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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*

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ABSTRACT

The sequence motif 5′ TYTTCACATGY 3′ functions as an upstream activation site common to both yeast fatty acid synthase genes, *FAS1* and *FAS2*. In addition, this UAS_{FAS} element is shared by all so far characterized genes of yeast phospholipid biosynthesis. We have investigated the influence of a functional *INO4* gene previously described as a regulator of inositol biosynthesis on the expression of *FAS1* and *FAS2*. In a *Δino4* null allele strain, both genes are expressed at only 50% of wild type level. Using individual UAS_{FAS} sequence motifs inserted into a heterologous test system, a drastic decrease of reporter gene expression to 2 – 10% of the wild type reference was observed in the *Δino4* mutant. In gel retardation assays, the protein-DNA complex involving the previously described FAS binding factor 1, Fbf1, was absent when using a protein extract from the *Δino4* mutant. On the other hand, this signal was enhanced with an extract from cells grown under conditions of inositol/choline derepression. Subsequent experiments demonstrated that *INO4* expression is itself affected by phospholipid precursors, mediated by an UAS_{FAS} element in the *INO4* upstream region. Thus, in addition of being an activator of phospholipid biosynthetic genes, *INO4* is also subject to a positive autoregulatory loop in its own biosynthesis.

INTRODUCTION

Phospholipids are key components of biological membranes and therefore of central importance for establishing the structural and functional characteristics thereof. The complex interplay of many different enzymes of distinct intracellular localization contributing to phospholipid biosynthesis as a whole suggests some kind of common genetic control ensuring the balanced synthesis of membrane components at varying metabolic conditions. The yeast *Saccharomyces cerevisiae* has proven as an especially suitable model organism to study this control, genetically. Thus, a considerable number of genes involved in phospholipid biosynthesis has been cloned and sequenced from *Saccharomyces cerevisiae*. Among them, the genes *FAS1* and *FAS2* encoding subunits β and α, respectively, of the $(\alpha\ \beta)_6$ fatty acid synthase complex are necessary for the *de novo* synthesis of long chain saturated fatty acids (reviewed in 1). Studying the expression of these genes, we have previously identified an upstream activation site (UAS_{FAS}) common to *FAS1* and *FAS2* (2). Very similar UAS_{FAS} elements were also found in the upstream regions of at least ten other genes encoding different enzymes of phospholipid biosynthesis, thus leading to a consensus sequence, 5′ TYTTCACATGY 3′, derived from 18 different UAS_{FAS}-like motifs (2). A nonamer motif, 5′ ATGTGAAAT 3′, has been proposed to be involved in the regulated expression of *INO1* (encoding inositol-1-phosphate synthase) and *CHO1* (encoding phosphatidylserine synthase) genes (3; 4). The reverse complement of this nonamer motif is highly related to and partially contained within UAS_{FAS}. However, this nonamer element was unable to activate a heterologous reporter gene (3) and, therefore, contrasted strikingly to UAS_{FAS} which confers a 40 - 100-fold orientation-independent activation on an UAS-less test system. This activation was suppressible by exogenously added inositol + choline, leading to the definition of UAS_{FAS} as an inositol/choline responsive element (ICRE; 2). In contrast, intact *FAS* promoters showed only a weak response to inositol/choline repression.

The presence of similar *cis*-acting elements upstream of a variety of phospholipid biosynthetic genes suggests the involvement of common *trans*-acting factors in yeast. Indeed, pleiotropic regulatory mutations, *ino2* and *ino4*, have been shown to cause an inability to derepress at least *INO1* and *CHO1* (5; 6). The positively acting regulatory gene *INO4* has been recently sequenced (7) and proposed to encode a member of the basic helix-loop-helix (bHLH) family of DNA-binding proteins. A similar structural motif has been described for the gene product of *INO2* (8). Interestingly, the UAS_{FAS} element contains the CANNTG core motif common to all so far characterized binding sites of bHLH proteins (2; 9). Thus, it appears reasonable to consider Ino4p as a possible candidate necessary for UAS_{FAS}-dependent gene activation. In this paper, we used null mutants to characterize the influence of *INO4* on the expression of *FAS* genes. We show that *INO4* indeed is a *trans*-activator of *FAS1*

and *FAS2* responsible for about 50% of *FAS* gene activation. In addition to its role as a regulator of phospholipid biogenesis, *INO4* is itself subject to inositol/choline repression, thus creating an autoregulatory circuit leading to amplification and maintenance of the regulatory signal.

