from the respective transformants was confirmed to contain the authentic *INO2* gene by DNA sequencing. Plasmid pSS77 (*INO2 INO4 URA3* 2 μm) allowing an over-expression in yeast of both *INO2* and *INO4* was constructed by insertion of a 2.7 kb *Pst*I–*Hin*dIII fragment containing *INO2* and a 1.2 kb *Kpn*I–*Sma*I fragment containing *INO4* into the episomal yeast/*E.coli* shuttle vector YEplac195 (14). For subsequent experiments, reading frame cassettes for *INO2* (0.93 kb *Bam*HI–*Hin*dIII fragment in pSS33) and *INO4* (0.47 *Eco*RI–*Bam*HI fragment in pKR21) were constructed by PCR. To obtain an *INO2* size variant, the respective cassette controlled by the *ADH1* promoter was fused in-frame to the *lacZ* gene in YEp357 (15), giving pSS36 (*ADH1–INO2–lacZ URA3* 2 μm). Similarly, a *lexA–INO4* fusion was constructed by insertion of the *INO4* reading frame into plasmid pSH2-1 (16), leading to pKR23 (*ADH1–lexA*$_{DBD}$*–INO4 HIS3* 2 μm). The same reading frame cassettes were used for the construction of *GST–INO2* and *GST–INO4* fusions in the plasmid pGEX-2TK (17) to give pSS56 and pJS391, respectively, allowing heterologous expression in *E.coli* after IPTG induction. The bHLH domain encoded by *INO2* (codons 213–304) was amplified by PCR and subsequently ligated into pDBD31 (18) as a 0.29 kb *Cla*I–*Hin*dIII fragment. The resulting plasmid pSS39 carries a *GAL1–INO2*$_{bHLH}$*–VP16* fusion in an *ARS1 CEN4 LEU2* vector. As a control, the *INO2* wild-type gene was cloned into the *ARS1 CEN4 LEU2* plasmid YCplac111 (14) giving pSS55. In order to assay for *in vivo* protein–protein interaction between Ino4p and the bHLH domain of Ino2p, the respective DNA fragments were ligated into plasmids pGBT9 (*ADH1–GAL4*$_{DBD}$ *TRP1* 2 μm) and pGAD424 (*ADH1–GAL4*$_{TAD}$ *LEU2* 2 μm; 19). The resulting plasmids were pSS47 (*ADH1-GAL4*$_{DBD}$*-INO4*), pSS49 (*ADH1–GAL4*$_{DBD}$*–INO2*$_{bHLH}$), pSS50 (*ADH1–GAL4*$_{TAD}$*–INO4*) and pSS54 (*ADH1–GAL4*$_{TAD}$*–INO2*$_{bHLH}$). For the construction of $GAL4_{DBD}$ gene fusions present in single-copy vectors, plasmids pY1, pY2 and pY3 were used (20). Full-length fusions of *INO2* and *INO4* to $GAL4_{DBD}$ (pRE2 and pKR25, respectively) were constructed using the reading frame cassettes described above. Plasmid pRE2 was subsequently utilized for the construction of N- or C-terminal deletions of the *INO2* reading frame by a combined exonuclease III/S1 nuclease treatment (21). In addition, natural restriction sites were used to obtain internal fragments of *INO2* fused in-frame to $GAL4_{DBD}$ of pY vectors. Plasmids resulting from exonuclease digestion or PCR mutagenesis were characterized by DNA sequencing. For plasmid mutagenesis, pJS385 containing the *INO4* gene as a 1.2 kb *Kpn*I–*Sma*I fragment subcloned into YCplac33 (14) was treated with hydroxylamine (22). Plasmids unable to complement a Δ*ino4* null mutant were recovered and analysed by DNA sequencing. To obtain suitable two-hybrid constructs reading frame cassettes of the mutant *ino4* alleles were amplified by PCR and subcloned into pGAD424.

### Gel retardation assays

Gel retardation experiments using protein extracts from yeast strains have been described (2). As an ICRE probe, the synthetic DNA fragment FBF56 ($UAS_{INO1}$[–240]; 2) was used. In order to confirm a similar expression of various $GAL4_{DBD}$*–INO2* fusion constructs, protein extracts prepared from the respective transformants of strain YJOZ were assayed for their binding to the $UAS_{GAL}$ probe GALAB (23). Protein extracts from *E.coli* strain RR1 transformed with either pSS56 (*GST–INO2*) or pJS391 (*GST–INO4*) were prepared as follows. IPTG-induced cells were harvested, resuspended in a 1/10 vol of denaturation buffer (6 M urea, 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 8 mM dithiothreitol) and broken by sonication until the cell suspension became translucent. Insoluble material was removed by centrifugation. The supernatant was dialyzed against renaturation buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) for a total time of 2 h with three changes of buffer. Subsequently, proteins were precipitated by addition of 3 vol of buffer A (200 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 7 mM β-mercaptoethanol, 10% glycerol), saturated with ammonium sulfate. Following centrifugation, the precipitate was resolved in a minimal volume of buffer B (20 mM HEPES, pH 7.8, 5 mM EDTA, 7 mM β-mercaptoethanol, 10% glycerol) and finally dialyzed against the same buffer for 2 h with two changes. Removal of the GST domain from the fusion proteins was accomplished by thrombin cleavage in buffer B (0.01 thrombin cleavage units/mg total *E.coli* protein for 1 h). Protein concentrations were determined by the protein dye assay (24). In order to reconstitute a functional GST–Ino2p/GST–Ino4p heterodimer,

