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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

Hans-Joachim Schüller, Angela Hahn, Fritz Tröster, Armin Schütz and Eckhart Schweizer

Institut für Mikrobiologie und Biochemie, Lehrstuhl Biochemie, Staudstrasse 5, W-8520 Erlangen, FRG

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A systematic search for upstream controlling elements necessary for efficient expression of the yeast fatty acid synthase genes *FAS1* and *FAS2* revealed identical activation sites, UAS_{FAS}, in front of both FAS genes. The individual element confers, in a heterologous yeast test system, an ~40-fold stimulation of basal gene expression. The UAS_{FAS} motifs identified have the consensus sequence TYTTCACATGY and function in either orientation. The same sequence motif is found in the upstream regions of all so far characterized yeast genes encoding enzymes of phospholipid biosynthesis. In gel retardation assays, a protein factor, Fbfl (FAS binding factor), was identified which interacted with UAS_{FAS}. The UAS_{FAS} motif proved to be an inositol/choline responsive element (ICRE) conferring strict repression by exogenous inositol and choline on a heterologous reporter gene. Its core sequence perfectly matches the CANNTG motif typical of basic helix–loop–helix DNA-binding proteins. In contrast to the individual UAS_{FAS} element, the intact yeast FAS promoters are not significantly influenced by inositol and choline, and thus allow nearly constitutive fatty acid synthase production. Available evidence suggests that additional *cis*- and *trans*-acting elements, other than UAS_{FAS} and Fbfl, are involved in this constitutive FAS gene expression.

Key words: fatty acid synthase/gene regulation/helix–loop–helix proteins/upstream activation site/yeast

Introduction

Fatty acids are essential components of phospholipids and are therefore necessary for the formation of biological membranes. Thus, fatty acid synthases represent typical 'house-keeping' enzymes fulfilling basic functions in cellular metabolism and proliferation (reviewed by Singh *et al.*, 1985; Schweizer, 1989). Despite their essentially identical reaction mechanisms, the molecular organization of fatty acid synthases from different organisms varies considerably. In the yeast *Saccharomyces cerevisiae*, saturated fatty acids are synthesized by a 2.4 MDa multifunctional enzyme complex exhibiting an $(\alpha\beta)_6$ subunit composition. The subunits are encoded by two unlinked genes, *FAS1* (β subunit, 229 kDa; Schweizer *et al.*, 1986; Köttig *et al.*, 1991) and *FAS2* (α subunit, 206 kDa; Schweizer *et al.*, 1987) which were mapped to yeast chromosomes XI and XVI, respectively (Siebenlist *et al.*, 1990).

Eukaryotic genes encoding functionally cooperating enzymes may underlie a common genetic control mediated by similar *cis*- and *trans*-acting elements. In yeast, regulatory systems such as the general control of amino acid biosynthesis (reviewed by Hinnebusch, 1990) and the *GAL4* dependent induction of galactose-metabolizing enzymes (Johnston, 1987) are in support of this view. In lipid metabolism, formation of the heteromultimeric yeast fatty acid synthase (FAS) complex requires the expression of *FAS1* and *FAS2* at comparable rates. It appears reasonable that this coordinate control is accomplished by a common mechanism of *FAS1* and *FAS2* gene activation. In accordance with this view, we describe in this work the identification and functional characterization of an upstream activation site (UAS) common to *FAS1* and *FAS2*. Since the same sequence element is also present in the upstream regions of many other yeast genes involved in phospholipid biosynthesis, a general control mechanism affecting most components of yeast membrane biogenesis is suggested.

Results

Functional analysis of FAS1 and FAS2 upstream regions

Using the FAS promoter–β-galactosidase fusion gene constructs pJS200 (*FAS1-lacZ*; for details see Materials and methods) and pJS203 (*FAS2-lacZ*), we undertook a deletion analysis of both the *FAS1* and *FAS2* upstream regions. Two clusters of TATA elements in the *FAS1* promoter, at positions −110/−40 (T1) and −405/−340 (T2) relative to the translational start codon, were shown to be functionally redundant, since deletion of either one did not significantly affect *lacZ* gene expression (Δ20 and Δ16 in Figure 1). However, removal of T1 and T2 at the same time, drastically reduced the activity of the fusion gene product (Δ28 in Figure 1). Besides these elements responsible for basal TATA/TFIID-mediated gene expression (Hahn *et al.*, 1989), the deletion analysis also disclosed two putative activation sites in the far upstream region of *FAS1*, i.e. UAS1 at position −866/−838 (cf. Δ21 and Δ25 in Figure 1) and UAS2 at position −713/−670 (cf. Δ36 and Δ37 in Figure 1). In both cases, the removal of a rather short DNA segment of 28 and 43 bp, respectively, led to a reduction of the reporter gene expression to ~5% of wild type level.

In order to confirm these data, successively shortened *FAS1* upstream fragments were inserted into the *CYC1-lacZ* derived promoter test plasmid pJS205BXX (ΔUAS-*CYC1-lacZ*) which was devoid of its own, *CYC1* specific upstream activation elements. Using this test system, a striking drop of the gene activation potency was observed when the constructs pJS235 and pJS237 were compared (Figure 2a). Therefore, the 28 bp DNA sequence by which the *FAS1* promoter inserts in these two plasmids differ, is

