DNA binding site of the yeast heteromeric Ino2p/Ino4p basic helix-loop-helix transcription factor: structural requirements as defined by saturation mutagenesis

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Abstract The inositol/choline-responsive element (ICRE) is an 11 bp *cis*-activating sequence motif with central importance for the regulated expression of phospholipid biosynthetic genes in the yeast *Saccharomyces cerevisiae*. The ICRE containing the CANNTG core binding sequence (E-box) of basic helix-loop-helix (bHLH) regulatory proteins is recognized by the heteromeric bHLH transcription factor Ino2p/Ino4p. In this study, we define the Ino2p/Ino4p consensus binding sequence (5′-WYTTCAYRTGS-3′) based on the characterization of all possible single nucleotide substitutions. Interestingly, this analysis also identified a single functional deviation (CACATTC) from the CANNTG core recognition element of bHLH proteins. The DNA binding specificities of different yeast bHLH proteins may now be explained by distinct nucleotide preferences especially at two positions immediately preceding the CANNTG core motif.

Key words: bHLH transcription factor; Phospholipid biosynthesis; Saturation mutagenesis; UAS element; *Saccharomyces cerevisiae*

1. Introduction

In the yeast *Saccharomyces cerevisiae*, many structural genes of phospholipid biosynthesis are coordinately regulated by the availability of the precursor molecules inositol and choline in the growth medium (reviewed in ref. [1]). Our studies on the transcriptional control of fatty acid synthase genes *FAS1* and *FAS2* led to the identification of a UAS element (upstream activation site) apparently involved in this general regulatory mechanism [2]. The respective UAS sequence was designated as inositol/choline-responsive element (ICRE) since gene activation mediated by this motif turned out to be synergistically repressed by the two phospholipid precursors, inositol and choline [2]. The ICRE functions as a binding site for the transcription factor Fbf1 which is composed of the two basic helix-loop-helix DNA-binding proteins, Ino2p and Ino4p [3,4]. After the identification of a common sequence motif, CANNTG, in the core of almost all known bHLH binding sites (E-box), it remained to be shown how different bHLH proteins distinguish between their individual targets. For the two yeast bHLH proteins Pho4p and Cpf1p both binding to the same central CACGTG element, nucleotides flanking this core motif were shown to be essential for their recognition specificity [5]. *PHO4* encodes a transcriptional activator of genes involved in phosphate supply, while *CPF1* functions as a centromere DNA binding factor and is, in addition, required for methionine biosynthesis. However, not only the peripheral positions but also the central part of the CANNTG element contributes to differential bHLH protein/DNA-interactions, as it was shown for the Myc/Max (binding to CACGTG; [6]) and MyoD proteins (preferred binding to CAGCTG; [7]).

From naturally occurring ICRE-like motifs as identified upstream of various phospholipid biosynthetic genes, we derived the preliminary consensus sequence, 5′-TYTTCACATGY-3′. Other authors have suggested distinct variants of this ICRE to function as UAS elements. For instance, the octamer motif TTCAYATG was described as a UAS element in the phosphatidylserine synthase gene (*PSS1/CHO1*) promoter [8]. Actually, this motif is entirely contained within the above ICRE sequence. Similarly, several copies of the nonamer element, ATTTCACAT, were proposed to function as regulatory sites upstream of the inositol-1-phosphate synthase gene *INO1* [9]. In order to characterize the minimal structural requirements of a functional ICRE with respect to its length and nucleotide sequence, we subjected this element to a saturation mutagenesis. As will be reported in this study, the data thereby obtained define, by functional criteria, an ICRE consensus sequence and quantify the relevance of distinct sequence variants.

2. Materials and methods

2.1. Yeast strains and media

The *Saccharomyces cerevisiae* wild-type strain JS91.15-23 (*MATα ura3 leu2 his3 trp1 can1 MAL3 SUC3*) and the isogenic *Δino2::LEU2* mutant SS92.3-1 were used as recipients for transformation with reporter plasmids. Synthetic complete media (SCD) suitable for the selection of yeast transformants have been described [3]. Growth of transformants of the *ino2* mutant was supported by the addition of a non-repressing amount of inositol+choline (final concentration 5 μM each).

