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Influence of gene dosage and autoregulation of the regulatory genes *INO2* and *INO4* on inositol/choline-repressible gene transcription in the yeast *Saccharomyces cerevisiae*

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Abstract Expression of structural genes of phospholipid biosynthesis in yeast is mediated by the inositol/choline-responsive element (ICRE). ICRE-dependent gene activation, requiring the regulatory genes *INO2* and *INO4*, is repressed in the presence of the phospholipid precursors inositol and choline. *INO2* and, to a less extent, *INO4* are positively autoregulated by functional ICRE sequences in the respective upstream regions. However, an *INO2* allele devoid of its ICRE functionally complemented an *ino2* mutation and completely restored inositol/choline regulation of Ino2p-dependent reporter genes. Low-level expression of *INO2* and *INO4* genes, each under control of the heterologous *MET25* promoter, did not alter the regulatory pattern of target genes. Thus, upstream regions of *INO2* and *INO4* are not crucial for transcriptional control of ICRE-dependent genes by inositol and choline. Interestingly, over-expression of *INO2*, but not of *INO4*, counteracted repression by phospholipid precursors. Possibly, a functional antagonism between *INO2* and a negative regulator is the key event responsible for repression or de-repression.

Key words Transcriptional regulation · Phospholipid biosynthesis · *Saccharomyces cerevisiae* · *INO2*

Introduction

In the yeast *Saccharomyces cerevisiae* most structural genes of phospholipid biosynthesis are regulated at the transcriptional level by the precursor molecules inositol and choline (repression in the presence of inositol and choline, de-repression in their absence; reviewed by Greenberg and Lopes 1996). We have previously identified a regulatory sequence, designated ICRE (for **i**nositol/**c**holine-**r**esponsive **e**lement), which mediates the differential transcriptional activation of the respective target genes (Schüller et al. 1992 a). ICRE motifs (also described as UAS_{INO}; Bachhawat et al. 1995) were identified upstream of the structural genes *INO1* (encoding inositol-1-phosphate synthase; Lopes et al. 1991), *PSS1*/*CHO1* (encoding phosphatidylserine synthase; Kodaki et al. 1991), and *FAS1* and *FAS2* (encoding the subunits of the fatty acid synthase complex; Schüller et al. 1992 a). ICRE-dependent structural genes are regulated by phospholipid precursors to varying degrees (about 40-fold repression of *INO1* in contrast to a less than 2-fold repression of *FAS1* and *FAS2*). This weak regulation of *FAS1* and *FAS2* was shown to be the result of a composite promoter, containing binding sites of constitutive activators Rap1p, Abf1p and Reb1p in addition to ICRE sequences (Schüller et al. 1994). In contrast, synthetic minimal promoters containing an ICRE as the sole upstream activation site (UAS) mediated a significant inositol/choline regulation (de-repression factors of 12–30, depending on the sequence variant tested; Schüller et al. 1992 a, 1995). The ICRE functions as the binding site of a heterodimeric transcription factor encoded by the regulatory genes *INO2* and *INO4* (Schüller et al. 1992 b; Ambroziak and Henry 1994; Schwank et al. 1995). Ino2p and Ino4p are members of the basic helix-loop-helix family (bHLH) of DNA-binding proteins (Hoshizaki et al. 1990; Nikoloff et al. 1992), forming exclusively heterodimers via their HLH regions. In addition, Ino2p, but not Ino4p, also contains two separate transcriptional-activation domains (Schwank et al. 1995).

Although *cis*- and *trans*-acting elements of the inositol/choline (IC) regulation of phospholipid biosynthetic genes have been identified, the regulatory pathway of this metabolic control is still unknown. A negative regulator of IC repression is encoded by the *OPI1* gene, containing a leucine zipper motif (White et al. 1991). In the respective *opi1* mutants, *INO1* is constitutively expressed at a high level, supporting the hypothesis of Opi1p as a repressor of transcriptional regulation. We have previously shown that

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INO4 is positively autoregulated, possibly mediated by an ICRE-like motif in its upstream region (Schüller et al. 1992 b). Since its promoter contains a related sequence, a similar regulation appeared plausible also for the *INO2* gene. Thus, autoregulation of the positive regulators Ino2p and Ino4p may be considered as an important step of the IC derepression response (Ashburner and Lopes 1995 a, b). We therefore wished to determine the significance of *INO2* and *INO4* autoregulation for the differential expression of Ino2p/Ino4p-dependent structural genes by phospholipid precursors. In the present work we clearly demonstrate ICRE-dependent autoregulation of *INO2* and *INO4*. However, autoregulation of *INO2* and *INO4* is identified as a dispensable mechanism of IC-mediated transcriptional control, at best being involved in metabolic fine-tuning.