MATERIALS AND METHODS

Strains and media

The following strains of *Saccharomyces cerevisiae* were used in this work: JS89.27-3 (*MATα* ura3 leu2 trp1 can1 MAL3 SUC3 INO4), RSH3 (*MATα* ura3 leu2 trp1 can1 MAL3 SUC3 Δ*ino4::LEU2*), C13-ABY.S86 (*MATα* ura3 leu2 his pra1 prb1 prc1 cps1 INO4; 10), RSH2 (*MATα* ura3 leu2 his pra1 prb1 prc1 cps1 Δ*ino4::LEU2*) and JS91.11-32 (*MATα* ura3 leu2 ino1; obtained by back-crossing of strain MC3, received from Yeast Genetic Stock Center; Berkeley, CA, USA). Plasmids were amplified in *E.coli* strain RR1 (11). Yeast transformants were grown in selective synthetic complete media (SCD; 12). To allow growth of Δ*ino4* mutants without causing inositol/choline repression in correspondingly cultivated wild type cells, media were brought to 3 μM inositol + 3 μM choline (derepressed growth conditions). On the other hand, inositol/choline repression was achieved by 200 μM inositol + 2 mM choline.

Enzyme assays

For enzyme assays, yeast cells were harvested in the mid-log phase ($2-3\times10^7$ cells/ml), washed and stored at −20°C until used. Protein extracts were prepared by cell disruption with glass beads. Fatty acid synthase activity was determined according to Lynen (13) by measuring the oxidation of NADPH at 366 nm. β-Galactosidase was assayed as previously described (2). Assays with *INO4-lacZ* transformants were performed using turbid cell homogenates to prevent removal of a considerable part of nuclear protein associated with cellular debris. Assay mixtures were cleared by centrifugation prior to photometric determination of absorbance.

Yeast transformation

Yeast transformation was performed according to (14) with slight modifications (2). Transformants were grown under selective conditions on appropriate omission media. If required, plates were supplemented with 50 μM inositol + 50 μM choline.

Isolation of *INO1* and *INO4*

INO1 was cloned by complementation of the inositol auxotrophy in strain JS91.11-32 (*ura3 ino1*). After transforming the recipient strain with a YEp24-based gene library (15), Ura^+ transformants were collected and spread onto a selective medium lacking inositol. Plasmid DNA was isolated from Ura^+ Ino^+ transformants according to (16). From plasmid YEp24-INO1 thus obtained, a 2.8 kb *Pst*I/*Kpn*I fragment was subcloned into YEplac181 (17) giving pJS302. This plasmid was also able to complement the *ino1* mutation in strain JS91.11-32, in agreement with subcloning data previously described (18). Partial DNA sequencing finally proved the identity of the cloned DNA with the *INO1* gene.

INO4 was isolated from the YEp24 gene library by colony hybridization (11) with an end-labelled 22-meric synthetic oligonucleotide derived from the published sequence (7). Positive clones YEp24-INO4.1 and YEp24-INO4.2 contained overlapping sequences in agreement with the restriction map of *INO4*. Authenticity of the cloned *INO4* gene was confirmed by DNA sequencing.

Construction of Δ*ino4* null mutants

A 1.3 kb *Kpn*I/*Sma*I fragment from YEp24-INO4.1 carrying the complete *INO4* reading frame together with about 400 bp of the noncoding region on both sides was subcloned into pBlueskript KS (Stratagene) giving pRS1. A 320 bp *Hin*dIII fragment representing the N-terminal half of Ino4p was substituted by a 2.8 kb *LEU2* restriction fragment (see Fig. 1 b). The resulting plasmid pRS5 contains the desired Δ*ino4::LEU2* construction. Prior to integrative transformation of *INO4 leu2* strains, a 3.8 kb *Kpn*I/*Sma*I fragment was released from pRS5. Transformants exhibiting the Δ*ino4::LEU2* gene disruption were selected on SCD-Leu medium, supplemented with 50 μM inositol + 50 μM choline. The inositol-requiring phenotype of Δ*ino4::LEU2* disruptants could be complemented by transformation with pJS314 (*LEU2* 2μm INO4), which was obtained by transfer of the 1.3 kb *Kpn*I/*Sma*I *INO4* fragment into YEplac181 (17).

Recombinant DNA and plasmid constructions

All DNA manipulations followed established protocols (11; 12). Reporter gene plasmids pJS200 (*FAS1-lacZ*), pJS203 (*FAS2-lacZ*), pJS234N (UAS_{RPG}-*CYC1-lacZ*), pJS246N (UAS_{FAS1}[−860]-CYC1-lacZ), pJS263N (UAS_{INO1}[−240]-CYC1-lacZ), pJS264N (UAS_{FAS1}[−700]-CYC1-lacZ) and pFT3N (UAS_{FAS2}[−230]-CYC1-lacZ) have been previously described (2). To obtain an *INO1-lacZ* fusion, a 1.0 kb *Pst*I/*Bgl*II fragment from pJS302 was subcloned into *Pst*I/*Bam*HI cleaved YEp357R (19). The resulting plasmid pJS325 (*INO1-lacZ* 2μm URA3) contains about 550 bp of the *INO1* upstream region together with the first 157 codons fused in frame to *lacZ*. Similarly, the *INO4-lacZ* fusion in pJS345 (see Fig. 1 c) was constructed by insertion of a 720 bp *Kpn*I/*Hin*dIII fragment from pRS1 into YEp357 (19), digested with the appropriate enzymes.