2.2. Construction of ICRE sequence variants

ICRE sequence variants were generated by the insertion of double-stranded synthetic DNA fragments derived from the reference sequence 5′-tcgagaaTTTTCACATGCagatct-3′ (capital letters) into the *Xho*I restriction site of reporter plasmid pJS205 (*ΔUAS-CYC1-lacZ URA3* 2 μm; [2]). Chemically synthesized oligonucleotides (MWG Biotech; Ebersberg, Germany) were mutated by using, at successively shifted dinucleotide positions, the four different nucleotide precursors with equal probability. The complementary strand was synthesized correspondingly. Annealing of both oligonucleotides created cohesive ends suitable for insertion into an *Xho*I site. The constructs were amplified in *E. coli* and characterized by DNA sequencing. A few variants not obtained by this method were synthesized, specifically.

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Abbreviations: bHLH, basic helix-loop-helix; CDE, centromere DNA element; ICRE, inositol/choline-responsive element; UAS, upstream activation site.

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2.3. Miscellaneous procedures

Procedures of recombinant DNA, yeast transformation, preparation of protein extracts and β-galactosidase assay have been described previously [3].

3. Results and discussion

3.1. Importance of individual nucleotides for ICRE activity and specificity

In order to distinguish between functional and non-functional ICRE variants, a saturation mutagenesis of the highly efficient, naturally occurring ICRE sequence 5′-TTTTCACATGC-3′ was performed. The experimental strategy was based on the analysis of reporter gene activation upon insertion of the mutated synthetic DNA fragments into a ΔUAS-*CYC1-lacZ* promoter test plasmid [10]. This reporter construct allows by itself only basal gene expression and may be activated about 40-fold by insertion of the functional ICREs present in the *FAS1*, *FAS2* or *INO1* promoters [2]. The sequence variants analysed in this study exhibited single nucleotide substitutions at positions N_1–N_{11} of the above sequence. The activation factors observed with 44 different sequence variants in a *S. cerevisiae* wild type strain are listed in Fig. 1. Quite generally, stimulation of basal gene expression was, with all ICRE variants tested, independent of their orientation (not shown). The synergistic effect of more than one ICRE in the same promoter has already been reported previously [2]. In order to rule out an unspecific gene activation by factors other than Ino2p/Ino4p which may bind to some of the sequences artificially created by the mutagenesis procedure, all insertion constructs showing at least 5-fold activation rates in the wild type were also transformed into an *ino2* null mutant. In this mutant, ICRE-dependent gene activation is specifically abolished [4]. It was found that in all cases functional ICRE variants were indeed inactive in the *ino2* mutant. In contrast, gene activation caused by a different UAS element (UAS_{RPG}=Rap1p binding site; [11,12]) remained unaffected by the *ino2* mutation (data not shown). In accordance with these results, the reporter constructs proved to be repressible by inositol+choline (not shown). Both kinds of control experiments confirmed for all ICRE variants tested the specificity of their regulation by the two phospholipid precursors and the dependence of this regulation on a functional *INO2* gene.

Table 1
Influence of selected double substitutions on ICRE-dependent gene activation

(a) Substitutions at positions N_7 N_8 (TTTTCAYRTGC):

Sequence	Activation factor
TTTTCACATGC	40
TTTTCACGTGC	20
TTTTCATATGC	24
TTTTCATGTGC	35

(b) Substitutions at positions N_{10} N_{11} (TTTTCACATTN):

Sequence	Activation factor
TTTTCACATTG	1
TTTTCACATTA	1
TTTTCACATTT	1
TTTTCACATTC	17
TTTTCACATGC	40
TTTTCACATGG	40

Activation factors were calculated as described in the legend of Fig. 1.

position	N_1	N_2	N_3	N_4	N_5	N_6	N_7	N_8	N_9	N_{10}	N_{11}
G	18	5	1	5	1	1	3	20	6	40	40
A	64	7	15	3	1	40	1	40	8	1	13
T	40	40	40	40	1	5	24	1	40	17	20
C	17	44	1	9	40	4	40	2	2	1	40
reference sequence	T	T	T	T	C	A	C	A	T	G	C
consensus sequence	W	Y	T	T	C	A	Y	R	T	G	S
optimal sequence	W	Y	T	T	C	A	C	A	T	G	S

Fig. 1. Activation factors determined for reporter constructs containing ICRE sequence variants. Plasmids carrying single nucleotide substitutions within the ICRE reference sequence upstream of a *CYC1-lacZ* reporter gene were transformed into *S. cerevisiae* wild-type strain JS91.15-23 [4]. Transformants were subsequently assayed for specific β-galactosidase activities. The activation factors were calculated with respect to the enzyme activity determined in pJS205 transformants (ΔUAS-*CYC1-lacZ* fusion construct). The ICRE consensus sequence was derived from sequence variants leading to a more than 20-fold gene activation. R: G or A; Y: C or T; W: A or T; S: G or C.