Materials and methods

Yeast strains used in this work, media and growth conditions. The strains of *S. cerevisiae* listed in Table 1 were derived from the regulatory wild-type JS91.15-23 (Schwank et al. 1995). Strain SIRP3 contains a *CYC1-lacZ* reporter gene controlled by an ICRE-dependent synthetic minimal promoter (Schwank et al. 1995). Integrative plasmids pJS386 and pSS72 with *INO2-lacZ* or *INO2*(ICRE$_{Mut}$)-*lacZ* fusions (see below) were transformed into JS91.15–23 to give JS93.19-1 and SS95.5-1, respectively. Yeast transformants were grown at 30°C in synthetic complete medium selecting for the plasmid marker(s). The composition of medium has been described by Schüller et al. 1992 b). Inositol/choline repression (r) was achieved by 200 μM inositol + 2 mM choline; 5 μM inositol + 5 μM choline allowed growth of inositol-requiring mutants without causing repression in the wild-type (de-repressing conditions, d).

Synthetic oligonucleotides. Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany). Synthetic DNA fragments used for insertion studies and gel-retardation analyses were PRINO2 (5′ tcgaagatctAA**AATTCACATG**TGTGTTG 3′) and INO4AB (5′ tcgaagatctAA**TATTCACATG**TTTTTC 3′; authentic promoter sequences shown in capital letters; ICRE in bold). FBF56 contains an ICRE from the *INO1* promoter (5′ tcgaagatctAC**TTTTCACATGC**CGCATT 3′; Schüller et al. 1992 a).

Plasmid constructions. Plasmids pSS20 (Schwank et al. 1995) and pRS5 (Schüller et al. 1992 b) were used to introduce null mutations Δ*ino2::LEU2* and Δ*ino4::LEU2*, respectively. Plasmids pJS406N and pRK1N were obtained by inserting synthetic DNA fragments PRINO2 or INO4AB into pJS205 (ΔUAS-*CYC1-lacZ URA3* 2 μm; Schüller et al. 1992 a). Integrative plasmid pJS386 (*URA3*) contains 57 codons of the *INO2* reading frame together with about 1.9 kb of the upstream region fused to *lacZ*. Mutagenesis of the ICRE(*INO2*) core sequence CACATG into GGATCC by PCR led to pSS72 which is otherwise identical to pJS386. Plasmid pJS409 carries the entire *INO2* gene under natural promoter control (530-bp upstream sequence), inserted as a 2.7-kb *Pst*I/*Xba*I fragment into YCplac22 (*ARS CEN TRP1*; Gietz and Sugino 1988). Similarly, pJS413 is an isogenic variant, mutated at the ICRE(*INO2*) motif. The single-copy plasmid pSS69 contains the entire *INO4* gene (450-bp upstream sequence) as a 1.3-kb *Kpn*I/*Sma*I fragment, inserted into pRS413 (*ARS CEN HIS3*; Sikorski and Hieter 1989). Reading frame cassettes of *INO2* (0.93 kb) and *INO4* (0.47 kb), previously described by Schwank et al. (1995), were used for the construction of *GAL1-INO2* (pEH4) and *GAL1-INO4* fusions (pEH3) in the *ARS CEN TRP1 GAL1* promoter plasmid YCpIF16 (Foreman and Davis 1994). Plasmids pSS108 (*ARS CEN TRP1 MET25-INO2*) and pSS107 (*ARS CEN HIS3 MET25-INO4*) were similarly obtained from parental vectors p414-MET25 and p413-MET25, respectively (Mumberg et al. 1994).

RNA isolation and Northern-blot hybridization. Total RNA was isolated from mid-log phase cultures (about 2×10^7 cells/ml) as previously described (Schüller et al. 1992 b). Twenty micrograms of RNA were size-fractionated in a vertical 1% agarose gel containing 2.2 M formaldehyde and subsequently transferred to a nylon membrane. A 0.9-kb *Bam*HI/*Xba*I *INO2* reading frame cassette labelled by nick-translation was used as a hybridization probe. A 2.5-kb *Pst*I/*Bam*HI fragment containing the constitutively transcribed *CAT1* gene (Schüller and Entian 1987) was used as an internal reference probe.

Gel-retardation assay. The synthetic DNA fragments PRINO2 and INO4AB were end-labelled by a fill-in reaction using α-[^{32}P]dATP and Klenow enzyme. The labelled probes (5000 cpm; about 0.2–0.3 ng) were incubated with 5 μg of protein from an extract containing GST-Ino2p+GST-Ino4p fusion proteins and subsequently separated on a native 4% polyacrylamide gel. Preparation of the extract and reconstitution of a functional Ino2p/Ino4p heterodimer has been reported previously (Schwank et al. 1995).

Miscellaneous procedures. Yeast transformation and β-galactosidase assays followed established procedures and were described previously (Schüller et al. 1992 b). Specific enzyme activities represent the average of at least three independent transformation procedures and at least five independent assays (in duplicate) of the transformants obtained. Polymerase chain reaction (PCR) experiments were done with reagents from Boehringer Mannheim under standard conditions.