Northern hybridization

RNA was isolated from mid-log phase cultures as previously described (20). 20 μg of total RNA were size-fractionated in a vertical 1% agarose gel containing 2.2 M formaldehyde. Transfer of RNA to a nylon membrane (Pall Biodyne B) and subsequent hybridization using nick-translated probes followed established procedures (11).

EMSA experiments

Preparation of protein extracts and electrophoretic mobility shift assays (EMSA) were performed as previously described (2). Yeast strains grown in various media were harvested at a density of about 5×10^7 cells/ml. The extracts used in the binding reaction represent total cellular protein of the 10−60% $(NH_4)_2SO_4$ fraction.

RESULTS

Influence of a Δ*ino4* null mutation on *FAS* gene expression

The identification of two UAS_{FAS} sequence elements in the upstream region of *INO1* suggested a common *trans*-factor involved in expression of the *INO1* and *FAS* genes. Two regulatory genes, *INO2* and *INO4*, have been described to be indispensable for *INO1* expression (21; reviewed in 22). We cloned the *INO4* gene by colony hybridization using an oligonucleotide designed according to its published sequence (7).

Table 1. Expression of *lacZ* fusion gene constructs in wild type strain JS89.27-3 (*INO4*) and Δ*ino4* mutant RSH3.

Plasmid	Reporter construct[a]	Specific β-galactosidase activities[b] (U/mg)		
		INO4, derep.[c]	*INO4*, rep.[d]	Δ*ino4*. derep.[c]
pJS200	*FAS1-lacZ*	1260	950	610
pJS203	*FAS2-lacZ*	900	700	440
pJS325	*INO1-lacZ*	420	10	5
pJS345	*INO4-lacZ*	20	6	4
pJS205	Δ*UAS-CYC1-lacZ*	14	14	15
pJS246N	UAS_{FAS1}[−860]-*CYC1-lacZ*	550	60	45
pJS264N	UAS_{FAS1}[−700]-*CYC1-lacZ*	1640	60	20
pFT3N	UAS_{FAS2}[−230]-*CYC1-lacZ*	580	50	20
pJS263N	UAS_{INO1}[−240]-*CYC1-lacZ*	650	65	50
pJS234N	UAS_{RPG}[−860]-*CYC1-lacZ*	240	260	270

[a] Reporter constructs pJS246N, pJS264N, pFT3N, pJS263N and pJS234N, respectively, were derived from pJS205 by insertion of the following oligonucleotide sequences into a XhoI site at −245 (with respect to the translational start site) of the Δ*UAS-CYC1-lacZ* fusion (see also 2): pJS246N: ACGGCAGCATGTGAAAAAACCCGTAGAAGGTCCGCA (nucleotides −873/−838 from *FAS1*; oligonucleotide FBF12); pJS264N: AACTTCACATGCCG (nucleotides −709/−696 from *FAS1*); pFT3N: GCGTTTTCACATGCTACCTCA (nucleotides −239/−219 from *FAS2*); pJS263N: AATGCGGCATGTGAAAAGT (nucleotides −250/−232 from *INO1*; oligonucleotide FBF56); pJS234N: AACACCCATACATTT (Rap1p consensus binding site; 23).

[b] Specific β-galactosidase activities are given in nmol oNPG hydrolyzed per minute per mg of protein. Data presented are mean values from at least ten independent transformants.

[c] For a direct comparison of wild type and Δ*ino4* mutant, transformants were grown under selective conditions (SCD-Ura) at a final concentration of 3 μM inositol + 3 μM choline. This addition allows sufficient growth of the Δ*ino4* mutant without causing inositol/choline repression in the wild type.

[d] To achieve inositol/choline repression, transformants were grown in SCD-Ura, brought to 200 μM inositol + 2 mM choline.

To obtain a Δ*ino4* null mutation, a 320 bp HindIII fragment representing the N-terminus of Ino4p was replaced by the *LEU2* gene (Fig. 1 b). Yeast Leu$^+$ transformants of *leu2* recipient strains had lost the ability to grow without inositol, a deficiency which could be complemented upon introduction of YEp24-INO4.1. Southern hybridization experiments confirmed the intended gene disruption at the chromosomal level (not shown).