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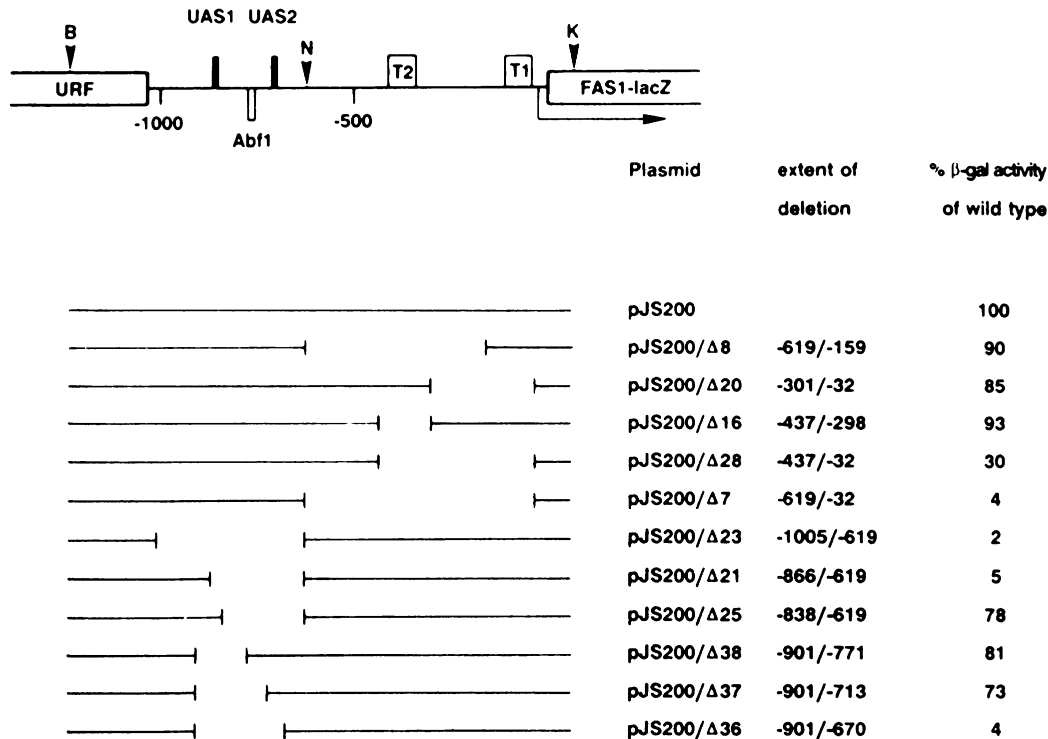

Fig. 1. Deletion analysis of *FAS1* upstream region. The arrow indicates start and direction of transcription. T1 and T2 represent clusters of TATA boxes. Positions of UAS_{FAS} elements are marked by dark boxes; the Abf1 binding site is shown as an open box. The limits of deleted upstream regions are indicated with respect to the translational initiation codon. URF, unassigned reading frame; B, *Bgl*II; N, *Nru*I; K, *Kpn*I.

suggested to be responsible, at least in part, for *FAS1* gene activation. A similar combination of promoter deletion and promoter fragment insertion analyses was performed with the *FAS2* upstream region. As shown in Figure 2b, a 56 bp sequence between nucleotides −243 and −187 upstream of the translational start codon was thereby identified mediating a pronounced activation of the heterologous promoter test construct (cf. plasmids pAF7 and pAF12 in Figure 2b). As is also seen from Figure 2b, another activating element located between positions −279 and −377 of the *FAS2* promoter also confers a significant stimulation of *lacZ* gene expression (cf. pAF9 and pAF14). Interestingly, three of the four sites thus identified as putative upstream activation elements in *FAS1* and *FAS2* gene expression contain essentially the same sequence motif of 15 almost completely conserved base pairs, TYTTCACATGCYRCC. Hence, this sequence appeared as a likely candidate for an UAS_{FAS} element mediating the coordinate activation of both *FAS* genes.

Functional characterization of the UAS_{FAS} sequence motif

In order to test their UAS function, we inserted distinct oligonucleotide fragments representing the core sequences of the three supposed UAS_{FAS} elements (see Materials and methods) into the promoter test plasmid pJS205. Without UAS insertion, this plasmid allows only basal, TATA-mediated expression of the *CYC1-lacZ* reporter gene. With all three oligonucleotide insertions tested, a significant stimulation of this basal gene expression by factors between 40 and >100 was obtained (Table I). As is also evident from Table I, the extent of activation was independent of the orientation of the UAS sequences relative to the reporter gene. Insertion of two copies of the same sequence element led to a synergistic, i.e. higher than additive, activation. For instance, a second copy of the FBF12 oligonucleotide conferred a 4-fold higher activation of *CYC1-lacZ* gene expression than a single FBF12 fragment. Interestingly, the three UAS_{FAS} elements tested, though very similar in sequence (see Table IIa), differed significantly in their activation potency: while single copy insertions of FBF12 or FBF34 led, in either orientation, to ~40-fold activation of basal gene expression, FBF78 conferred a >100-fold activation effect (Table I).

The three oligonucleotide fragments used in the above expression studies were also examined in electrophoretic mobility shift assays (EMSA). As expected for DNA fragments carrying transcriptional activation sites, distinct retardation signals indicating specific protein−DNA interactions were observed (Figure 3). With both the UAS_{FAS1}[−860]- and UAS_{FAS2}[−230]-specific oligonucleotides FBF12 and FBF34, respectively, three differently migrating protein−DNA complexes, C1, C2 and C3, were observed. According to the relative signal intensities indicating complexed or free DNA, a rather low cellular abundancy of the UAS_{FAS} binding factor(s) is suggested. Competition assays using an excess of unlabelled homologous or heterologous DNA fragments proved the identity of the proteins interacting with UAS_{FAS1} and UAS_{FAS2} (lanes 3 and 4 in Figure 3). Similarly, the retardation bands obtained with the labelled UAS_{FAS1}[−700] sequence as a probe were efficiently competed by both UAS_{FAS1}[−860] and UAS_{FAS2}[−230] (data not shown). These findings clearly demonstrate the binding of identical *trans*-acting protein factor(s) to the UAS_{FAS1} and UAS_{FAS2} sequence elements.