Results

Regulation of *INO2* and *INO4* by inositol/choline-responsive elements (ICRE)

We have previously shown that expression of the regulatory gene *INO4* necessary for ICRE-dependent gene acti-

Table 1 Strains of *S. cerevisiae* used in this work

Strain	Genotype
JS91.15-23	*MATα ura3 leu2 his3 trp1 can1 MAL3 SUC3*
SS92.3-1	*MATα ura3 leu2 his3 trp1 can1 MAL3 SUC3 Δino2::LEU2*
RSH3	*MATα ura3 leu2 trp1 can1 MAL3 SUC3 Δino4::LEU2*
MBY1	*MATα ura3 leu2 his3 trp1 can1 MAL3 SUC3 Δopi1::HIS3*
JS93.19-1	*MATα leu2 his3 trp1 can1 MAL3 SUC3 ura3::INO2-lacZ::URA3*
JS93.25-1	*MATα leu2 his3 trp1 can1 MAL3 SUC3 ura3::INO2-lacZ::URA3 Δopi1::HIS3*
SS95.5-1	*MATα leu2 his3 trp1 can1 MAL3 SUC3 ura3::INO2*(ICRE$_{Mut}$)*-lacZ::URA3*
SS95.5-7	*MATα leu2 his3 trp1 can1 MAL3 SUC3 ura3::INO2*(ICRE$_{Mut}$)*-lacZ::URA3 Δopi1::HIS3*
SIRP3	*MATα leu2 his3 trp1 can1 MAL3 SUC3 ura3::ICRE-CYC1-lacZ::URA3*
SIRP3.Δino2	*MATα leu2 his3 trp1 can1 MAL3 SUC3 ura3::ICRE-CYC1-lacZ::URA3 Δino2::HIS3*
SIRP3.Δino4	*MATα leu2 his3 trp1 can1 MAL3 SUC3 ura3::ICRE-CYC1-lacZ::URA3 Δino4::LEU2*

Table 2 Inositol/choline-regulated activation of ICRE(*INO2*)- and ICRE(*INO4*)-dependent reporter genes in the wild-type and in regulatory mutants. Plasmids pJS406N and pRK1N contain a single copy of an ICRE(*INO2*) or ICRE(*INO4*) insert upstream of the basal promoter of a ΔUAS-*CYC1-lacZ* fusion (pJS205; Schüller et al. 1992 a). Specific enzyme activities (U/mg) assayed in transformants are given in nmoles of ONPG hydrolyzed per min per mg protein. A basal expression level of 20 U/mg β-galactosidase activity was observed with pJS205 transformants under either conditions. *r*, repressing growth conditions (200 μM inositol+2 mM choline); *d*, de-repressing growth conditions (5 μM inositol + 5 μM choline). The standard deviations (SD) of mean values are given in parentheses

Strain (genotype)	Specific β-galactosidase activity in transformants of			
	pJS406N [ICRE(*INO2*)-*CYC1-lacZ*]		pRK1N [ICRE(*INO4*)-*CYC1-lacZ*]	
	r (SD)	*d* (SD)	*r* (SD)	*d* (SD)
JS91.15-23 (wild-type)	30 (5)	310 (45)	25 (3)	110 (20)
SS92.3-1 (Δ*ino2*)	25 (4)	30 (4)	25 (3)	40 (8)
RSH3 (Δ*ino4*)	25 (4)	35 (5)	20 (4)	30 (7)
MBY1 (Δ*opi1*)	380 (45)	350 (60)	150 (20)	140 (30)

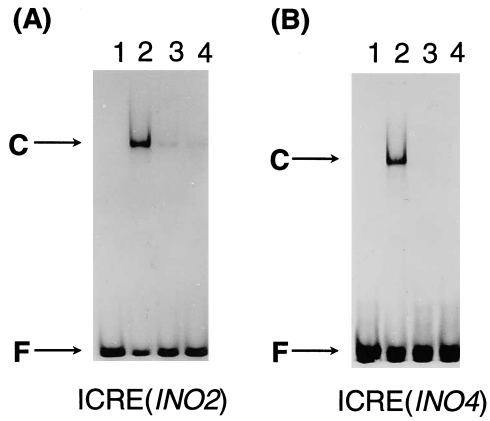


Fig. 1 A, B Gel-retardation analysis of Ino2p/Ino4p binding to ICRE(*INO2*) or ICRE(*INO4*) probes. (**A**) Use of the labelled synthetic DNA fragment PRINO2 containing ICRE(*INO2*). (**B**) Use of the labelled synthetic DNA fragment INO4AB containing ICRE (*INO4*). *Lanes 2–4* contained 5 μg of total *E.coli* protein with the reconstituted Ino2p/Ino4p complex. In *lanes 3*, a 100-fold molar excess of the respective unlabelled DNA fragments was added [ICRE(*INO2*) in (**A**) and ICRE(*INO4*) in (**B**)]. In *lanes 4*, the DNA fragment FBF56 containing a high-affinity ICRE from the *INO1* promoter was used for competition. *C* Ino2p/Ino4p-DNA complex; *F* free DNA

vation is itself regulated by the phospholipid precursors inositol and choline (Schüller et al. 1992 b). Upstream of *INO4*, we identified a sequence motif (TATTCACATGT) significantly similar to the ICRE consensus sequence, arguing for an autoregulation of *INO4* expression. Since an almost identical element is also present upstream of *INO2* (AATTCACATGT) we became interested in the possible importance of *INO2*/*INO4* autoregulation for the de-repression of inositol/choline (IC)-regulated structural genes.