To investigate the influence of a functional *INO4* gene on the expression of *FAS1* and *FAS2*, we transformed various *lacZ* fusion constructs into the Δ*ino4* disruptant strain RSH3 (*ura3* Δ*ino4::LEU2*) and into the isogenic wild type strain JS89.27−3. Plasmids pJS200, pJS203 and pJS325 carrying gene fusions *FAS1-lacZ*, *FAS2-lacZ* and *INO1-lacZ*, respectively, were designed to reflect the effect of *ino4* deficiency on the activating potential of the respective *INO1* and *FAS* control regions. In contrast, plasmids pJS246N, pJS264N, pFT3N and pJS263N contain insertions of the four activating sequence elements UAS_{FAS1}[−860], UAS_{FAS1}[−700], UAS_{FAS2}[−230] and UAS_{INO1}[−240], respectively, into the Δ*UAS-CYC1-lacZ* promoter test system pJS205 (2). As a control, a similar plasmid (pJS234N) containing the unrelated UAS_{RPG} insertion (binding site of repressor/activator protein Rap1p; 23) was used. Wild type transformants were grown in selective media, either under conditions of inositol/choline repression (200 μM inositol, 2 mM choline) or in the presence of 3 μM inositol and 3 μM choline allowing growth of Δ*ino4* mutants without causing repression of reporter gene expression in the *INO4* strain. The specific β-galactosidase activities obtained for the various test plasmids under different genetic and physiological conditions are shown in Table 1. In the absence of a functional *INO4* gene, expression of both *FAS-lacZ* gene fusions becomes reduced to about 50% while *INO1* expression is almost completely abolished. In accordance with published data (summarized in 22), inositol/choline strongly repressed *INO1* expression even in wild type cells while the two *FAS* genes were only weakly influenced by these conditions. In contrast, the 11 bp UAS_{FAS} sequence motifs transferred to the heterologous Δ*UAS-CYC1-lacZ* context dramatically responded to both inositol/choline repression and to

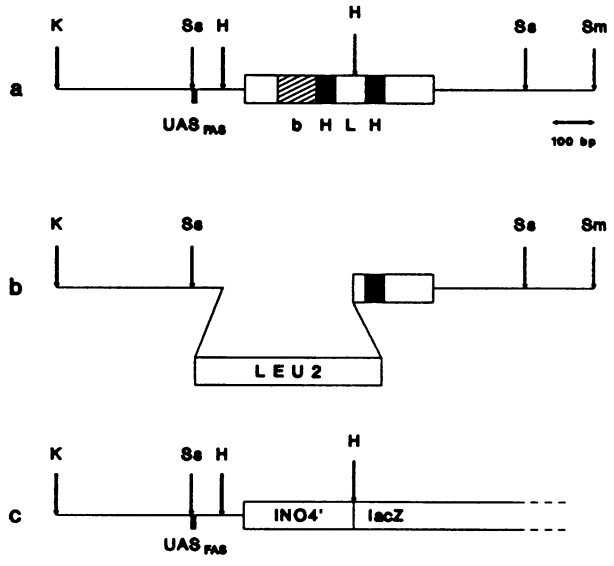

Figure 1. Construction of the Δ*ino4::LEU2* gene disruption (b) and the *INO4-lacZ* gene fusion (c). In (a), the *INO4* reading frame (open bar) is shown indicating the helical (black) and basic (hatched) regions of the bHLH domain in Ino4p. UAS_{FAS} depicts the position of the activating motif presumably responsible for *INO4* autoregulation. Abbreviations: H, *Hin*dIII; K, *Kpn*I; Sm, *Sma*I; Ss, *Ssp*I.

the Δ*ino4* mutation. This result clearly shows that inositol/choline repression is mediated by the UAS_{FAS} site with Ino4p serving as a *trans*-activator in the absence of inositol and choline. Under all conditions tested, the control plasmid pJS234N carrying the UAS_{RPG} element led to similar β-galactosidase activities.

Our studies on *FAS* gene expression in wild type and isogenic Δ*ino4* mutant strains were extended by measuring *FAS* transcript levels and specific FAS activities as elicited by intact chromosomal *FAS1* and *FAS2* genes rather than by extrachromosomal reporter constructs. Fig. 2 presents Northern blot hybridizations of total RNA isolated from wild type and Δ*ino4* mutant cells when probed against *FAS1* (Fig. 2 a) and