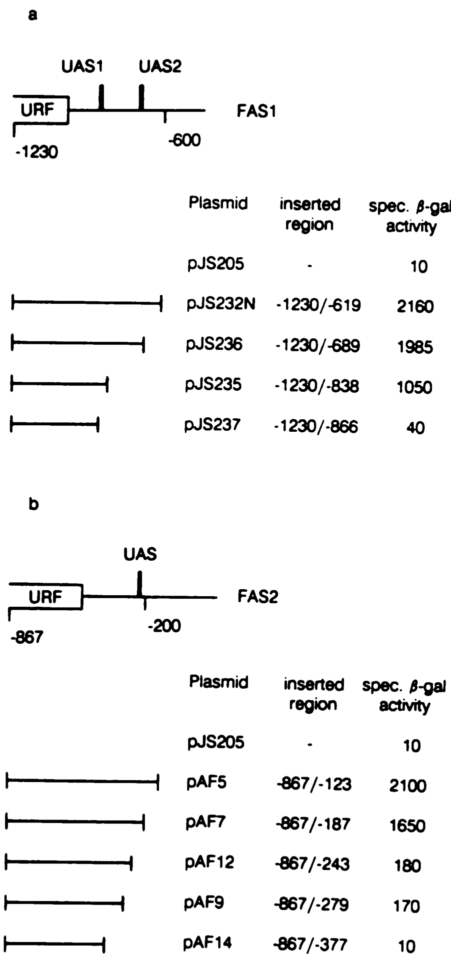

Fig. 2. Insertion of *FAS1* (**a**) and *FAS2* (**b**) promoter fragments into the UAS-less promoter test plasmid pJS205BXX. Putative UAS_{FAS} elements are indicated by dark boxes. Endpoints of deletions were generated by Bal31 nuclease digestion, followed by *Xho*I linker insertion. URF, unassigned reading frame.

General occurrence of the UAS_{FAS} sequence motif in connection with genes of yeast phospholipid metabolism

The core sequence of the three UAS_{FAS} elements, TTCACATGC, was used in a database search for similar or identical sequence motifs upstream of other yeast genes. The matches thereby obtained represented all so far characterized genes of yeast phospholipid synthesis. A compilation of the 18 UAS_{FAS}-like elements thus identified in the promoters of 13 different yeast genes involved in membrane biogenesis is listed in Table IIa. A preliminary consensus sequence, TYTTCACATGY, may be derived from this list. The functional equivalence of the three UAS_{FAS} elements and of the corresponding element upstream of *INO1* was demonstrated by EMSA competition experiments (Figure 4). The $UAS_{INO1}[-240]$ oligonucleotide FBF56 was able to release the radiolabelled $UAS_{FAS1}[-860]$ probe FBF12 from the protein–DNA complex C2 (Figure 4a, lane 4). Conversely, the single retardation signal obtained with the FBF56 probe can be competed by an excess of $UAS_{FAS1}[-860]$ (Figure 4b, lane 4). Therefore, we will refer to the protein factor engaged in formation of the C2 complex with $UAS_{FAS1}[-860]$ as Fbf1 (*FAS* binding factor 1). Obviously, the widespread occurrence of the UAS_{FAS}

Table I. Gene activation conferred by distinct oligonucleotide fragments inserted into the promoter test plasmid pJS205

Plasmid	Construct	Specific β-gal activity (nmol/min/mg)
pJS205	ΔUAS-*CYC1-lacZ*	10
pJS246N	$(FBF12)_N$-*CYC1-lacZ*	390
pJS246R	$(FBF12)_R$-*CYC1-lacZ*	430
pJS246NR	$(FBF12)_N$-$(FBF12)_R$-*CYC1-lacZ*	1690
pJS264N	$(FBF78)_N$-*CYC1-lacZ*	1150
pJS264R	$(FBF78)_R$-*CYC1-lacZ*	1030
pFT3N	$(FBF34)_N$-*CYC1-lacZ*	410
pFT3R	$(FBF34)_R$-*CYC1-lacZ*	480
pJS263N	$(FBF56)_N$-*CYC1-lacZ*	370
pJS263R	$(FBF56)_R$-*CYC1-lacZ*	400
pJS238	$(BAF12)_N$-*CYC1-lacZ*	14
pJS239	$(BAF12)_R$-*CYC1-lacZ*	12

Indices 'N' (normal) or 'R' (reverse) indicate the orientation of the inserted fragment with respect to the direction of transcription. Yeast transformants were grown under selective conditions and harvested in the mid log phase. Yeast strain JS89.11-8 was used as a recipient. FBF12 = $UAS_{FAS1}[-860]$; FBF34 = $UAS_{FAS2}[-230]$; FBF56 = $UAS_{INO1}[-240]$; FBF78 = $UAS_{FAS1}[-700]$. BAF12 corresponds to the oligonucleotide fragment carrying the Abf1 binding site from *FAS1* upstream region (−774/−762).

consensus sequence suggests that the biological function of Fbf1 is not restricted to *FAS* gene expression but probably extends to most or even all genes of phospholipid biosynthesis. The functional similarity of UAS_{FAS} and UAS_{INO1} is further supported by expression studies using insertions of the oligonucleotide FBF56 (=$UAS_{INO1}[-240]$) into the promoter test plasmid, pJS205. After transformation into yeast, the UAS_{INO1} element led to an ~40-fold, orientation-independent activation of basal *CYC1-lacZ* fusion gene expression (cf. plasmids pJS263N and pJS263R in Table I).

As is evident from Table IIb, additional genes obviously not involved in phospholipid biosynthesis also contain the Fbf1 consensus sequence in their upstream regions. As these genes do not share any obvious regulatory characteristics, the possible meaning of these matches remains unclear, as yet.

Inositol/choline repression of Fbf1-mediated gene expression

It has been reported by Donahue and Henry (1981) that, in yeast, expression of the *INO1* gene encoding inositol-1-phosphate synthase is subjected to strict repression by inositol and choline. These findings, together with the identification of very similar UAS motifs in the upstream regions of *INO1* and both *FAS* genes, prompted us to investigate the influence of inositol and choline on the *FAS1* and *FAS2* promoter activities. To this end, the expression of several fusion gene constructs was studied which contained either the complete *FAS* promoters or the individual UAS_{FAS} sequence motifs inserted into the heterologous ΔUAS-*CYC1-lacZ* context. Both the *FAS1-lacZ* and *FAS2-lacZ* promoter fusions displayed only a slight (25%) reduction of β-galactosidase activities upon addition of 200 μM inositol/2 mM choline to the growth medium. In contrast, constructs carrying only a single UAS_{FAS} element instead of the intact *FAS* upstream region exhibited a strong (90–95%) inositol/choline repression (Figure 5). As a

Table II. Compilation of genes exhibiting an upstream sequence motif similar to UAS_{FAS}