Synthetic DNA fragments PRINO2 and INO4AB containing the putative ICREs upstream of *INO2* and *INO4*, respectively, were individually inserted into the upstream region of a suitable promotor test-construct devoid of its natural UAS (pJS205; ΔUAS-*CYC1-lacZ* reporter gene). The resulting plasmids pJS406N [ICRE(*INO2*)] and pRK1N [ICRE(*INO4*)] each containing a single insert were transformed into a wild-type strain and regulatory mutants. In the absence of IC, a 15-fold and 5–6-fold activation, respectively, of the basal expression level of pJS205 transformants was observed (Table 2). Thus, the ICRE of the *INO2* promoter is a stronger UAS than the corresponding sequence found in the *INO4* control region. However, both elements are significantly weaker than ICREs upstream of structural genes (activation factors of about 40; Schüller et al. 1995). Nevertheless, gene activation observed for ICRE(*INO2*)-*CYC1-lacZ* and ICRE(*INO4*)-*CYC1-lacZ* reporter genes was IC-repressible in the wild-type strain (Table 2). Repression could no longer be observed in the *opi1* null mutant, lacking a negative-regulatory protein of phospholipid biosynthesis. On the other hand, activation by ICRE(*INO2*) and ICRE(*INO4*) motifs was eliminated in *ino2* or *ino4* mutants.

In order to confirm the binding of the Ino2p/Ino4p heterodimer to the ICREs of *INO2* and *INO4* promoters, synthetic DNA fragments PRINO2 and INO4AB were used for gel-retardation experiments. As is shown in Fig. 1, a reconstituted Ino2p/Ino4p complex derived from proteins separately expressed in *E. coli* indeed bound efficiently to both promoter probes. No binding was observed with the individual proteins Ino2p or Ino4p (data not shown). We therefore conclude that both genes *INO2* and *INO4* encoding the subunits of the heteromeric transcription factor Ino2p/ino4p are directly affected by positive autoregulation.

Inositol/choline-regulated expression of *INO2* in the wild-type and the *opi1* mutant

In order to confirm the IC regulation of *INO2* at the transcriptional level, we performed a Northern-blot hybridization with total RNA isolated from cells grown under conditions of IC repression or de-repression. As is shown in Fig. 2, the *INO2* mRNA level in the wild-type indeed increased with respect to the constantly expressed *CAT1* transcript (Schüller and Entian 1987) under de-repressing conditions. No *INO2* transcript could be detected in RNA isolated from an *ino2* null mutant (Fig. 2).

Since regulation of *INO2* by IC is more severe than we have previously found for *INO4* (13-fold IC repression of *INO2* in comparison with a 3-fold repression of *INO4*; cf. Table 3 of this work and Schüller et al. 1992 b), we wished to determine the importance of the ICRE(*INO2*) motif for the IC regulation of *INO2*. We therefore constructed a translational *INO2-lacZ* fusion (in plasmid pJS386) which was subsequently integrated at the *ura3* locus of a regulatory wild-type strain. As shown in Table 3, repression of *INO2* by IC in the wild-type was no longer observed in an

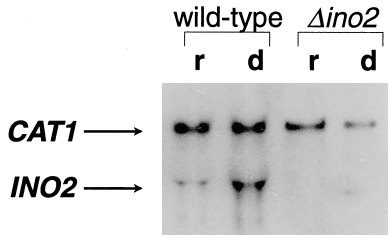


Fig. 2 Detection of *INO2* and *CAT1* transcripts in wild-type and mutant strains by a Northern-blot hybridization. Strains JS91.15-23 (wild-type) and SS92.3–1 (Δ*ino2*) were grown under repressing (*r*) or de-repressing (*d*) conditions and subsequently used for RNA preparation. About 20 μg of total RNA was separated on each lane by agarose-gel electrophoresis under denaturing conditions. DNA fragments containing the entire *INO2* and *CAT1* reading frames were used as ^{32}P-labelled probes. The weakly expressed *CAT1* gene served as an internal hybridization standard. The size of *CAT1* and *INO2* transcripts is about 2.4 kb and 1.2 kb, respectively

Table 3 Expression of *INO2-lacZ* reporter constructs containing functional or mutated ICRE promoter motifs in a wild-type strain and an *opi1* regulatory mutant. Plasmid pJS386 contains an *INO2-lacZ* fusion with about 1.9 kb of the wild-type upstream region in an integrative *URA3* plasmid. pSS72 is identical to pJS386 but has been mutated at the ICRE of the *INO2* promoter. The wild-type strain JS93.19–1 resulted from targeting of pJS386 to the *ura3* locus of JS91.15–23. Similarly, SS95.5–1 was obtained by integration of pSS72. The respective *opi1* mutants were derived from these strains by subsequent gene disruptions. Specific enzyme activities are given in nmoles of ONPG hydrolyzed per min per mg protein. *r*, repressing growth conditions (200 μM inositol+2 mM choline); *d*, derepressing growth conditions (5 μM inositol + 5 μM choline). The standard deviations (SD) of mean values are given in parentheses