Obviously, position N_1 is not of critical importance but merely distinguishes between ICREs of high and intermediate strength; at N_2, however, a pyrimidine residue is clearly preferred to a purine. Similarly, functional ICREs should contain T residues at both positions, N_3 and N_4. Within the subsequent CANNTG core element (pos. N_5–N_{10}), only the sequence CAYRTG mediates strong gene activation (Table 1a lists the activation factors obtained for the 4 possible CAYRTG elements). Any deviation from this consensus leads to a significantly reduced efficiency. Thus, the core sequence CACGTG present in the Pho4p and Cpf1p binding sites should also allow recognition and effective activation by the Ino2p/Ino4p complex. According to the CANNTG rule of bHLH binding sites, no T nucleotide should be expected to be tolerated at position N_{10}. Nevertheless, it may be effectively used at this site provided that it is followed by C at N_{11} (Table 1b). Otherwise, position N_{11} resembles N_1 in being obviously not very critical, either. A similar deviation from the CANNTG rule has been observed for one of the Pho4p binding sites in the upstream region of the acid phosphatase gene *PHO5* [13]. While the bHLH protein Pho4p binds efficiently to a CACGTG core element (UAS_{P2}) of the *PHO5* promoter, the sequence CACGTT (UAS_{P1}) is recognized with only moderate affinity. Using a binding site selection technique, interaction of the c-Myc/Max heterodimer complex with non-canonical sites such as CACGCG, CACGAG and CACGTT could also be demonstrated [14]. In summary, the data of Fig. 1 allow the definition of two different ICRE consensus sequences, WYTTCAYRTGS and WYTTCACATGS, exhibiting intermediate (at least 20-fold) and maximal (at least 40-fold) transcriptional activation rates, respectively.

The results of the ICRE saturation mutagenesis clearly allow to discriminate between binding sites of the yeast bHLH proteins Pho4p and Ino2p/Ino4p. Binding of Pho4p to a CACGTG motif was shown to be strictly inhibited by a T residue 5′ to this core element (corresponding to position N_4 in our analysis; [5]). In contrast, binding of Cpf1p or Ino2p/Ino4p is definitely favoured by this arrangement. Thus, no interference between Pho4p and either Cpf1p or Ino2p/Ino4p is expected to occur,

Table 2
Compilation of *Saccharomyces cerevisiae* genes containing ICRE sequences in their upstream regions

Phospholipid biosynthesis:

Gene	Function	Sequence
FAS1	Fatty acid synthase, β subunit	**TTTTCACATGC**
FAS1	Fatty acid synthase, β subunit	**ACTTCACATGC**
FAS2	Fatty acid synthase, α subunit	**TTTTCACATGC**
ACC1	Acetyl-CoA carboxylase	**TCTTCACATGG**
FAA1	Acyl-CoA synthetase	TATTCACATGG
FAA1	Acyl-CoA synthetase	TCTTCATATTC
ACB1	Acyl-CoA binding protein	ATTTCACATGT
ITR1	Inositol permease	**TCTTCACATGC**
ITR1	Inositol permease	**TTTTCACATGC**
CTR1	Choline permease	**TTTTCACATGC**
INO1	Inositol-1-phosphate synthase	**TTTTCACATGC**
INO1	Inositol-1-phosphate synthase	AATTCACATGG
INO1	Inositol-1-phosphate synthase	ATTTCACATTC
INO1	Inositol-1-phosphate synthase	TCATCATATGC
CHO1	Phosphatidylserine synthase	CTTTCACATGG
CPT1	Diacylglycerol cholinephosphotransferase	**TTTTCACATGC**
PSD1	Phosphatidylserine decarboxylase	ATATCACATGC
PSD1	Phosphatidylserine decarboxylase	TTTCCACATGC
CKI1	Choline kinase	TATTCACATGG
PEM1	Phosphatidylethanolamine N-methyltransferase I	AATTCACATGT
PEM1	Phosphatidylethanolamine N-methyltransferase I	TTTTCATATGC
PEM1	Phosphatidylethanolamine N-methyltransferase I	TCTTCACATGA
PEM2	Phosphatidylethanolamine N-methyltransferase II	TTTCCACATGC
PEM2	Phosphatidylethanolamine N-methyltransferase II	TCTTCATATGC
PEM2	Phosphatidylethanolamine N-methyltransferase II	TCTTCATATGT
PIS1	Phosphatidylinositol synthase	ACTTCATATGC
SLC1	Long-chain acyl-CoA transferase	CCTTCATATGA
SLC1	Long-chain acyl-CoA transferase	GCTTCACATTC
GUT1	Glycerol kinase	**TTTTCACATGG**
URA8	CTP synthetase	TCTTCACATTC
INO2	Positive regulator of phospholipid biosynthesis	AATTCACATGT
INO4	Positive regulator of phospholipid biosynthesis	TATTCACATGT
OPI1	Negative regulator of phospholipid biosynthesis	TCTTCATATGC