Gene	Function	Upstream sequence (5′ → 3′)											Position
a) Genes of lipid biosynthesis													
FAS1	Fatty acid synthase, β subunit	T	T	T	T	C	A	C	A	T	G	C	−857/−867
FAS1	Fatty acid synthase, β subunit	A	C	T	T	C	A	C	A	T	G	C	−708/−698
FAS2	Fatty acid synthase, α subunit	T	T	T	T	C	A	C	A	T	G	C	−236/−226
ITR1	Inositol permease	G	T	C	T	C	A	C	A	T	G	A	−396/−386
ITR1	Inositol permease	T	C	T	T	C	A	C	A	T	G	C	−286/−276
CTR1	Choline permease	T	T	T	T	C	A	C	A	T	G	C	−271/−261
INO1	Inositol-1-phosphate synthase	T	T	T	T	C	A	C	A	T	G	C	−234/−244
INO1	Inositol-1-phosphate synthase	A	A	T	T	C	A	C	A	T	G	G	−182/−172
CHO1	Phosphatidylserine synthase	C	T	T	T	C	A	C	A	T	G	G	−163/−153
CPT1	Diacylglycerol cholinephosphotransferase	T	T	T	T	C	A	C	A	T	G	C	−113/−123
EPT1	Diacylglycerol ethanolaminephosphotransferase	T	T	C	T	C	A	C	A	T	G	C	−543/−553
CKI1	Choline kinase	T	A	T	T	C	A	C	A	T	G	G	−197/−187
PEM1	Phosphatidylethanolamine *N*-methyltransferase I	A	A	T	T	C	A	C	A	T	G	T	−327/−337
PEM1	Phosphatidylethanolamine *N*-methyltransferase I	T	C	T	T	C	A	C	A	T	G	A	−258/−268
PEM2	Phosphatidylethanolamine *N*-methyltransferase II	T	T	T	C	C	A	C	A	T	G	C	−203/−213
PEM2	Phosphatidylethanolamine *N*-methyltransferase II	T	C	T	T	C	A	T	A	T	G	C	−166/−156
PIS1	Phosphatidylinositol synthase	A	C	T	T	C	A	T	A	T	G	C	−266/−276
FPP1	Farnesylpyrophosphate synthetase	T	T	T	T	C	A	C	A	T	G	T	−424/−414
b) Additional genes of unrelated functions													
ARG4	Argininosuccinate lyase	T	T	T	T	C	A	C	A	T	G	T	−134/−144
COX4	Cytochrome oxidase, subunit IV	T	T	T	T	C	A	C	A	T	G	A	−747/−737
MFα1	α-Factor precursor	T	T	T	T	C	A	C	A	T	G	G	−233/−243
SWI5	Regulatory gene of mating type switch	T	T	T	T	C	A	C	A	T	G	C	−681/−671
TPI1	Triosephosphate isomerase	A	T	C	T	C	A	C	A	T	G	C	−302/−312
Consensus		T	Y	T	T	C	A	C	A	T	G	Y	
	G	1	–	–	–	–	–	–	–	–	23	4	
	A	5	3	–	–	–	23	–	23	–	–	3	
	T	16	15	20	22	–	–	2	–	23	–	3	
	C	1	5	3	1	23	–	21	–	–	–	13	

Positions of sequence elements are given with respect to the translational initiation codon. Both possible orientations of the consensus motif appear with similar frequency. Sequence data were taken from: *FAS1* (Schweizer *et al.*, 1986), *FAS2* (Schweizer *et al.*, 1987), *CTR1* (Nikawa *et al.*, 1990), *ITR1* (Nikawa *et al.*, 1991), *INO1* (Dean-Johnson and Henry, 1989), *CHO1* (Kiyono *et al.*, 1987), *CPT1* (Hjelmstad and Bell, 1990), *EPT1* (Hjelmstad and Bell, 1991), *CKI1* (Hosaka *et al.*, 1989), *PEM1*, *PEM2* (Kodaki and Yamashita, 1987), *PIS1* (Nikawa *et al.*, 1987), *FPP1* (Anderson *et al.*, 1989), *ARG4* (Beacham *et al.*, 1984), *COX4* (Schneider and Guarente, 1987), *MFα1* (Inokuchi *et al.*, 1987), *SWI5* (Stillman *et al.*, 1988), *TPI1* (Alber and Kawasaki, 1982).

control, UAS_{RPG}/Rap1 (Shore and Nasmyth, 1987) dependent β-galactosidase expression was studied and found to be unaffected by the addition of inositol/choline (pJS234N in Figure 5). The strikingly different regulatory characteristics of the complete *FAS* control regions, compared with individual UAS_{FAS} motifs derived from them, strongly argues for a 'composite' structure of the two *FAS* promoters with additional *cis*-acting elements involved in relieving the inositol/choline repression observed with the individual UAS_{FAS} sequences.

Effect of ubiquitous yeast transcription factors on FAS gene expression

In a search for binding sites of known transcriptional activators in the *FAS* upstream regions, we and others (Dorsman *et al.*, 1990) identified a sequence element within the *FAS1* promoter region (ATCACGATACACG; positions −774/−762) perfectly matching the Abf1/Baf1 consensus motif (RTCRYNNNNNACG; Dorsman *et al.*, 1989; Halfter *et al.*, 1989). Indeed, the corresponding oligonucleotide fragment gave a strong retardation signal in EMSA experiments which could be competed by an excess of an authentic Abf1 binding site (not shown). Nevertheless, this element did not stimulate β-galactosidase expression after

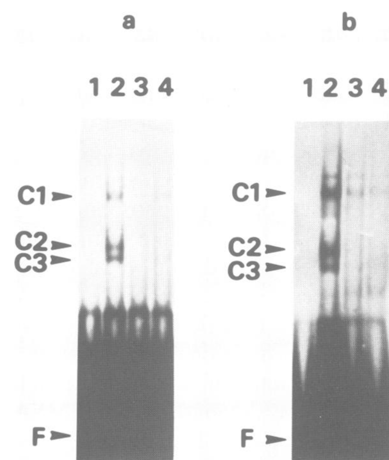

Fig. 3. Gel retardation patterns of UAS_{FAS1} (**a**) and UAS_{FAS2} (**b**). For competition, an ~200-fold molar excess of unlabelled fragment was used. Lanes 1: no protein extract added; lanes 2–4: addition of protein extract (15 μg of total cellular protein); lanes 3: competition with excess of unlabelled FBF12; lanes 4: competition with excess of unlabelled FBF34. FBF12 = UAS_{FAS1}[−860]; FBF34 = UAS_{FAS2}[−230]; C1, C2, C3 = protein–DNA complexes; F = free DNA.