Strain (genotype)	Specific β-galactosidase activities	
	r (SD)	*d* (SD)
Integrated *INO2-lacZ*:		
JS93.19-1 (wild-type)	1.5 (< 0.3)	20.0 (2.5)
JS93.25-1 (Δ*opi1*)	30.0 (2.0)	27.0 (3.0)
Integrated *INO2*(ICRE$_{Mut}$)-*lacZ*:		
SS95.5-1 (wild-type)	1.0 (< 0.3)	1.5 (< 0.3)
SS95.5-7 (Δ*opi1*)	1.5 (< 0.3)	1.0 (< 0.3)

isogenic *opi1* null mutant. Site-directed mutagenesis of the ICRE(*INO2*) motif resulted in an *INO2-lacZ* reporter construct (in plasmid pSS72) which was expressed at a low constant level in the regulatory wild-type and the *opi1* null mutant (Table 3). This result demonstrates that the influence of Opi1p on *INO2* expression is mediated exclusively via the ICRE.

Influence of *INO2* autoregulation on ICRE-dependent gene expression

Biosynthetic de-repression of *INO2* in the absence of phospholipid precursors no longer occured with an allele mutated at its upstream ICRE. Thus, we wished to determine whether an *INO2* gene mutated at its upstream ICRE [*INO2*(ICRE$_{Mut}$) mutant allele] still allows de-repression of an ICRE-dependent reporter gene. In order to focus entirely on ICRE-mediated transcriptional effects, and to exclude a possible interference with further regulatory elements, we used a reporter gene controlled by a synthetic minimal promoter instead of a complex control region. Wild-type strain SIRP3 and its derivatives used for the following analyses all contain a ICRE-*CYC1-lacZ* reporter gene stably integrated at the *ura3* locus.

Table 4 Inositol/choline-mediated gene regulation by an *INO2* promoter mutant. Strains SIRP3 and SIRP3.Δ*ino2* contain an ICRE-*CYC1-lacZ* reporter construct targeted to the *ura3* locus. Effector plasmids pJS409 and pJS413 were derived from the single-copy vector YCplac22 (*ARS CEN TRP1*; Gietz and Sugino 1988) and contain the complete *INO2* reading frame together with about 530 bp of its upstream region. The mutant allele lacking the ICRE motif is indicated by *INO2*[ICRE$_{Mut}$]. Specific enzyme activities are given in nmoles of ONPG hydrolyzed per min per mg protein. *r*, repressing growth conditions (200 μM inositol + 2 mM choline); *d*, de-repressing growth conditions (5 μM inositol + 5 μM choline). The standard deviations (SD) of mean values are given in parentheses

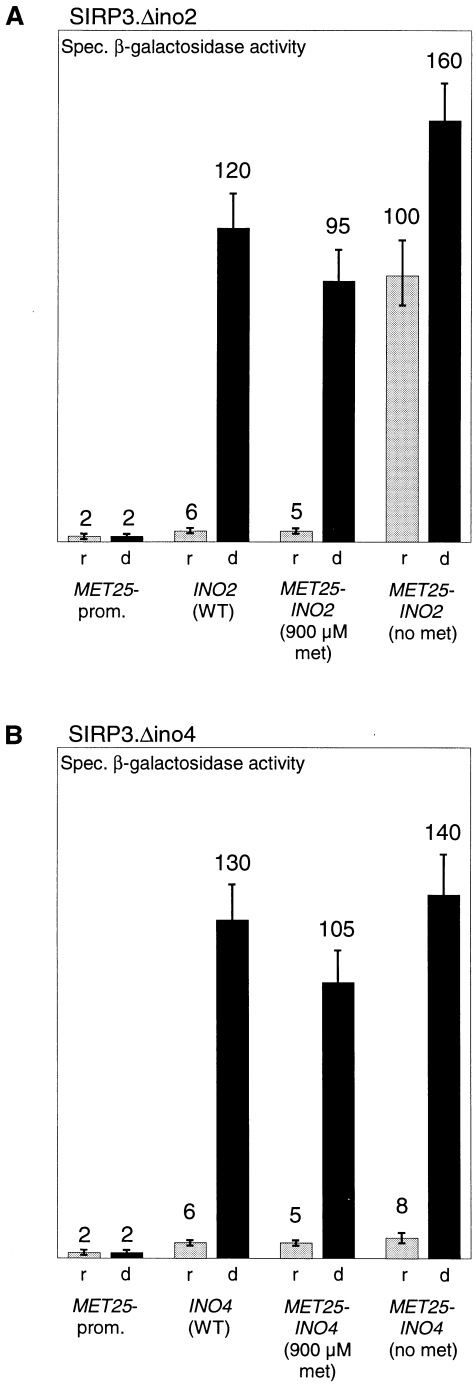
Strain (genotype)	Effector plasmid (genetic markers)	Spec. β-gal. activity	
		r (SD)	*d* (SD)
SIRP3 (wild-type)	YCplac22	6 (1)	150 (20)
SIRP3.Δino2 (Δ*ino2*)	YCplac22	2 (1)	3 (1)
SIRP3.Δino2 (Δ*ino2*)	pJS409 (*INO2*)	5 (1)	125 (18)
SIRP3.Δino2 (Δ*ino2*)	pJS413 (*INO2*[ICRE$_{Mut}$])	6 (2)	120 (15)