equal amounts of the respective resuspended *E.coli* transformants were mixed prior to cell lysis.

### Far-Western analysis

For the detection of *in vitro* interaction of Ino2p and Ino4p, 50 µg total protein from *E.coli* transformants expressing either *GST–INO2* or *GST–INO4* were size-fractionated by SDS–polyacrylamide gel electrophoresis on a 15% resolving gel. Proteins were transferred to nitrocellulose and renatured by overnight incubation in Blotto G (50 mM Tris–HCl, pH 7.5, 1% non-fat milk powder, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol; 25, modified) at room temperature. Filters were then rinsed twice in Hyb 75 buffer (20 mM HEPES, 1% non-fat milk powder, 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% Triton X-100; 17) and subsequently hybridized in Hyb75 against $^{32}$P-labelled GST fusion proteins (100 000 c.p.m./ml) at 4°C with gentle shaking overnight. GST fusion proteins were labelled by *in vitro* phosphorylation of the protein kinase A recognition site encoded by the pGEX-2TK vector using [$\gamma$-$^{32}$P]ATP (17). To avoid protein labelling due to GST homodimer formation, a crude extract from pGEX-2TK transformants synthesizing solely the GST domain was also added in excess to the hybridization mixture. Filters were washed three times with Hyb 75 buffer and then subjected to autoradiography.

### Miscellaneous procedures

RNA isolation from yeast, Northern blot hybridization, yeast transformation and β-galactosidase assay followed established procedures and have been described previously (12). Polymerase chain reaction (PCR) amplifications were performed with the Perkin Elmer Cetus GeneAmp PCR reagent kit under standard conditions.

## RESULTS

### Influence of *INO2* on ICRE-dependent gene expression and on DNA-binding of Fbf1

The importance of *INO4* for the formation of Fbf1 was demonstrated previously by the construction of a Δ*ino4* null mutant (12). In order to determine a similar function of *INO2* for the activation of ICRE-containing promoters, we constructed a Δ*ino2* null mutant lacking essentially the entire *INO2* reading frame. Comparative expression studies using various reporter constructs containing synthetic minimal promoters revealed a complete loss of ICRE-dependent gene activation in the Δ*ino2* mutant, when compared to the isogenic wild-type strain (Table 1). A similar effect of the Δ*ino2* mutation was observed with the intact *INO1* promoter, while the expression of *FAS–lacZ* reporter constructs was only partially affected (cf. plasmids pJS200 and pJS203 in Table 1). This latter result agrees with the identification of additional and Fbf1-independent upstream activation sites in both *FAS* promoters (26). Thus, derepression defects caused by the Δ*ino2* null mutation are fully consistent with those previously observed in a corresponding Δ*ino4* mutant (12). Reporter gene expression in a *ino2 ino4* double mutant was identical with that measured in the respective single mutants (not shown), indicating the participation of *INO2* and *INO4* in the same regulatory pathway.