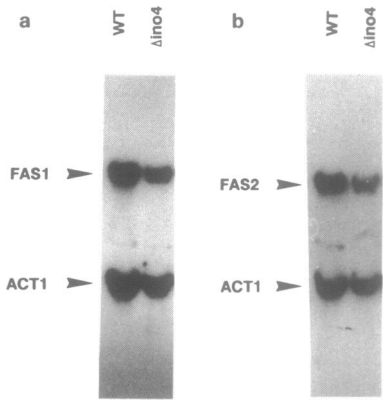

Figure 2. Northern blot hybridization of *FAS* transcripts in wild type (WT) and *Δino4* mutant. Strains used for RNA isolation were grown in SCD + 3 μM inositol/choline until mid-log phase. A nick-translated 2.3 kb *Pvu*II fragment representing the 3'-terminal part of the *FAS1* transcript was used as a hybridization probe (a). Correspondingly, the *FAS2* mRNA was probed against a 2.3 kb *Eco*RV fragment representing the 3'-end of *FAS2* (b). Yeast actin mRNA hybridized against a 1.0 kb *Hin*dIII/*Xho*I fragment from *ACT1* served as an internal reference.

Table 2. Specific FAS activities measured in a wild type strain and a *Δino4* mutant.

Strain[a]	Genotype	Spec. FAS activities (mU/mg prot.)[b]		
		YEPD	SCD,rep.[c]	SCD,derep.[c]
C13-ABY.S86	wild type	14.3	19.8	28.6
RSH2	*Δino4*	7.1	13.8	14.6

[a] Strains are deficient in vacuolar proteinases yscA, yscB, yscY and yscS (10).
[b] Specific FAS activities in mU indicate nmol NADPH oxidized per minute per mg of protein. Mean values from three independent assays are presented.
[c] Repressed and derepressed growth conditions were as defined in 'Materials and Methods'.

FAS2 (Fig. 2 b). Using the hybridization signal of actin mRNA as an internal standard, the steady state levels of both *FAS* transcripts were reduced to about 50% in the *Δino4* mutant with respect to the wild type (densitometric analysis not shown). A similar result was obtained when the specific FAS activities in both strains were compared (Table 2). Again, only a moderate reduction of specific FAS activity was observed under conditions of inositol/choline repression. Interestingly, an additional and *INO4* independent reduction of FAS activity by another 50% was observed with protein extracts from cells grown in rich media, when compared to those grown in synthetic media.

Taken together, these results show that about half of the activation potency of both *FAS* genes depends on a functional *INO4* gene. However, growth of a *Δino4* mutant does not require exogenously added fatty acids and, thus, the *FAS* promoters obviously guarentee, even in the absence of *INO4*, a gene expression rate sufficient for appropriate *de novo* synthesis of fatty acids.

Gene dosage studies with *INO4* multi-copy transformants

The molecular properties of Ino4p as predicted by its amino acid sequence suggested it to be a member of the basic helix-loop-helix family of DNA binding regulatory proteins. Thus, gene dosage-dependent overexpression of *INO4* might cause similar dosage effects on its target genes, as it has been previously shown for other DNA-binding proteins such as *GAL4* (24) or *GCN4*

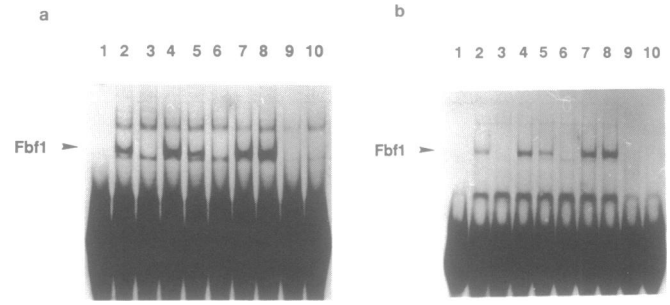

Figure 3. EMSA experiments with UAS_{FAS1} (a) and UAS_{INO1} (b) probes, respectively. Binding reactions contained 10000 cpm (about 1 ng) of ^{32}P-labelled oligonucleotide fragments FBF12 (=UAS_{FAS1}[−860]) or FBF56 (=UAS_{INO1}[−240]) and 10 μg of total cellular protein. For sequences of oligonucleotides FBF12 and FBF56, respectively, see also the legend to Table 1. Protein extracts were prepared from wild type strain C13-ABY.S86 (proteinase-deficient) and its isogenic *Δino4* counterpart RSH2, grown under various conditions of inositol/choline availability. Lanes 1: no protein added; lanes 2: wild type extract, grown in YEPD; lanes 3: *Δino4* mutant extract, grown in YEPD; lanes 4: wild type extract, grown in SCD (derepressed conditions); lanes 5: wild type extract, grown in SCD + 200 μM inositol + 2 mM choline (repressed conditions); lanes 6: *Δino4* mutant extract, grown in SCD + 3 μM inositol/choline; lanes 7: extract from wild type (transformed with YEp24), grown in SCD-Ura; lanes 8: extract from wild type (transformed with YEp24-INO4.1), grown in SCD-Ura; lanes 9 and 10: like lanes 4, with addition of 100-fold molar excess of unlabelled FBF12 and FBF56, respectively.