The *ino2* null mutant SIRP3.Δ*ino2* was transformed with single-copy plasmids containing either the *INO2* wild-type gene (pJS409) or the otherwise identical *INO2*(ICRE$_{Mut}$) variant (pJS413). With both plasmids, the inositol auxotrophy of the mutant could be entirely complemented. Both plasmids also restored wild-type regulation of the ICRE-*CYC1-lacZ* reporter gene. As shown in Table 4, a 20–25-fold IC repression of reporter gene activation was observed with the *INO2* wild-type gene as well as with the *INO2*(ICRE$_{Mut}$) allele. We also compared the de-repression kinetics with IC-repressed *INO2* or *INO2*(ICRE$_{Mut}$) transformants. However, the time course of de-repression was almost identical with both *INO2* alleles. These results show that autoregulation of *INO2* can be excluded as an essential regulatory parameter of ICRE-dependent transcriptional control, at least under the conditions investigated so far.

Variation of *INO2* and *INO4* gene dosage by expression under heterologous promoter control

Since ICRE-dependent gene activation requires both subunits of the Ino2p/Ino4p complex, we investigated the influence of gene-dosage variations of *INO2* and *INO4* on IC repression and de-repression. In order to uncouple IC regulation of *INO2* and *INO4* from the ICRE-dependent

control exerted by the Ino2p/Ino4p activator, both regulatory genes were fused individually to the heterologous *MET25* promoter. Depending on the concentration of methionine in the growth medium, this region allows a subtle control of the expression level of a gene (Mumberg et al. 1994). No influence of varying concentrations of methionine on the pattern of IC regulation was found. Moreover, the *MET25* promoter was not affected by IC repression (data not shown). The *ino2* mutant SIRP3.Δ*ino2* was transformed with the *MET25-INO2* fusion construct and subsequently grown in the absence or presence of methionine. As shown in Fig. 3 A, almost identical de-repression factors (ratio of enzyme activities under de-repressing vs repressing growth conditions; d/r) were observed with a weakly expressed *MET25-INO2* fusion (900 μM methionine; d/r = 19) and the *INO2* wild-type gene (d/r = 20). Thus, even with a heterologous promoter upstream of *INO2*, IC regulation of an Ino2p-dependent reporter gene was still obvious. Similarly, a *MET25-INO4* fusion restored wild-type regulation in the *ino4* null mutant SIRP3.Δ*ino4* (Fig. 3B). Essentially the same results were obtained with *GAL1-INO2* and *GAL1-INO4* fusions, respectively (data not shown).



Interestingly, maximal de-repression of the *MET25-INO2* fusion in strain SIRP3.Δ*ino2* by omitting methionine allowed an almost constitutive expression of the Ino2p-dependent reporter gene (d/r = 1.6; Fig. 3 A). By contrast, methionine de-repression of the *MET25-INO4* fusion in strain SIRP3.Δ*ino4* did not affect IC regulation of the reporter gene (Fig. 3 B). Thus, even when strongly expressed by the heterologous *MET25* promoter, *INO2* and *INO4* unequally influence regulation of Ino2p/Ino4p-dependent transcription. We conclude that *INO2*, but not *INO4*, should be considered as the ultimate target of the signal transduction pathway initially triggered by phospholipid precursors. The metabolic signal finally acting on *INO2* must not affect its upstream region since IC regulation of target genes occurs even with an *INO2* allele controlled by a weak heterologous promoter.

Discussion

In this work, we have investigated the influence of transcriptional autoregulation of regulatory genes *INO2* and *INO4* on inositol/choline repression of an Ino2p/Ino4p-dependent reporter gene. In a previous paper, we showed that *INO4* itself is about 3-fold repressed in the presence of IC (Schüller et al. 1992 b). Similarly, expression of *INO2* is even more strongly (about 13-fold) affected by phospholipid precursors. We were able to show by Northern-blot