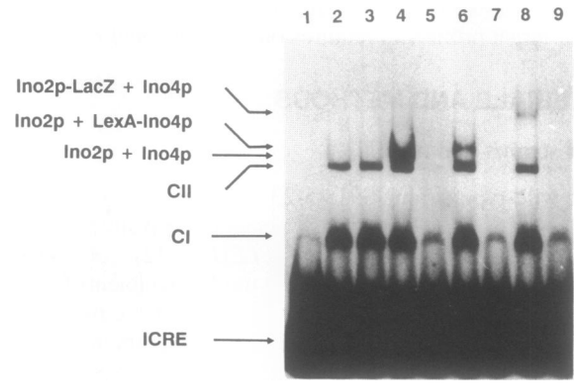


**Figure 1.** Alteration of the electrophoretic mobility of Fbf1 by the use of Ino2p and Ino4p size variants. As a probe, the ICRE-containing synthetic DNA fragment FBF56 was used. In lanes 2–9 40 µg total cellular protein prepared from various strains was added. Lane 1: no protein extract added; lane 2: Δ*ino2* mutant; lane 3: Δ*ino4* mutant; lanes 4, 5: wild-type; lanes 6, 7: Δ*ino4* mutant, transformed with a *lexA–INO4* fusion gene; lanes 8, 9: Δ*ino2* mutant, transformed with an *INO2–lacZ* fusion gene. In lanes 5, 7 and 9, protein binding to the labelled probe was competed for by a 200-fold molar excess of FBF56. Position of Fbf1 (Ino2p + Ino4p) and its size variants (Ino2p + LexA–Ino4p or Ino2p–LacZ + Ino4p) bound to the ICRE probe is indicated by arrows. Besides Fbf1, protein–DNA complexes CI and CII unrelated to Ino2p or Ino4p are also formed with the FBF56 probe.

**Table 1.** Influence of a Δ*ino2* null mutation on the activation of ICRE-containing promoters

| Plasmid[a] | Reporter construct | Specific β-galactosidase activity (nmol/min/mg) | |
|---|---|---|---|
| | | wild type | Δ*ino2* |
| pJS200 | *FAS1–lacZ* | 1300 | 690 |
| pJS203 | *FAS2–lacZ* | 950 | 500 |
| pJS325 | *INO1–lacZ* | 480 | 10 |
| pJS246N | $UAS_{FAS1}$(–860)–*CYC1–lacZ* | 550 | 30 |
| pFT3N | $UAS_{FAS2}$(–230)–*CYC1–lacZ* | 600 | 20 |
| pJS263N | $UAS_{INO1}$(–240)–*CYC1–lacZ* | 600 | 30 |
| pJS234N | $UAS_{RPG}$–*CYC1–lacZ* | 150 | 170 |

[a]Plasmids were transformed into strains JS91.15-23 (wild-type) and SS92.3-1 (Δ*ino2::LEU2*). Transformants were grown in SCD-Ura medium containing 5 µM inositol + 5 µM choline (derepressed conditions).

Gel retardation analyses employing an ICRE probe revealed that protein extracts of both the Δ*ino2* mutant (Fig. 1, lane 2) and an isogenic Δ*ino4* strain (lane 3) were devoid of the previously described Fbf1 complex (2). Thus, both *INO2* and *INO4* are necessary for the formation of the Fbf1–ICRE protein–DNA complex and, subsequently, for ICRE-dependent gene activation.

### Interaction of the Ino2p/Ino4p heterodimer with the ICRE

The absence of the Fbf1–ICRE complex in gel retardation experiments employing *ino2* or *ino4* null mutant extracts suggests that Ino2p and Ino4p are either controlled one by the other or both are indeed components of Fbf1. To confirm this latter idea, we constructed size variants of both *INO2* (as an *INO2–lacZ* fusion)

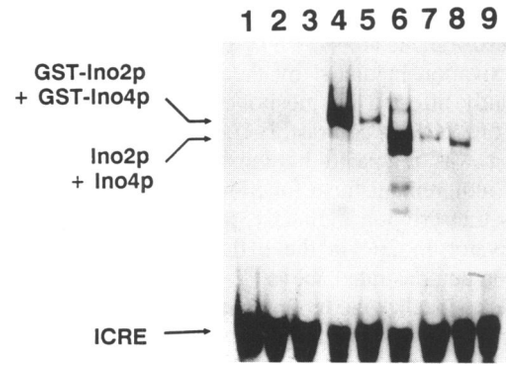


**Figure 2.** Reconstitution of an ICRE-binding factor by heterologous expression of *INO2* and *INO4*. The synthetic DNA fragment FBF56 was used as a probe in the gel retardation experiment. Lanes 2–7 contain 5 µg total protein prepared from *E.coli* transformants. Lane 1: no protein extract added; lane 2: GST–Ino2p; lane 3: GST–Ino4p; lanes 4, 5: GST–Ino2p + GST–Ino4p; lanes 6, 7: GST–Ino2p + GST–Ino4p, treated with thrombin. In lanes 8 and 9, 40 µg total yeast protein prepared from an (*INO2* + *INO4*) multi-copy transformant was added. In lanes 5, 7 and 9, protein binding to the ICRE was competed for by a 100-fold molar excess of FBF56. The positions of Fbf1 and its GST-containing size variant are indicated by arrows. Besides full-length Ino2p + Ino4p, smaller derivatives retaining ICRE-binding activity are released upon thrombin digestion of GST–Ino2p + GST–Ino4p.