(25). With *INO4*, however, the specific FAS activity observed in multi-copy YEp24-INO4.1 transformants (*URA3* 2μm INO4), was enhanced to only 110%, when compared to vector-transformed cells (not shown). Similarly, the expression of reporter plasmids containing the UAS_{FAS} motif in combination with *lacZ* was only slightly increased (about 10%) upon co-transformation with an *INO4* multi-copy plasmid (data not shown). These results suggest that *INO4* represents a necessary though obviously not sufficient regulatory component in UAS_{FAS}-mediated gene expression.

Influence of *INO4* on UAS_{FAS}-mediated protein-DNA interactions

Electrophoretic mobility shift assays (EMSA) employing UAS_{FAS}-containing oligonucleotides previously revealed a protein-DNA complex comprising Fbf1 (*FAS* binding factor 1). The UAS_{FAS}-Fbf1 complex could be competed by an excess of unlabelled UAS_{INO1} element (2). These data together with the characteristics of the *Δino4* null mutation suggested a possible function of *INO4* for the formation of Fbf1-dependent gel retardation complexes. As shown in Fig. 3, the Fbf1-containing protein-DNA complexes involving both oligonucleotide probes FBF12 (=UAS_{FAS1}[-860]; Fig. 3 a, lanes 3 and 6) and FBF56 (=UAS_{INO1}[-240]; Fig. 3 b, lanes 3 and 6) were absent when protein extracts from the *Δino4* mutant were used. Thus, the derepression deficiency of UAS_{FAS}-containing reporter constructs in *Δino4* mutants obviously coincides with the lack of a corresponding gel retardation complex.

In order to uncover the effect of inositol/choline on Ino4p-dependent derepression of UAS_{FAS}-containing reporter constructs, we studied the influence of growth conditions on EMSA signal intensities. Indeed, the signal of the Fbf1-dependent gel retardation complex is definitively more intensive with extract from derepressed cells (Fig. 3 a, b, lanes 4) than with those

obtained under repressing conditions (Fig 3 a, b, lanes 2 and 5). Overexpression of *INO4* on a multi-copy plasmid results in a further slight increase of the respective signal intensity (Fig 3 a, b, lanes 8). The latter result may indicate that inositol/choline repression is mediated, at least in part, by the control of the DNA-binding activity of Fbf1 via the supply with phospholipid precursors. The possible significance of the two additional retardation signals observed with FBF12 in Fig. 3 a has been discussed previously (2).

Positive autoregulation of *INO4*

Inositol/Choline repression reducing the amount of detectable Fbf1/UAS_{FAS} gel retardation complex may be explained easily by assuming a biosynthetic control of the protein component(s) involved. Alternatively, even at a constant cellular level of Fbf1, its DNA-binding characteristics may be modulated. In order to distinguish between these alternatives, we studied the effect of distinct growth conditions and genetic backgrounds on the expression of an *INO4-lacZ* fusion, pJS345, containing the upstream region up to −450 together with the first 90 codons of *INO4* fused to *lacZ* (see Fig. 1 c). Using this construct, the activity of the *INO4* control region itself turned out to be subject to inositol/choline repression, exhibiting 3-fold derepression in cells grown at non-repressing inositol/choline concentration (Table 1). This ratio is in good agreement with the results of the EMSA experiments shown in Fig. 3 and may therefore explain the diminished signal intensities of gel retardation complexes observed with extracts from repressed cells. It remains to be shown whether the low specific β-galactosidase activity observed in *INO4-lacZ* transformants even under derepression conditions (only 5% of the corresponding activity obtained with *INO1-lacZ* transformants; Table 1) is due to the absence of a canonical TATA element in the *INO4* promoter region.

Interestingly, the expression of the *INO4-lacZ* fusion requires a functional *INO4* gene (Table 1) and is ineffective in the Δ*ino4* null mutant (the *INO4-lacZ* fusion in pJS345 containing only a part of the *INO4* reading frame does not complement the Δ*ino4* null mutation by itself). This finding suggesting a positive regulatory feedback of *INO4* on its own expression is further supported by the identification of an UAS_{FAS}-like upstream element, 5′ TATTCACATGT 3′, in the *INO4* control region (positions −125/−115 with respect to the translational start codon). This element is presumably a target of the *INO4* dependent *FAS* binding factor 1, too.