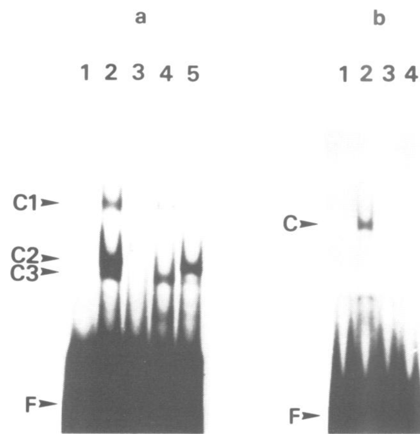

Fig. 4. Competitive electrophoretic mobility shift assays with UAS_{FAS1} (**a**) and UAS_{INO1} (**b**) oligonucleotide fragments. For protein binding, the radiolabelled fragments FBF12 (UAS_{FAS1}[−860]) and FBF56 (UAS_{INO1}[−240]) were used. Lanes 1: no protein extract added; lanes 2−5 (a) and lanes 2−4 (b): addition of 15 μg of whole cellular protein. For competition, a 200-fold molar excess of fragments FBF12 (a, lane 3; b, lane 4), FBF56 (a, lane 4; b lane 3) or CBF12 (consensus binding site of centromere promoter factor Cpf1; a, lane 5) was added. C, C1, C2, C3 = protein−DNA complexes; F = free DNA.

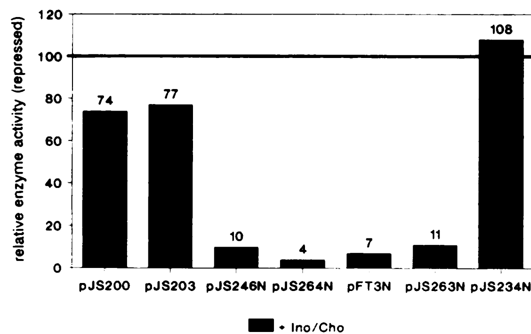

Fig. 5. Inositol/choline repression on *FAS* upstream sequences. The indicated plasmids were transformed into the yeast wild type strain JS89.11−8. Transformants were grown under selective conditions in the absence or presence (dark boxes) of 200 μM inositol + 2 mM choline. The β-galactosidase activities of transformants grown in the presence of inositol/choline (repressed conditions) are in reference to the respective derepressed enzyme activities (= 100%). pJS200: *FAS1-lacZ* (complete *FAS1* upstream region); pJS203: *FAS2-lacZ* (complete *FAS2* upstream region); pJS246N: UAS_{FAS1}[−860]-*CYC1-lacZ*; pJS264N: UAS_{FAS1}[−700]-*CYC1-lacZ*; pFT3N: UAS_{FAS2}[−230]-*CYC1-lacZ*; pJS263N: UAS_{INO1}[−240]-*CYC1-lacZ*; pJS234N: UAS_{RPG}−*CYC1-lacZ*.

insertion into the ΔUAS-*CYC1-lacZ* test plasmid, pJS205 (Table I). Correspondingly, site-directed mutagenesis of this Abf1 binding site did not alter the expression of a *FAS1-lacZ* fusion construct, either in the absence or in the presence of inositol/choline (data not shown). Hence, this sequence motif appears to be dispensable for *FAS1* gene expression.

After identifying the UAS_{FAS} consensus sequence in three FAS upstream elements, we realized its similarity to the binding site of the centromere promoter factor Cpf1 described by Mellor *et al.* (1990). This factor interacting with the consensus sequence, RTCACRTG, is synonymous with Cbf1 (Cai and Davis, 1990), CP1 (Baker *et al.*, 1989) and GF-II (Dorsman *et al.*, 1988). Oligonucleotide fragment CBF12 representing the Cpf1 binding site of the *RIP1*

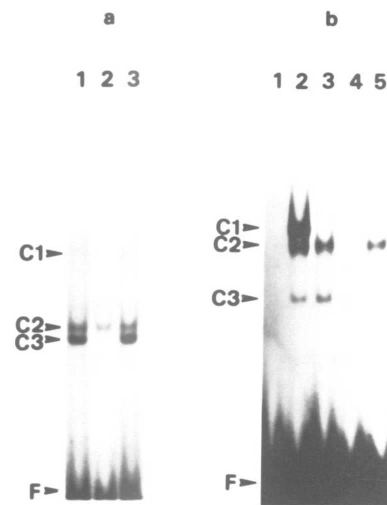

Fig. 6. EMSA characteristics of UAS_{FAS1} (**a**) and of the Cpf1 binding fragment (**b**) with extracts from a *cpf1* null mutant. The UAS_{FAS1} sequence of FBF12 and the Cpf1 consensus sequence of CBF12 (for details see Materials and methods) were used. (a) lanes 1 and 2: addition of protein extract from *CPF1* wild type strain YPH266; lane 2: addition of 200-fold excess of unlabelled CBF12 fragment; lane 3: addition of protein extract from *cpf1* null mutant strain YSS91. (b) lane 1: no protein extract added; lane 2: addition of protein extract from *CPF1* wild type strain; lanes 3−5: addition of protein extract from *cpf1* mutant strain; lane 4: competition by a 200-fold excess of unlabelled CBF12 fragment; lane 5: competition by a 200-fold excess of unlabelled FBF12 fragment.