Other functions:

Gene	Function	Sequence
ARG4	Argininosuccinate lyase	TTTTCACATGT
MET2	Homoserine O-transacetylase	TTTTCACGTGA
MET8	Unknown	ATTTCACGTGT
MET14	ATP:adenylylsulfate-3'-phosphotransferase	ATTTCACGTGA
MET16	3'-Phosphoadenylylsulfate reductase	ATTTCACGTGA
SAM2	S-adenosylmethionine synthetase	TCTTCACATGT
SAM2	S-adenosylmethionine synthetase	TCTTCATATGC
MSW1	Mitochondrial tryptophanyl-tRNA synthetase	TTTTCACGTGA
ADE12	Adenylosuccinate synthetase	TTTTCATATGT
CDC8	Thymidylate kinase	ACTTCATATGC
CDC8	Thymidylate kinase	TTTTCATATGA
CYC7	Iso-2-cytochrome c	GTTTCACATGC
COX4	Cytochrome oxidase, subunit IV	TTTTCACATGA
SOD1	Cu,Zn-Superoxiddismutase	ATTTCACATGG
MAL61	Maltose permease	TTTTCATGTGT
FPP1	Farnesylpyrophosphate synthetase	TTTTCACATGT
MFα1	α-factor precursor	**TTTTCACATGG**
STE2	α-factor receptor	TCTTCATATGT
STE4	β-subunit of receptor-coupled G-protein	CCTTCACATGA
CCL1	Negative regulator of pheromone response	TCTTCATGTGT
SWI5	Regulatory gene of mating type switch	**TTTTCACATGC**
KAR1	Nuclear fusion	TCTTCACATTC
KSS1	Protein kinase	TTTTCACATTC
YCK1	Casein kinase I	TTTTCATGTGG
NUF1	Component of nucleoskeleton	ATTTCATATGT
SAC6	Fimbrin (Actin binding protein)	TTTTCACATGA
MSB1	Morphogenesis	ATTTCATGTGC
RAD9	Cell cycle arrest protein	ATTTCATATGT
RAD23	Excision repair protein	ACTTCATATGT
RAD52	Recombination protein	TTTTCATATGT
GCR2	Glycolysis regulator	TCTTCACATTC
MCM3	Minichromosome maintenance	TTTTCATGTGC
CAP1	Capping protein, α-subunit	TTTTCACATTC
YEF3	Elongation factor 3	TTTTCACGTGC
PMA2	Transport ATPase	ACTTCATATGT
AFG2	Putative ATPase	TCTTCATATGT
PEP12	Sorting of vacuolar proteinases	TTTTCATGTGA
PTP2	Tyrosine phosphatase	CTTTCACATGC

Bold letters indicate UAS elements with an at least 40-fold activation capacity. Open reading frames of unknown function identified by systematic sequencing of the *S. cerevisiae* genome were not considered for this compilation.

in vivo. On the other hand, discrimination between Cpf1p and Ino2p/Ino4p binding is less clear although some specificity may be accomplished by nucleotides N_1–N_3. This is evident from the Cpf1p consensus sequence RTCACGTG (centromere DNA element, CDEI; [15,16]) being unlikely to interact efficiently with Ino2p/Ino4p due to the purine residue corresponding to position N_3 of the ICRE. However, analysis of the in vitro DNA-binding capacity of Cpf1p and of the in vivo mitotic stability of mutant CDEI sequences revealed for the *CEN6* CDEI variant TCTTCACGTGC only a moderate loss of function (wild-type *CEN6* CDEI: TCATCACGTGC; [17]). Since the mutant CDEI sequence is expected to mediate about 20-fold *INO2/INO4*-dependent gene activation (cf. Table 1a), the DNA binding specificities of Cpf1p and Ino2p/Ino4p may indeed overlap to a certain extent. Thus, an ICRE with A at position N_3 should be recognized by both, Cpf1p and Ino2p/Ino4p, while another element with G at this place would exclude Ino2p/Ino4p binding (cf. Fig. 1). It is therefore concluded that some ICRE sequence variants with ambivalent bHLH protein binding characteristics may acquire their functional specificity by additional criteria. Interactions with specific regulatory proteins or other functional differences between Cpf1p and Ino2p/Ino4p may guarantee their specificities despite some ambiguity of binding site recognition. In support of this view, Cpf1p obviously lacks a transcription activation domain [18], while Ino2p is a genuine activator even in the absence of Ino4p [4].