DISCUSSION

Fatty acid synthase, considered as a 'house keeping' enzyme in most organisms, provides the cell with fatty acids necessary for phospholipid biosynthesis and acylation reactions. In the course of our studies on the regulation of yeast fatty acid synthase, we recently identified a sequence motif, UAS_{FAS} (2), which is involved in the efficient expression of yeast fatty acid synthase genes, *FAS1* and *FAS2*. In this paper, we now describe the regulatory gene *INO4* as one of the *trans*-acting components of this UAS_{FAS}-mediated gene expression. Although UAS_{FAS}-controlled *lacZ* reporter constructs indicated a severe drop of β-galactosidase expression in Δ*ino4* disruptant cells, overexpression of *INO4* caused no significant increase in reporter gene expression. This suggested *INO4* to be a necessary though not sufficient component of the UAS_{FAS}-mediated activation system. As supposed by Nikoloff et al. (8), the bHLH protein Ino4p may be involved in heterodimer formation with Ino2p, another member of this family of DNA-binding proteins. This aggregation could explain the inadequacy of *INO4* overexpression alone for UAS_{FAS}-dependent gene activation. The identity of Ino4p with the UAS_{FAS} binding factor Fbf1, or at least with a component thereof, is strongly suggested by the presence of a CANNTG motif within UAS_{FAS}. This motif was found in the core of several so far described bHLH binding sites (9). However, as long as this identity is not confirmed by more direct (e. g. immunological) evidence, an indirect effect of *INO4* on the functioning or on the biosynthesis of Fbf1 cannot be ruled out.

Upon computer-aided analysis of the upstream regions of several phospholipid biosynthetic genes, Carman and Henry (26) postulated a 9 bp sequence ('nonamer motif': 5′ ATGTGAAAT 3′) as a common regulatory element in these genes. The reverse complement of this nonamer actually represents an incomplete form of the 11 bp UAS_{FAS} consensus sequence defined by our studies. In contrast to the UAS_{FAS} element, however, the nonamer motif failed to activate transcription of a heterologous reporter gene (3). As we have shown previously, this incompetence may be overcome by using an extended element which then functions quite efficiently as UAS_{FAS} (2). At least the addition of a single G to the 3′-end of the nonamer appears to be indispensable, as this will restore the CANNTG core motif of bHLH DNA-binding sites (9). For the same reason, we propose that only two of the seven nonamer repeats identified in the yeast *INO1* upstream region (3) are of regulatory importance. In accordance with the functional inefficiency of the isolated nonamer motif, the nonamer binding factor (NBF) described in (27) was unaffected by mutations in any of the known regulatory genes of phospholipid biosynthesis, i. e. *INO2*, *INO4* or *OPI1*. In contrast, the EMSA experiments which we performed with an oligonucleotide containing the complete CANNTG core motif of one of the *INO1* upstream elements clearly exhibited an *INO4* dependency (Fig. 3 b).

In EMSA experiments using chromosomal fragments of *INO1* comprising the two CANNTG-containing UAS_{INO1} upstream elements (see above), Lopes and Henry (27) observed the disappearence of corresponding retardation complexes upon mutation of either one of the two regulatory genes, *INO2* or *INO4*. These results are in good agreement with our hypothesis that Ino2p and Ino4p are *trans*-acting factors cooperating in UAS_{FAS}-dependent gene activation. Heterodimer formation between Ino2p and Ino4p may reasonably be assumed to occur by the hydrophobic interaction of the amphipathic α-helical stretches of the two bHLH proteins. This would be analogous to the mammalian tissue-specific regulators E12/MyoD, E47/MyoD (E12 and E47 representing alternative splice products of the E2A gene primary transcript; 28) and c-myc/Max (29). As would be expected from this hypothesis, formation of an *INO2/INO4* dependent complex was not observed with a chromosomal *INO1* DNA fragment containing an incomplete CANNTG motif (27).

An UAS_{FAS}-like octamer motif, 5′ TTCAYATG 3′, has been described also by Kodaki et al. (30; 31) in the upstream regions of genes *PEM1*, *PEM2* (= *CHO2*, *OPI3*; encoding phospholipid N-methyltransferases) and *PSS1* (= *CHO1*, encoding phosphatidylserine synthase). Obviously, this motif is completely contained within UAS_{FAS}. However, the octamer was found to be a weak UAS element exhibiting only 4-fold transcriptional activation in a heterologous context. On the other hand, the undecameric UAS_{FAS}-element of this study provided a

40–100-fold stimulation depending on which of the known variants of the TYTTCACATGY consensus sequence was tested (cf. Table 1). Thus, the sequence context around the CANNTG core obviously influences the activation potential decisively and, as is evident from previously published data (2), an element which matches the consensus sequence best confers not necessarily a maximum of gene activation. Exact knowledge on the optimal length and sequence properties of UAS_{FAS} should thus be obtained by saturation mutagenesis studies.