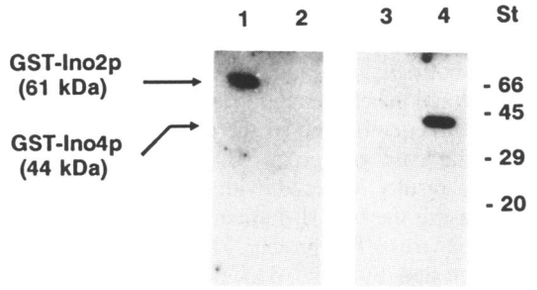


**Figure 3.** Far-Western analysis of Ino2p–Ino4p interaction. 50 µg *E.coli* protein prepared from *GST–INO2* (lanes 1, 3) or *GST–INO4* transformants (lanes 2, 4) was size-fractionated by SDS–PAGE, transferred to nitrocellulose filters and subsequently incubated with $^{32}$P-labelled GST–Ino2p (lanes 3, 4) or GST–Ino4p probes (lanes 1, 2). The calculated molecular weights of the respective fusion proteins are shown in parentheses. Note the increased apparent molecular weight of GST–Ino2p. The protein size standard is indicated.

and *INO4* (as a *lexA–INO4* fusion) and transformed these into the respective null mutants. Protein extracts from the resulting transformants were assayed in gel retardation experiments. As is shown in Figure 1, both fusion genes *lexA–INO4* (lane 6) and *INO2–lacZ* (lane 8) led to Fbf1 variants of reduced electrophoretic mobility, demonstrating the participation of both Ino2p and Ino4p in the formation of Fbf1. In order to prove Ino2p and Ino4p as the only components of Fbf1, *INO2* and *INO4* were expressed in *E.coli* as glutathione S-transferase (GST) fusion proteins. When extracts of these *E.coli* transformants were employed for gel retardation experiments, no protein factor interacting with the ICRE could be detected with either extract alone (Fig. 2, lanes 2 and 3). However, mixing of both extracts with subsequent denaturation and renaturation led to the formation of an ICRE-binding factor (Fig. 2, lane 4) which could be released from the DNA probe by specific competition (lane 5). The GST moiety of the fusion proteins could be cleaved off with thrombin. After this proteolytic treatment, the mixture of the two extracts contained an Ino2p/Ino4p heterodimer which gave rise to a protein–DNA complex exhibiting an electrophoretic retardation pattern essentially identical to that of the genuine Fbf1 from *Saccharomyces cerevisiae* (lanes 6 and 8 of Fig. 2). Thus, the two Fbf1 components obviously do not need any post-translational modification in order to bind to DNA. From these results we conclude that Ino2p and Ino4p are necessary and sufficient to constitute the ICRE-associated Fbf1 heterodimer.

### Ino2p–Ino4p interaction is mediated by the bHLH domains

The *in vitro* reconstitution of the ICRE-binding factor with DNA sequence requirements indistinguishable from those of Fbf1 suggested the possibility of direct protein–protein interactions between Ino2p and Ino4p. We thus used $^{32}$P-labelled GST–Ino2p and GST–Ino4p fusion proteins to directly demonstrate the DNA-independent interaction of Ino2p and Ino4p by Far-Western experiments. As is shown in Figure 3 (lane 1), a GST–Ino4p probe interacts exclusively with the GST–Ino2p fusion protein. Vice versa, a GST–Ino2p probe specifically recognizes filter-fixed GST–Ino4p (lane 4). With both probes, no signals indicative of homodimer formation were obtained (lanes 2 and 3). This result is in agreement with the reconstitution studies described above allowing ICRE-binding factor formation only in the presence of both Ino2p and Ino4p.

Since both Ino2p and Ino4p belong to the basic helix–loop–helix family of DNA-binding proteins, their heterodimerization and DNA-binding properties may be determined by the respective bHLH domains. In order to demonstrate Ino2p/Ino4p heterodimer formation in yeast, we constructed two-hybrid gene fusions (27) suitable for the study of protein–protein interactions *in vivo*. An artificial *INO2*$_{bHLH}$ domain (encoding amino acids 213–304 of Ino2p) was constructed using PCR and subsequently fused in-frame to the Gal4p DNA-binding (DBD) and transcriptional activation domains (TAD), respectively. Similar fusion genes were obtained with the complete *INO4* reading frame (151 amino acids). All pairwise combinations of these four plasmids comprising one DNA-binding and one *trans*-activating domain of Gal4p were co-transformed into a *gal4 gal80 ino2* recipient strain (YJOZ.Δino2) containing an integrated *GAL1–lacZ* reporter gene. As is shown in Table 2, only the heteromeric combinations of Ino2p and Ino4p fusion constructs led to a significant increase in basal reporter gene expression. Obviously, only in these cases was the Gal4p TAD integrated into a functional transcriptional activator complex. Again, no evidence for homodimeric interactions between either Ino2p or Ino4p was obtained. Interestingly, reporter gene activation by the reciprocal fusion combinations *GAL4*$_{DBD}$*–INO2*$_{bHLH}$/*GAL4*$_{TAD}$*–INO4* and *GAL4*$_{DBD}$*–INO4*/*GAL4*$_{TAD}$*–INO2*$_{bHLH}$ differs by one order of magnitude. This indicates that the efficiency of a transcriptional activation domain depends also on structural parameters of the protein as a whole. Two missense mutants of *INO4* mapping to the basic region (R47H) or to helix I (P73S) of the bHLH domain were also analysed for their two-hybrid interactions. As is also shown in Table 2, substitution of the proline residue terminating helix I (P73S) results in a complete abrogation of Ino2p–Ino4p interaction. In contrast, mutation of an arginine residue present in the basic domain of Ino4p (R47H mutant) does