Fig. 3 Influence of single-copy *MET25-INO2* (**A**) and *MET25-INO4* (**B**) fusion genes on inositol/choline regulation. Recipient strains SIRP3.Δino2 and SIRP3.Δino4 contain an ICRE-*CYC1-lacZ* reporter construct targeted to the *ura3* locus. The single-copy *TRP1* plasmids p414-MET25 (*MET25*-prom.; no *INO2* insert), pJS409 (*INO2* under control of its wild-type promoter) and pSS108 (*MET25-INO2* fusion) were transformed into SIRP3.Δ*ino2* (**A**). Similarly, single-copy *HIS3* plasmids p413-MET25 (*MET25*-prom.; no *INO4* insert), pSS69 (*INO4* under control of its wild-type promoter) and pSS107 (*MET25-INO4* fusion) were transformed into SIRP3.Δino4 (**B**). Transformants were grown under selective conditions in the presence of the methionine (*met*) concentration indicated. The *MET25* promoter was completely de-repressed in the absence of methionine while maximal repression was achieved in the presence of 900 μM of methionine. Specific enzyme activities are given in nmoles ONPG hydrolyzed per min per mg protein. *r* repressing growth conditions (200 μM inositol + 2 mM choline; *grey*); *d* de-repressing growth conditions (5 μM inositol + 5 μM choline; *black*). *Error bars* represent the standard deviation of the mean value

hybridization that regulation of *INO2* by IC indeed acts at the transcriptional level (Fig. 2). The upstream regions of *INO2* and *INO4* both contain ICRE sequence variants which are bound by the Ino2p/Ino4p complex in vitro (Fig. 1) and function as UAS elements in vivo (Table 2). Recently, Ashburner and Lopes (1995 a) also proved IC repression of an integrated *INO2-cat* fusion gene. In contrast to our data, these authors reported a constitutive expression of *INO4*. In our hands, the 3-fold de-repression of an *INO4-lacZ* fusion was completely absent in *ino2* and *ino4* mutants, while constitutive expression could be observed in an *opi1* mutant (Schüller et al. 1992 b; data not shown). These differing results may be explained by the possible influence of additional activating factors, leading to a strain-dependent expression of *INO4*.

Since *INO2* is more severely affected by IC repression than *INO4*, we wished to investigate the importance of the ICRE(*INO2*) motif for the differential expression of the Ino2p-dependent ICRE-*CYC1-lacZ* reporter gene. We, and others, have previously suggested that autoregulation of *INO2* and *INO4* might be important as a mechanism of self-amplification of an input signal generated elsewhere, finally determining the magnitude of the regulatory response (Schüller et al. 1992 b; Ashburner and Lopes 1995 a, b). Site-directed mutagenesis of the ICRE(*INO2*) motif led to a constant expression of *INO2* at a low level even under conditions of IC limitation (Table 3). Nevertheless, even an *INO2* allele devoid of its ICRE could functionally replace the wild-type gene under all conditions tested (complementation of an *ino2* null mutation, maximal de-repression of an ICRE-dependent reporter gene, time course of de-repression). Autoregulation may be considered as a redundant mechanism of IC de-repression which may have some minor importance under conditions yet to be defined.

This conclusion was further supported by the finding that a weakly expressed *INO2* gene under control of the heterologous *MET25* promoter could mediate IC regulation of an Ino2p-dependent reporter gene, as did *INO2* under natural promoter control (Fig. 3 A). A similar result was obtained with a *MET25-INO4* fusion and the wild-type *INO4*, respectively (Fig. 3 B). Thus, the natural upstream regions of *INO2* and *INO4* are not essential for a differential regulation of Ino2p/Ino4p-dependent target genes by IC. Interestingly, maximal activation of the *MET25-INO2* fusion by omitting methionine from the medium led to a strong activation of the ICRE-dependent reporter gene even in the presence of repressing amounts of phospholipid precursors (Fig. 3 A). A similar result was obtained with an *INO1-lacZ* reporter gene (data not shown). This finding differs from a recent report of Ashburner and Lopes (1995 b) who tested the influence of a *GAL1-INO2* fusion on the *INO1* transcript level. Even with a completely induced *GAL1* promoter, these authors found a strong IC regulation of the *INO1* mRNA. However, Hosaka et al. (1994) reported that *SCS1* (= *INO2*) present on an episomal plasmid could strongly elevate the level of *INO1*, *PSS1*/*CHO1*, *PEM1* and *PEM2* transcripts under repressing conditions. These results completely agree with our data obtained with a reporter gene controlled by an ICRE-activated synthetic minimal promoter.

On the other hand, a maximally expressed *MET25-INO4* fusion was unable to overrule IC repression which could be observed with the *MET25-INO2* construct. Under either condition, no significant influence of an increased *INO4* expression on the regulatory response was observed. This result argues for *INO2* as a sensitive target of the IC signal. Possibly, elevating the *INO2* gene dosage under repressing conditions (either by a multi-copy situation or by heterologous promoter control) causes an imbalance between Ino2p and a negative factor counteracting Ino2p function. Such a negative factor could act as an antagonist of *INO2* under repressing, but not under de-repressing, conditions. A possible candidate for the repressor may be Opi1p. Previous work identified *OPI1* as a crucial negative regulator of phospholipid biosynthetic enzymes (White et al. 1991). However, the molecular function of its gene product is presently unknown. Since Opi1p contains a basic leucine zipper motif, it might function as a DNA-binding factor. Nevertheless, a target sequence of Opi1p has not yet been defined. Promoter-swapping experiments argue against the importance of the *INO2* upstream region for Opi1p function. It remains to be shown whether the coding region of *INO2*, its corresponding transcript, or Ino2p are affected by the regulatory signal initiated by phospholipid precursors.