A puzzling feature of several UAS_{FAS}-controlled structural genes of phospholipid biosynthesis is their quite different response to exogenously added inositol/choline. While *INO1* (5; this study) and *CHO1* (6) show repression factors of 30 and 4–5, respectively, expression of the two *FAS* genes is almost unaffected by the two phospholipid precursors (cf. Table 1). Nevertheless, the UAS_{FAS}-elements *per se* proved to be highly responsive to inositol/choline repression when placed in a heterologous context. This demonstrates that inositol/choline repression is an inherent feature of the UAS element itself. With respect to the mechanism of this regulation, it was interesting to note that actually the amount of *INO4* dependent Fbf1 protein-DNA complex was affected by inositol/choline repression. A similar result has been also reported for the *INO2/INO4* dependent retardation complex observed with a fragment from the *INO1* promoter carrying both CANNTG nonamer elements (27). Among several means which may account for this effect, the following three models appear to be of particular interest: Firstly, the hypothetical Ino2p/Ino4p complex may be dissociated by an inhibitory HLH (iHLH) protein being triggered in some way by the phospholipid precursor signal and consequently leading to the loss of DNA binding activity. iHLH proteins such as Id1, Id2 (32; 33) and emc (34) have been shown to lack the basic motif necessary for DNA binding. Thus, bHLH/iHLH heterodimers are unable to interact with DNA. Another possibility would be the covalent interconversion of regulatory proteins by reversible phosphorylation. This represents a general theme not only in metabolic but also in eukaryotic gene regulation (reviewed in 35). Phosphorylation of transcriptional activators has been shown for Gal4p (36), yeast heat shock transcription factor (encoded by *HSF1*; 37) and the mammalian CREB family (cAMP responsive element binding factor; 38). However, in all these cases phosphorylation affects the functional rather than the DNA binding properties of the respective activator proteins. Interestingly, potential phosphorylation sites with protein kinase C or casein kinase II specificities were identified within the Ino4p sequence (7). This view is further supported by the recent analysis of the *IRE1* gene necessary for inositol biosynthesis. The sequence of *IRE1* predicts a putative protein kinase with a membrane-spanning region resembling the general structure of the EGF receptor (39). Further work is required to elucidate the role of *IRE1* in the inositol/choline regulatory pathway. Finally, reduced amounts of the *INO4* dependent gel retardation complex may also be due to the biosynthetic regulation of the regulator itself. This has been described for the *GCN4* encoded activator of yeast amino acid biosynthetic genes which is controlled at the translational level (reviewed in 40) and the glucose-repressible Gal4p activator (41). Using an *INO4-lacZ* gene fusion, we have shown in this study, that the *INO4* regulator itself is repressed by inositol/choline at the biosynthetic level. The presence of an UAS_{FAS}-like activation motif in the *INO4* upstream region together with the *INO4* dependent expression of an *INO4-lacZ* fusion strongly suggests a positive autoregulatory loop as a key element in the inositol/choline-controlled signal transduction pathway of yeast phospholipid biosynthesis. In support of this idea, a similar sequence is also present in the upstream region of the *INO2* regulatory gene (5′ AATTCACATGT 3′; pos. −135/−145 with respect to the translational start codon; 8; unpublished). The proposed mechanism might contribute to a self-amplifying activation response following moderate alterations of a signal input of currently unknown origin. Similar autoregulatory circuits have been described for several mammalian gene activators like c-jun (42), MyoD (43), Pit-1/GHF-1 (44) and the estrogen receptor (45). To our knowledge, autoregulation of *INO4* is the first example demonstrated in yeast, although a positive feedback for the expression of the ARS binding factor 1 / Bidirectional activating factor 1 gene *ABF1/BAF1* was inferred from several potential Abf1/Baf1 binding sites in the upstream region of this gene (46).

Although the primary target and the molecular details of inositol/choline-dependent gene regulation still remain to be elucidated, the reduction of *INO4* expression mediated by these metabolites may sufficiently explain the experimentally observed decrease of UAS_{FAS}-containing retardation complexes, under repressive conditions. Nevertheless, the differential response of individual phospholipid biosynthetic genes to inositol/choline repression indicates that additional regulatory means may complement or modulate this effect. This concerns especially the two fatty acid synthase genes, *FAS1* and *FAS2*, which are only marginally affected by inositol/choline repression. Accordingly, *INO4* contributes only to about half of the *FAS* gene activation potential and, consequently, *Δino4* mutants are not auxotrophic for external fatty acids. Thus, expression of the yeast *FAS1* and *FAS2* genes obviously follows several different routes of transcriptional activation, including the autoregulation of one of the regulators.

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