not significantly interfere with binding to Ino2p, although DNA recognition is completely abolished (not shown). In summary, experiments both *in vitro* and *in vivo* confirm the exclusive heteromer formation between Ino2p and Ino4p. The specificity of this interaction is determined by the respective bHLH domains, at least in the case of Ino2p. This conclusion was further supported by results obtained with an artificial transcription factor comprising the bHLH domain of Ino2p and the TAD of herpes simplex virus VP16 protein. The *INO2*$_{bHLH}$–*VP16* fusion gene as controlled by the *GAL1* promoter (plasmid pSS39) perfectly complemented an *ino2* null mutation in a galactose-dependent manner and efficiently activated an ICRE-containing minimal promoter (Table 3). Obviously, the bHLH domain completely determines the DNA-binding characteristics of Ino2p. Gene activation by the *INO2*$_{bHLH}$–*VP16* construct is not significantly affected by inositol/choline repression, arguing against a regulatory modulation of the bHLH domain *per se*.

## Identification of two distinct transcriptional activation domains within Ino2p

Besides DNA-binding, transcriptional activation is an inherent functional characteristic of eukaryotic transcription factors. Since deletion of either *INO2* or *INO4* led to a loss of ICRE-dependent gene activation, it remained to be shown whether Ino2p or Ino4p or the combination of both were responsible for the gene activation potency of Fbf1. To study this question, *INO2* and *INO4* were separately fused in-frame to the DNA-binding domain of Gal4p. A *gal4 gal80* strain (YJOZ) containing an integrated *GAL1–lacZ* reporter gene as well as its isogenic *ino2* and *ino4* derivatives were transformed with the *GAL4*$_{DBD}$–*INO2* (pSS23) and *GAL4*$_{DBD}$–*INO4* (pKR25) fusion constructs, respectively. The specific β-galactosidase activities determined in these transformants are shown in Table 4. Obviously, transcriptional activation mediated by the Ino2p fusion protein is not significantly affected by inositol/choline repression or by the absence of *INO4*. In contrast, *GAL4*$_{DBD}$–*INO4*-dependent gene activation was regulated by inositol/choline and showed an absolute requirement for a functional *INO2* gene. Thus, Ino4p activates transcription indirectly, presumably by recruiting the true activator Ino2p via the bHLH-mediated protein–protein interaction demonstrated above.

In order to localize the Ino2p transcriptional activation domain more precisely, we constructed several C-terminal truncations of the *GAL4*$_{DBD}$–*INO2* fusion by exonuclease digestion. In addition, internal subdomains of Ino2p were fused in-frame to *GAL4*$_{DBD}$. As is shown in Figure 4, the C-terminus of Ino2p is completely dispensable for transcriptional activation. Moreover, removal of the C-terminal domain necessary for interaction with Ino4p increases the activation potency of Ino2p by a factor of about 2. Two separate activation domains, TAD1 (amino acids 1–33) and TAD2 (amino acids 32–106), each of them functioning independently of the other, may be defined by this analysis. The N-terminus of Ino2p representing TAD1 carries a negative charge of –7 within 33 amino acids, being reminiscent of the acidic activation domains described for Gal4p, Gcn4p and VP16 (28–30). However, this model has recently been challenged by a detailed analysis of mutants constructed within the Gal4p TAD (13). No obvious similarity to previously described patterns of activation domains could be observed with TAD2. A more precise definition of the Ino2p minimal domains required for transcriptional activation together with conformational considerations may lead to a better understanding of interaction surfaces between activators and the basal transcriptional system.