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References

Ambroziak J, Henry SA (1994) *INO2* and *INO4* gene products, positive regulators of phospholipid biosynthesis in *Saccharomyces cerevisiae*, form a complex that binds to the *INO1* promoter. J Biol Chem 269:15344–15349

Ashburner BP, Lopes JM (1995 a) Autoregulated expression of the yeast *INO2* and *INO4* helix-loop-helix activator genes effects cooperative regulation on their target genes. Mol Cell Biol 15: 1709–1715

Ashburner BP, Lopes JM (1995 b) Regulation of yeast phospholipid biosynthetic gene expression in response to inositol involves two superimposed mechanisms. Proc Natl Acad Sci USA 92: 9722–9726

Bachhawat N, Ouyang Q, Henry SA (1995) Functional characterization of an inositol-sensitive upstream activation sequence in yeast. J Biol Chem 270:25087–25095

Foreman PK, Davis RW (1994) Cloning vectors for the synthesis of epitope-tagged, truncated and chimeric proteins in *Saccharomyces cerevisiae*. Gene 144:63–68

Gietz RD, Sugino A (1988) New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74:527–534

Greenberg ML, Lopes JM (1996) Genetic regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae*. Microbiol Rev 60:1–20

Hosaka K, Nikawa J-i, Kodaki T, Yamashita S (1994) Cloning and characterization of the *SCS1* gene required for the expression of genes in yeast phospholipid synthesis. J Biochem 115:131–136

Hoshizaki DK, Hill JE, Henry, SA (1990) The *Saccharomyces cerevisiae INO4* gene encodes a small, highly basic protein required for de-repression of phospholipid biosynthetic enzymes. J Biol Chem 265:4736–4745

Kodaki T, Nikawa J-i, Hosaka K, Yamashita S (1991) Functional analysis of the regulatory region of the yeast phosphatidylserine synthase gene, *PSS*. J Bacteriol 173:7992–7995

Lopes JM, Hirsch JP, Chorgo PA, Schulze KL, Henry SA (1991) Analysis of sequences in the *INO1* promoter that are involved in its regulation by phospholipid precursors. Nucleic Acids Res 19: 1687–1693

Mumberg D, Müller R, Funk M (1994) Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res 22:5767–5768

Nikoloff DM, McGraw P, Henry SA (1992) The *INO2* gene of *Saccharomyces cerevisiae* encodes a helix-loop-helix protein that is required for activation of phospholipid synthesis. Nucleic Acids Res 20:3253

Schüller H-J, Entian K-D (1987) Isolation and expression analysis of two yeast regulatory genes involved in the de-repression of glucose-repressible enzymes. Mol Gen Genet 209:366–373

Schüller H-J, Hahn A, Tröster F, Schütz A, Schweizer E (1992 a) Coordinate genetic control of yeast fatty acid synthase genes *FAS1* and *FAS2* by an upstream activation site common to genes involved in membrane lipid biosynthesis. EMBO J 11:107–114

Schüller H-J, Schorr R, Hoffmann B, Schweizer E (1992 b) Regulatory gene *INO4* of yeast phospholipid biosynthesis is positively autoregulated and functions as a *trans*-activator of fatty acid synthase genes *FAS1* and *FAS2* from *Saccharomyces cerevisiae*. Nucleic Acids Res 20:5955–5961

Schüller H-J, Schütz A, Knab S, Hoffmann B, Schweizer E (1994) Importance of general regulatory factors Rap1p, Abf1p and Reb1p for the activation of yeast fatty acid synthase genes *FAS1* and *FAS2*. Eur J Biochem 225:213–222

Schüller H-J, Richter K, Hoffmann B, Ebbert R, Schweizer E (1995) DNA-binding site of the yeast heteromeric Ino2p/Ino4p basic helix-loop-helix transcription factor: structural requirements as defined by saturation mutagenesis. FEBS Lett 370:149–152

Schwank S, Ebbert R, Rautenstrauß K, Schweizer E, Schüller H-J (1995) Yeast transcriptional activator *INO2* interacts as an Ino2p/Ino4p basic helix-loop-helix heterodimer with the inositol/choline-responsive element necessary for expression of phospholipid biosynthetic genes in *Saccharomyces cerevisiae*. Nucleic Acids Res 23:230–237

Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122:19–27

White MJ, Hirsch JP, Henry SA (1991) The *OPI1* gene of *Saccharomyces cerevisiae*, a negative regulator of phospholipid biosynthesis, encodes a protein containing polyglutamine tracts and a leucine zipper. J Biol Chem 266:863–872