Table III. Gene activation potency of UAS_{FAS1}[−860] in a yeast *cpf1* null mutant

Strain	Genotype	Specific β-gal activity (nmol/min/mg)
YPH266	*CPF1* (wild type)	210
YSS91	Δ*cpf1::LEU2*	200

The plasmid pJS246N (see Table I) was transformed into yeast strains YPH266 (*CPF1*) and YSS91 (Δ*cpf1::LEU2*), respectively.

promoter (Dorsman *et al.*, 1988) was used in an EMSA experiment for competition with a labelled UAS_{FAS1}[−860] probe. As seen in Figure 4a, lane 5, the intensities of complexes C1 and C3 were, indeed, significantly reduced upon addition of CBF12, while the Fbf1-specific C2 complex remained unaffected. Interestingly, however, incubation of UAS_{FAS1}[−860] with protein extracts prepared from a *cpf1* null mutant yielded retardation patterns identical to that of an isogenic wild type strain (Figure 6a, lanes 1 and 3). These results suggest the existence of a family of Cpf1-like DNA-binding proteins, interacting non-specifically, also with the Fbf1 binding site. This idea is supported by the observation that in EMSA experiments with CBF12 as a probe, the most prominent of the three protein−DNA complexes, C1, is absent, when the *cpf1* null mutant extract is employed (Figure 6b lane 3). Therefore, DNA-binding proteins different from Cpf1 but exhibiting recognition specifities related to Fbf1 and Cpf1 are probably responsible for the formation of the UAS_{FAS1}[−860] retardation complexes C1 and C3 (Figures 4a and 6a) and for the C2 and C3 complexes observed with CBF12 as a probe (Figure 6b). Cpf1 itself is obviously not necessary for *FAS* gene expression, since

activation of the *CYC1-lacZ* fusion, in pJS246N, carrying the $UAS_{FAS}[-860]$ insert, is identical in a *CPF1* wild type strain and in the isogenic *cpf1* null mutant (Table III).

Discussion

In this paper a sequence motif, UAS_{FAS}, was characterized, which is necessary for efficient expression of both yeast fatty acid synthase genes, *FAS1* and *FAS2*. The motif fulfils the known criteria for defining a typical upstream activation site: (i) Insertion into a heterologous, UAS-free promoter context results in a pronounced gene activation; (ii) its distance to the TATA box(es) may be varied, within a wide range; (iii) activation takes place in an orientation-independent manner; (iv) the UAS element may be present in a single or in multiple copies in the upstream region of a gene. The UAS_{FAS} element, TYTTCACATGY, is also present in the upstream regions of all so far characterized yeast genes involved in phospholipid biosynthesis, indicating a general function of this *cis*-acting motif in regulating membrane biogenesis in yeast. It remains to be shown whether the ergosterol biosynthetic pathway also underlies this control, since only one of its genes, *FPP1* (see Table IIa), was found to contain the motif while others, such as *ERG8* (Tsay and Robinson, 1991) and *ERG12* (Oulmouden and Karst, 1990), at least in the regions sequenced thus far, did not. A sequence element almost identical to UAS_{FAS} has been proposed to be involved in *INO1* gene regulation (Carman and Henry, 1989; Lopes *et al.*, 1991). However, the 9 bp sequence investigated by these authors, ATTTCACAT, was unable to activate transcription when inserted into a heterologous test system (Lopes *et al.*, 1991). This negative result becomes understandable when the sequence motif studied by Lopes *et al.* (1991) is compared with the upstream consensus element shared by the various genes of yeast phospholipid biosynthesis (see Table IIa). Obviously, the two sequence motifs differ by the 3′-terminal G nucleotide which is absent from the proposed *INO1* regulatory site. Based on the efficient gene activation observed with certain natural variants of the UAS_{FAS} consensus sequence (see Table I), the different 5′ ends of the two motifs appear less critical. The decisive role of the 3′-terminal G was confirmed by the pronounced activation effect exhibited by an *INO1* upstream element to which this G nucleotide had been added (Table I). Thus, the interpretation that the ATTTCACAT motif investigated by Lopes *et al.* (1991) is an upstream induction site (UIS) required for stimulation of a distinct UAS yet to be identified is presumably due to the lack of the 3′-terminal guanine, i.e., to the use of an incomplete activation site, in these studies. In combination with the EMSA cross-competition experiments shown in Figure 5a and b, our results clearly demonstrate the functional identity of the so far investigated UAS motifs from three different genes of yeast phospholipid synthesis, i.e. *INO1*, *FAS1* and *FAS2*. These results completely agree with those obtained by Kodaki *et al.* (1991) when analysing the upstream regions of the two phosphatidylethanolamine *N*-methyltransferase genes, *PEM1* and *PEM2*. The octameric sequence motif TTCAYATG identified as an activation site of these genes is completely contained within the UAS_{FAS} element.

It has been shown in this work that the UAS_{FAS}-binding protein, Fbf1, has DNA recognition properties similar to the centromere promoter-binding factor Cpf1/Cbf1 (Mellor *et al.*, 1990; Cai and Davis, 1990). Additional yeast proteins possibly identical to Cpf1/Cbf1 have been characterized using the immunoglobulin heavy chain enhancer, μE3 (Beckmann and Kadesch, 1989) and the UEF/USF/MLTF binding sites of the advenovirus major late promoter (Moncollin *et al.*, 1990). The DNA-binding sites of all these factors contain the core motif, CANNTG, typical for basic helix–loop–helix proteins (Blackwell and Weintraub, 1990). Indeed, protein sequence data available for the μE3 binding factor TFE3 (Beckmann *et al.*, 1990), the adenovirus major late transcription factor USF (Gregor *et al.*, 1990) and the yeast centromere promoter factor Cpf1 clearly confirm the existence of a basic helix–loop–helix (bHLH) structure which is supposed to be essential for dimerization and DNA-binding. Recently, a similar structure has been proposed for the *PHO4* activator of yeast phosphatase genes (Ogawa and Oshima, 1990). Indeed, a CACGTG core sequence was identified in the UAS region of the *PHO8* gene encoding repressible alkaline phosphatase (Hayashi and Oshima, 1991). Thus, the *PHO4* gene product might be responsible for non-specific protein–DNA interactions with UAS_{FAS} or the Cpf1 binding site (Figures 4a and 6). Based on the core recognition motif of UAS_{FAS}, we conclude that the *FAS* binding factor Fbf1 identified in this work also belongs to the increasing group of basic helix–loop–helix DNA-binding proteins. In previous studies of Donahue and Henry (1981) two regulatory genes, *INO2* and *INO4*, necessary for efficient expression of *INO1* have been identified. The sequence predicted for the *INO4* encoded protein shows similarity to the bHLH family (Hoshizaki *et al.*, 1990) and, thus, may be related or even identical to Fbf1. The isolation and characterization of the UAS_{FAS} binding protein(s) is currently under investigation in our laboratory.