**Table 2.** Detection of *in vivo* interaction between the bHLH domain of Ino2p and Ino4p by two-hybrid

| Hybrid constructs[a] | Specific β-galactosidase activity (nmol/min/mg) | | |
|---|---|---|---|
| | *GAL4*$_{DBD}$ | *GAL4*$_{DBD}$–*INO2*$_{bHLH}$ | *GAL4*$_{DBD}$–*INO4* |
| *GAL4*$_{TAD}$ | 0.3 | 0.3 | 0.3 |
| *GAL4*$_{TAD}$–*INO2*$_{bHLH}$ | 0.3 | 0.3 | 5 |
| *GAL4*$_{TAD}$–*INO4* | 0.3 | 74 | 0.3 |
| *GAL4*$_{TAD}$–*INO4*(R47H) | 0.3 | 46 | n.t. |
| *GAL4*$_{TAD}$–*INO4*(P73S) | 0.3 | 0.9 | n.t. |

[a]The indicated combinations of hybrid constructs were co-transformed into the *gal4 gal80 ino2* strain YJOZ.Δino2 containing an integrated *GAL1–lacZ* reporter gene. Transformants were grown under selective conditions in $SCD_{0.2}Lac_2$-Leu-Trp medium containing 5 μM inositol + 5 μM choline. DBD, DNA binding domain; TAD, transcriptional activation domain; n.t., not tested.

**Table 3.** Activation of an ICRE-containing reporter gene by an artificial *GAL1–INO2*$_{bHLH}$–*VP16* construct

| Plasmid construct[a] | Specific β-galactosidase activity (nmol/min/mg) | | | |
|---|---|---|---|---|
| | 2% glucose | | 2% galactose | |
| | IC rep. | IC derep. | IC rep. | IC derep. |
| pSS55 (*INO2*) | 2 | 61 | 2 | 45 |
| pSS39 (*GAL1–INO2*$_{bHLH}$–*VP16*) | 1 | 2 | 78 | 101 |

[a]Plasmids were transformed into strain SIRP3.Δino2 containing an integrated ICRE–*CYC1–lacZ* reporter gene. Transformants were grown in SCD-Leu (2% glucose) or SCGal-Leu (2% galactose), as indicated. Inositol/choline repression was achieved with 200 μM inositol + 2 mM choline. Derepression conditions have been defined in the legend to Table 1.