3.2. *Yeast structural genes possibly controlled by an ICRE*

In order to identify additional structural genes possibly being transcriptionally controlled by the Ino2p/Ino4p activator, we performed a *Saccharomyces cerevisiae* data base search using the ICRE consensus sequence defined in Fig. 1. Several of the numerous ICRE-like elements thus identified are listed in Table 2. This Table includes only those elements which are located in the 5'-flanking promoter regions of the respective genes and which mediate transcriptional activation by a factor of >7 (cf. Fig. 1). In addition to a large number of ICRE-like sequences mapping to upstream regions, several motifs perfectly matching the ICRE consensus were also found within coding regions (not

shown). The biological significance of ICREs within reading frames may be questioned, since UAS elements usually do not function from a downstream position [19]. Remarkably, the promoters of all currently known genes of phospholipid biosynthesis contain at least one copy of the ICRE. However, for only few of them, such as *FAS1* and *FAS2* [2], *INO1* [9], *PEM1*, *PEM2* [20], *PSS1/CHO1* [8,21], *ITR1* [22] and *ACC1/FAS3* [23,24], these elements were definitely shown to be important for the efficient transcriptional activation of the respective genes. In a previous study, we postulated the existence of two ICREs in the *INO1* upstream region [2]. According to the results of this work, two additional motifs (one of which is a non-canonical bHLH binding site) may also contribute to the marked inositol/choline regulation of *INO1* (cf. Table 2).

Interestingly, the promoter of the glycerol kinase gene *GUT1* also contains a perfect ICRE. Since glycerol-3-phosphate functions as a recipient of long-chain fatty acids from the corresponding acyl-CoA residues, *GUT1* may be under dual control: the transcriptional activator Adr1p has been shown to be responsible for the carbon source control of the gene [25] while Ino2p/Ino4p could mediate its regulation by phospholipid precursors. Similarly, the ICRE upstream of the CTP synthetase gene *URA8* indicates an Ino2p/Ino4p-dependent control of CTP biosynthesis which provides the coenzyme necessary for the production of activated metabolites such as CDP-choline or CDP-diacylglycerol. It should be emphasized that the promoters of several regulatory genes of phospholipid biosynthesis, such as *INO2*, *INO4* and *OPI1*, also contain weak ICRE sequences. This may be important for the autoregulatory control of these genes ([3]; unpublished).

Besides phospholipid biosynthetic genes, Table 2 also lists a considerable number of additional genes as candidates for an ICRE-dependent control without any common functional characteristics being evident among them. Interestingly, several genes of methionine biosynthesis contain an ICRE in their respective upstream regions. Although the T nucleotide at position N_3 for the ICRE-like elements of *MET2*, *MET8*, *MET14*, *MET16* and *SAM2* argues for an Ino2p/Ino4p rather than a Cpf1p binding site, no methionine requirement was observed with *ino2* or *ino4* null mutants. Similarly, overexpression of both *INO2* and *INO4* by appropriate increases of their gene dosages could not complement the methionine auxotrophy of a *cpf1* null mutant. Although the ICREs in the various *MET* promoters may be targets of both Ino2p/Ino4p and Cpf1p (see above), the Ino2p/Ino4p heterodimer may fail to act synergistically with additionally necessary components of the methionine activation system such as the genuine transcriptional activator Met4p [18]. Thus, the possible significance of *INO2/INO4* for the expression of a structural gene with an ICRE in its upstream region obviously requires individual testing. The impaired growth characteristics of *ino2/ino4* null mutants even on inositol/choline-supplemented rich media argue for an additional function of the Ino2p/Ino4p activator beyond its role in phospholipid biosynthesis. The data compiled in Table 2 may provide clues to the possible targets of these additional functions of the Ino2p/Ino4p transcription factor.

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