Since UAS_{FAS}, together with its corresponding *trans*-acting protein, Fbf1, is obviously of central importance for the control of phospholipid synthesis in yeast, we investigated the response of these elements to exogenous inositol and choline. Previously, a considerable repression of several enzymes of this pathway by these phospholipid precursors had been observed (summarized by Carman and Henry, 1989). In accordance with these earlier findings our UAS_{FAS} sequence motifs as well as the related UAS_{INO1} element mediated, when tested individually, a 10- to 20-fold repression of the reporter constructs by inositol/choline. We therefore suggest the designation ICRE (inositol/choline responsive element) for the TYTTCACATGY consensus element. Interestingly, this inositol/choline repression is significantly alleviated in the context of the intact *FAS1* and *FAS2* promoters. This effect is possibly attributable to the existence of additional *cis*-acting elements in the *FAS* upstream regions. These additional elements may contribute, either independently of or synergistically with Fbf1, to *FAS* gene expression. As a possible candidate for such an accessory element, the Abf1 binding site located approximately midway between both ICREs of the *FAS1* upstream region was inactivated by *in vitro* mutagenesis. However, in spite of the various roles so far attributed to Abf1 as a transcriptional activator (Halfter *et al.*, 1989), a transcription modulator (Brindle *et al.*, 1990), a silencer binding factor (Shore *et al.*, 1987) or a protein interacting with origins of replication (Diffley and Stillman, 1988), we were unable to detect any effect of the Abf1 binding site

on *FAS* gene expression either under conditions of inositol/choline repression or derepression. Therefore, other *cis*-acting elements which remain to be identified might turn the ICREs in *FAS* promoters into nearly constitutive activation signals. A reduced inositol/choline repression should also be expected for the synthesis of choline permease and choline kinase, since both enzymes are necessary for the utilization of external choline. Indeed, the repression of the corresponding genes *CTR1* and *CKI1* is much less pronounced (2- and 4-fold repression, respectively; Nikawa *et al.*, 1990; Hosaka *et al.*, 1990) compared with *INO1* (30-fold repression; Carman and Henry, 1989), although the upstream regions of all these genes contain ICREs. Thus, ICRE-dependent genetic control obviously varies between efficient repression by inositol/choline, as is observed with *INO1*, and almost constitutive expression of genes such as *FAS1* and *FAS2*. It remains to be shown how the same sequence element, UAS_{FAS}, when placed into a different promoter context or upon interaction with different *trans*-acting protein factors, is functionally altered from a highly repressible to a constitutive upstream activation site.

Materials and methods

Yeast strains

Yeast wild type strain JS89.11−8 (*MAT***a** *ura3 his3 can1*) was routinely used for transformation with episomal or integrative plasmids carrying *FAS* promoter deletions or insertion constructions (see below). Protein extracts for EMSA experiments were prepared from the proteinase-deficient strain C13-ABY.S86 (*MAT*α *ura3 leu2 pra1 prb1 prc1 cps1*) lacking vacuolar proteinases yscA, yscB, yscY and yscS (Teichert *et al.*, 1989). In addition, the *cpf1* null mutant YSS91 (*MAT***a** *ura3−52 lys2−801 ade2−101 leu2−1* Δcpf1::LEU2; Mellor *et al.*, 1990) and the isogenic wild type YPH266 (*CPF1*) were used for transformation with UAS_{FAS} reporter constructions and whole-cell protein extract preparations if indicated.

Oligonucleotide fragments used for insertion studies and EMSA experiments

The oligonucleotides indicated below were synthesized on an Applied Biosystems 381 A DNA synthesizer. DNA sequences representing distinct upstream regions are given in capital letters; sequences added for cloning or labelling purposes are shown in lower case letters. For all sequences, complementary strands were designed to give ends compatible with an *Xho*I site after hybridization. FBF12 (=UAS_{FAS1}[−860]; nucleotides −873/−838 from *FAS1*), 5′-tcgatctagACGGCAGCATGTGAAAAAACCCGT-AGAAGGTCCGCAgatct-3′; FBF34 (=UAS_{FAS2}[−230]; nucleotides −239/−219 from *FAS2*), 5′-tcgaGCGTTTTCACATGCTACCTCAgatc-t-3′; FBF56, (=UAS_{INO1}[−240]; nucleotides −250/−232 from *INO1*), 5′-tcgagAATGCGGCATGTGAAAAGTagatct-3′; FBF78 (=UAS_{FAS1}[−700]; nucleotides −709/−696 from *FAS1*); 5′-tcgagAACTTCACATGC-CGagatct-3′; CBF12 (Cpf1 binding site from *RIP1* upstream region; Dorsman *et al.*, 1988), 5′-tcgagTGGGTCACGTGCGGagatct-3′; RAP12 (Rap1 consensus binding site; Shore and Nasmyth, 1987); 5′-tcgagAAC-ACCCATACATTTagatct-3′; BAF12 (Abf1 binding site from *FAS1* upstream region, positions −777/−753), 5′-tcgagagatctCGCATCACGA-TACACGAGGTGCAG-3′.

Plasmid constructions

A *FAS1-lacZ* fusion was constructed by inserting a 2.9 kb *Hin*dIII−*Kpn*I fragment from *FAS1* subclone pMS3111 (Schweizer *et al.*, 1984) into the polylinker region of YEp357R (Myers *et al.*, 1986). The resulting plasmid pJS200 is a derivative of YEp352 (Hill *et al.*, 1986) exhibiting high mitotic stability even under non-selective conditions. The *FAS1-lacZ* fusion thus obtained contains, 5′ proximal to the *lacZ* reporter gene, ∼2.8 kb of the *FAS1* upstream region together with the first 23 codons of the *FAS1* reading frame. In pJS200X, the *Nru*I site at position −619 (relative to the ATG start codon) was converted into a *Xho*I site.