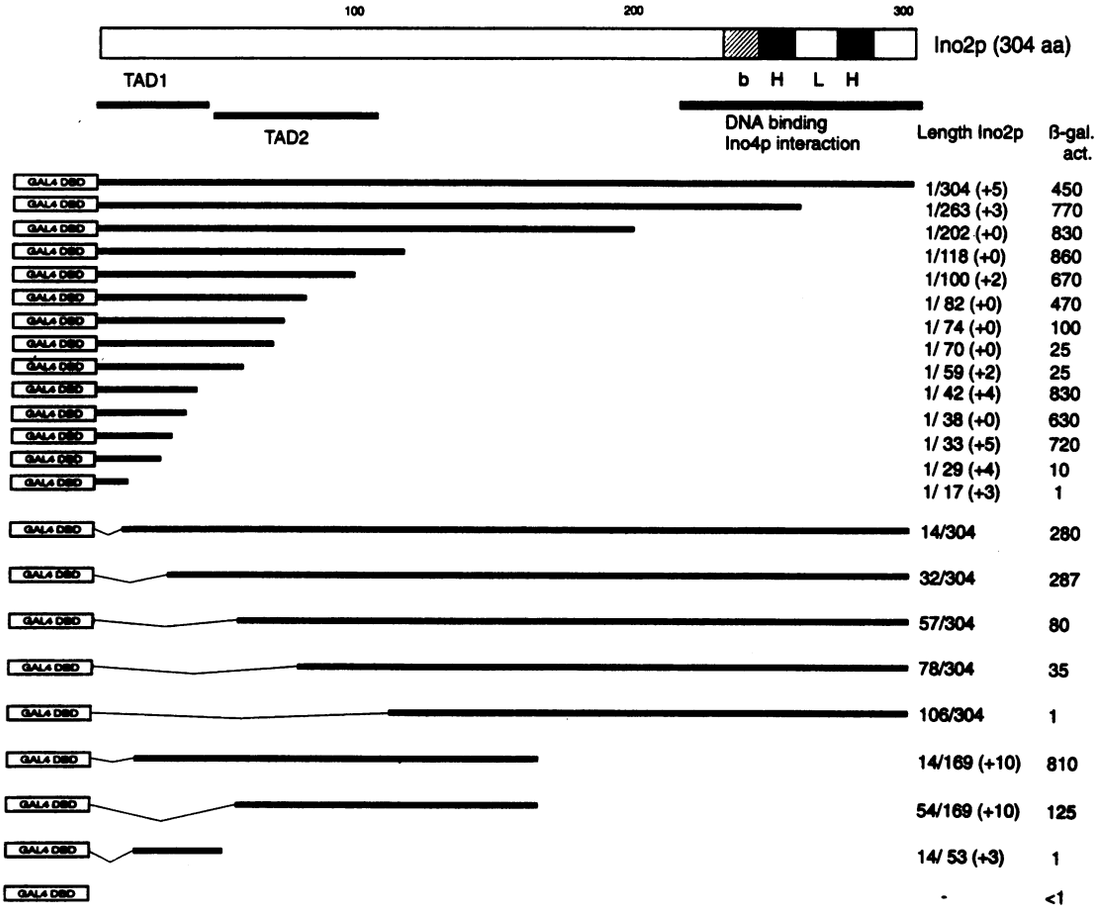


**Figure 4.** Identification of transcriptional activation domains within Ino2p. Various Ino2p fragments fused in-frame to the DNA-binding domain of Gal4p ($GAL4_{DBD}$) were transformed into the *gal4 gal80* strain YJOZ containing an integrated *GAL1–lacZ* reporter gene. Yeast transformants were grown in $SCD_{0.2}Lac_2$-Trp medium and subsequently assayed for specific β-galactosidase activities (right column). Parts of Ino2p present in the respective fusion proteins are indicated by bold lines and are shown also in precise positions (left column). The number of amino acids artificially added to the C-terminus of Ino2p by the cloning site is shown in brackets. The $INO2_{bHLH}$ domain sufficient for Ino4p interaction and DNA-binding as well as the positions of N-terminal transcriptional activation domains TAD1 and TAD2 are indicated by bars.

**Table 4.** Gene activation mediated by $GAL4_{DBD}$–*INO2* or $GAL4_{DBD}$–*INO4* fusion constructs

| plasmid[a] | Specific β-galactosidase activity (nmol/min/mg) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | YJOZ (wild-type) | | | YJOZ.Δino2 | | | YJOZ.Δino4 | | |
| | R | D | D/R | R | D | D/R | R | D | D/R |
| pY1 ($GAL4_{DBD}$) | <1 | <1 | 1 | <1 | <1 | 1 | <1 | <1 | 1 |
| pSS23 ($GAL4_{DBD}$–$INO2_{14/304}$) | 190 | 310 | 1.6 | 175 | 280 | 1.6 | 270 | 380 | 1.4 |
| pKR25 ($GAL4_{DBD}$–$INO4_{1/151}$) | 12 | 70 | 5.8 | <1 | <1 | 1 | 28 | 150 | 5.4 |

[a]Plasmids were transformed into the *gal4 gal80* strain YJOZ containing an integrated *GAL1–lacZ* reporter gene or into its isogenic derivatives YJOZ.Δino2 and YJOZ.Δino4. Transformants were grown in $SCD_{0.2}Lac_2$-Trp medium under conditions of inositol/choline repression (R) or derepression (D) as defined in the legend to Table 3.

## DISCUSSION

Members of the bHLH family of transcription factors play an outstanding role in the genetic control of differentiation and proliferation in eukaryotes (4–6). The ability of bHLH proteins to form homo- and/or heterodimers depending on the particular combination may account for their functional flexibility and wide occurence. For instance, the yeast bHLH protein Cpf1p/Cbf1p required for methionine prototrophy and binding to the centromere DNA consensus element CDEI was shown to homodimerize

efficiently (31). The Pho4p regulator of yeast phosphatase genes may act in a similar way (32). In contrast, the specificity of interaction between the Ino2p and Ino4p bHLH domains exclusively dictates heterodimer formation, as shown in this work *in vitro* by a Far-Western analysis and *in vivo* by two-hybrid fusion constructs. In a similar way, the *in vivo* interaction of mammalian bHLH proteins E12 and MyoD has been demonstrated (33). Although the data presented here do not allow discrimination between Ino2p/Ino4p heterodimer or higher order structures, the analogy to bHLH proteins of known molecular structures, such as Max (34), USF (35), E47 (36) and MyoD (37), strongly favours the heterodimeric arrangement. Interestingly, the bHLH–ZIP domain of USF may lead to a homotetramer dependent on the intact ZIP structure. A USF derivative with the ZIP sequence deleted retained the ability to form a homodimer due to its HLH stretches (35). The data presented in this paper show that the DNA-binding properties of Ino2p map to the bHLH domain which reconstitutes a functional transcription factor after fusion with the artificial VP16 activation domain. Our results confirm and extend the conclusions on the importance of the Ino2p bHLH domain derived from the analysis of *ino2* missense mutations mapping therein (38). Similarly, the importance of the Ino4p basic region is demonstrated by the R47H missense mutation described here, affecting a highly conserved arginine residue of all known bHLH proteins. Although the mutant Ino4p is unable to bind to the ICRE, its interaction with Ino2p is nearly unaffected. In contrast, the P73S mutation altering the proline residue at the start of the loop region also abolishes heterodimerization with Ino2p. This finding supports the importance of the loop region for the formation of a defined four-helix-bundle structure typical of bHLH interaction domains (34). Recently, the binding of *in vitro*-translated Ino2p + Ino4p to an *INO1* promoter fragment has been shown (39). In agreement with the results described here for an individual ICRE probe, no *INO1* promoter complex could be detected by these authors with either Ino2p or Ino4p alone. At present, there is no evidence for additional HLH interactions of either Ino2p or Ino4p with other protein factors, since only a single protein–DNA complex disappears in ICRE-dependent gel retardation experiments when extracts of wild-type and *ino2* or *ino4* mutant cells are compared. However, the combinatorial flexibility of bHLH oligomers may lead to the binding of one factor to a variety of similar, although distinct DNA sites. It remains to be shown whether either Ino2p or Ino4p may interact with additional partners possibly allowing recognition also of non-ICRE sites. Inhibitory HLH proteins such as Id1 and Id2 lack a basic region and may be responsible for sequestering bHLH transcription factors in an inactive state (40,41). The two-hybrid constructs used in this study provide a means for the isolation of possible interaction partners interfering with the binding of the Ino2p/Ino4p heterodimer complex to the ICRE.

Most eukaryotic transcription factors are multifunctional polypeptides containing distinct domains at least for DNA-binding and transcriptional activation that may be functionally separated from each other (42). Within a heteromeric activator protein, both properties may be unequally distributed, as it has been shown for the mammalian c-Myc/Max heterodimer (43). Interestingly, the molecular organization of the Ino2p/Ino4p complex closely resembles the c-Myc/Max system, with Ino2p or c-Myc being responsible for the transcriptional activation function. Moreover, the relative arrangement of DBD and TAD functions within both heteromeric systems is quite similar, with the exception of the additional leucine zipper present in c-Myc and Max. So far, three types of eukaryotic TADs have been recognized and classified according to their amino acid composition as acidic, glutamine-rich or proline-rich regions (44). The negative net charge of –7 within the 33 residues of TAD1 in Ino2p may argue for the importance of acidic amino acids for transcriptional activation mediated by Ino2p. Arrangement of residues 14–22 of Ino2p in a β-sheet structure would concentrate a negative charge on one side of the polypeptide chain (**DLDNDIDFE**, acidic amino acids shown in bold). Recently, transcriptional activation by at least two copies of the heptapeptide motif DDFDLDL derived from the VP16 TAD was demonstrated in mammalian cells (45). Although both sequences exhibit a similar pattern of acidic and hydrophobic residues, the significance of this comparison may remain controversial, since the $GAL4_{DBD}$–$INO2_{14/53}$ fusion construct carrying a negative charge of –8 is inactive in transcriptional activation. Nevertheless, several recent investigations argue for the importance of an orderly pattern of bulky hydrophobic amino acids interspersed with ionic residues within a TAD (13,46–48). In contrast to TAD1, the position of TAD2 within Ino2p is less well defined. A closer inspection of TAD2 did not reveal any sequence similarity to the known TAD motifs mentioned above. Successive removal of N-terminal residues led to a stepwise reduction in reporter gene expression, possibly indicative of redundant subdomains present in TAD2. Interestingly, C-terminal deletions within TAD2 in the presence of an intact TAD1 led to a drop in reporter gene expression which increased again following removal of additional amino acids (cf. deletion constructs 1/70 and 1/42 in Figure 4). A similar result was obtained previously in the course of the identification of Gal4p TADs (28). Although an inhibitory domain separating TAD1 and TAD2 cannot be ruled out, the local disorder of a destroyed protein subdomain may also affect neighbouring structures.

Transcription factors mediating differential gene expression exhibit regulatory responses to external stimuli which may act at several levels of the gene activation process. Since transcriptional activation of ICRE-containing promoters by the Ino2p/Ino4p heterodimer is regulated by the inositol/choline concentration in the medium, both the bHLH domains as well as the Ino2p TADs may be considered as possible targets of a signal transduction pathway. Although the bHLH domain of Ino2p *per se* is not affected by phospholipid precursors in the $GAL1$–$INO2_{bHLH}$–$VP16$ fusion construct, the presence of a negatively acting domain adjacent to the bHLH part of Ino2p cannot be ruled out. Similarly, both TAD1 and TAD2 of Ino2p are not significantly affected by inositol/choline repression when separately fused to a constitutively expressed DNA-binding domain (not shown). Instead of the functional modules present in Ino2p and Ino4p, the expression of the transcription factors themselves may also be considered as a target of inositol/choline repression. An important contribution to this biosynthetic control of *INO2* and *INO4* is indeed provided by ICRE sequences upstream of both genes, possibly leading to an autoregulatory response after a primary derepression signal has been generated (12, unpublished).

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