For linker deletion analysis, pJS200 was linearized at the unique *Bgl*II (−1230), *Nru*I (−619) and *Kpn*I (+67) restriction sites, respectively. The digest was subsequently treated with Bal31 nuclease, purified by gel electrophoresis and used for *Xho*I linker insertion into the shortened fragments. Linker concatemers were removed by an excess of *Xho*I. The positions of linker insertions were determined by DNA sequencing using plasmid templates and site-specific primers. Upstream deletion constructs lacking distinct internal regions were obtained by combining *Hin*dIII−*Xho*I fragments from various deletion plasmids of defined Bal31 termination sites. A subset of the pJS200/Δ plasmids thus obtained was converted into integrating plasmids by transfer of a *Hin*dIII−*Nco*I fragment carrying the *FAS1-lacZ* fusion into YIp352 (Hill *et al.*, 1986). A *FAS2-lacZ* fusion was obtained similarly by subcloning a 3.3 kb *Eco*RV−*Nhe*I fragment from pMS3246 (U.Hoya, unpublished) into pBR322. From the pJS201 construct thus obtained, a 3.5 kb *Eco*RI−*Nhe*I fragment was subsequently transferred into *Eco*RI−*Xba*I-cleaved YEp357 (Myers *et al.*, 1986). The fusion construct pJS203 contains, 5′ proximal to *lacZ*, 2.4 kb of the *FAS2* upstream region together with the first 307 codons of the *FAS2* reading frame. Prior to Bal31 digestion, pJS203 was linearized by cutting at the *Bgl*II (−867) and *Pvu*II (+912) restriction sites, respectively. Endpoints of deletion were converted into *Xho*I sites as described above.

To test the various shortened *FAS* upstream fragments for their UAS character, a *CYC1-lacZ* fusion gene lacking its natural UAS but retaining the TATA boxes was used. The *CYC1* upstream region from plasmid pLG669-Z (Guarente and Ptashne, 1981) was transferred as a 1.0 kb *Sal*I−*Bam*HI fragment into YEp356R (Myers *et al.*, 1986) to give pJS186. The 0.4 kb UAS_{CYC1} element was removed by cleavage with *Xho*I; by subsequent self-ligation of the remaining 8.5 kb fragment carrying the ΔUAS-*CYC1-lacZ* fusion construct pJS205 was formed. For reasons of cloning versatility, an oligonucleotide linker carrying recognition sites for *Bgl*II, *Xba*I and *Xho*I was ligated into the *Xho*I site of pJS205 at position −238 relative to the translational start codon. The resulting plasmids pJS205BXX and pJS205XXB differ by the orientation of the linker and were subsequently used for directed insertion of *Bgl*II−*Xho*I *FAS* upstream fragments (*Xho*I sites resulted from linkers ligated to the Bal31 termination sites). Insertion of synthetic oligonucleotide fragments into the *Xho*I digested promoter test plasmid pJS205 was performed according to established procedures (Ausubel *et al.*, 1987). The orientation and copy number of inserts were determined by DNA sequencing.

Site-directed mutagenesis

To remove the Abf1 binding site in the *FAS1* upstream region (positions −774/−762), an 1.07 kb *Eco*RI−*Pst*I fragment (−1497/−427) was subcloned from pJS200X into M13mp18 to give mp18/FAS1-1. Uridine-containing phage templates necessary for *in vitro* mutagenesis according to Kunkel *et al.* (1987) were isolated from mp18/FAS1−1 by transfection into *Escherichia coli* strain RZ1032 (*dut*$^-$ *ung*$^-$). A mutagenic 35mer primer with altered nucleotides in all conserved positions of the Abf1 binding site was used (Abf1 consensus sequence ATCACGATACACG was converted into GCATGCATACCAT). Finally, a 0.61 kb *Bgl*II−*Xho*I fragment from the resulting mutagenized RF phage was subcloned into pJS205BXX to give pSA4. With the exception of the mutated Abf1 binding site, pSA4 is identical to pJS232N.

Yeast transformation

Plasmids carrying the *lacZ* fusion constructs described above were transformed into yeast according to Ito *et al.* (1983), with the modification of using a 50% stock solution in order to adjust the polyethylene glycol 4000 concentration to 35% in the transformation mixture. Integrating plasmids derived from YIp352 were linearized with *Nco*I prior to transformation in order to direct their integration to the *URA3* locus. Transformants were selected on synthetic complete medium lacking uracil (SCD-Ura; Ausubel *et al.*, 1987).

β-galactosidase assay

Yeast transformants were grown up to mid-log phase under selective conditions in SCD-Ura liquid broth, harvested by centrifugation and stored frozen at −20°C. Protein extracts prepared by cell disruption with glass beads were assayed for β-galactosidase activity according to Guarente (1983). For protein determination, the microbiuret method of Zamenhoff (1957) was used.

EMSA experiments

Yeast total protein extracts used for electrophoretic mobility shift assays (EMSA) were prepared as described by Arcangioli and Lescure (1985). Binding reactions (20 μl) contained binding buffer (10 mM HEPES, pH 7.8; 50 mM NaCl; 5 mM $MgCl_2$; 0.5 mM EDTA; 10% glycerol), 2 μg of poly(dI−dC)/poly(dI−dC) (Boehringer, Mannheim), 10 000 c.p.m. of ^{32}P-labelled oligonucleotide fragment (1−2 ng) and 15 μg total cellular proteins. After incubation at 20°C for 20 min, binding mixtures were loaded onto a 4% polyacrylamide gel (acrylamide: bisacrylamide ratio of 19:1) in 0.5×TBE (45 mM Tris; 45 mM boric acid; 1 mM EDTA, pH 8.3),

which had been pre-electrophoresed for 1 h at 4°C and a voltage gradient of 12 V/cm. Electrophoresis was continued for 2–3 h (depending on the length of the labelled fragment) under identical conditions. Finally, gels were treated with 10% methanol + 10% acetic acid, dried and subjected to autoradiography.

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