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Interactions of the yeast centromere and promoter factor, Cpf1p, with the cytochrome c_1 upstream region and functional implications on regulated gene expression

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ABSTRACT

The upstream activation site (UAS) of the cytochrome c_1 gene, *CYT1,* contains sequences for DNA-binding of several transcription factors. Among them are the heme-dependent protein, Hap1p, and the multiprotein complex, Hap2/3/4/5, which mediate transcriptional induction under aerobic conditions and after exhaustion of glucose, respectively. The multiple interactions of nuclear proteins with the UAS region of *CYT1* observed in electrophoretic mobility shift experiments are influenced by carbon source and oxygen tension, but are independent of both regulators, Hap1p and Hap2/3/4/5. All protein–DNA complexes obtained are solely due to the association of the centromere and promoter factor 1 (Cpf1p) with the centromere determining element (CDE I)-like motif at the 5′ boundary of the UAS_{CYT1}. This motif overlaps with a consensus sequence for the binding of the general factor Abf1p. Functional analyses after the separate introduction of point mutations into both elements reveal no role for the latter protein and only a minor role for Cpf1p in the regulated expression of *CYT1/lacZ* chimaeric proteins. However, in *cpf1*-mutants, induction of *CYT1* reaches higher steady state levels and adaptation to aerobic conditions occurs faster than in wild-type. Thus, Cpf1p seems to reduce *CYT1* promoter activity under partly inducing conditions, e.g. when only one of the activators, Hap1p or the Hap2 complex, exerts its function.

INTRODUCTION

In the yeast, *Saccharomyces cerevisiae,* virtually all genes encoding polypeptides involved in oxidative energy metabolism are regulated by environmental conditions, in particular by carbon source and oxygen. The main factors conferring this kind of regulation on genes for respiratory enzymes are Hap1p and the Hap2/3/4/5 activator complex (1). Hap1p has both negative and positive effects on transcription (2,3). For activation Hap1p associates with its co-factor, heme (4). Since oxygen is absolutely required for the final steps in heme biosynthesis (5), the presence of heme is indicative of aerobic conditions and heme serves as the mediator to adapt the transcription machinery to the metabolic requirements of oxidative growth (6). Hap2/3/4/5 is composed of four subunits. Two of them, Hap2p and Hap3p, are responsible for DNA-binding. Hap4p carries activating function and is itself subject to glucose repression under the control of Mig1p (7). The recently discovered subunit, Hap5p, seems to be the nucleation element for the assembly of the multi-subunit Hap complex (8). As only the complete heteromeric protein is active, transcription of Hap2/3/4/5-dependent genes is exclusively induced in derepressed, glucose-free growth of yeast cells.

In addition, many of the yeast promoters of genes encoding respiratory enzymes contain recognition elements for one or several abundant nuclear proteins which bind constitutively and with high affinity to these DNA sequences. Some of these general factors fulfil multiple functions in the nucleus, e.g. in transcription, centromere and telomere structure and replication. Their function in transcriptional processes remains greatly unclear and is discussed controversially. Among these factors are Abf1p, Rap1p, Grf2p (Reb1p) and Cpf1p. Some of them are assumed to influence promoter structure by bending DNA and/or by positioning nucleosomes (9–14), processes suspected to influence promoter activity.

The *CYT1* gene, encoding the cytochrome c_1 protein, one out of nine subunits of the mitochondrial ubiquinol:cytochrome *c* oxidoreductase complex in yeast, contains an upstream activating sequence (UAS) with binding sites for at least four different nuclear factors. Functional significance of Hap1p and the Hap2/3/4/5 activator complex and their respective recognition sequences for the expression of *CYT1* has been established recently (15). Overlapping elements for the possible interaction of Cpf1p and Abf1p with the *CYT1* promoter are located at the 5′-border of UAS_{CYT1}. There are no indications for the association or a functional role of Abf1p, but here we present evidence that Cpf1p binds to its cognate sequences *in vitro.* Electrophoretic mobility shift assays (EMSA) yield complex patterns which vary dependent on carbon source and the availability of oxygen and heme. None of the Hap-regulators contributes to or is involved in the formation or regulation of these complexes. Rather, all of them are due exclusively to Cpf1p-binding to the CDE I-like element in the UAS_{CYT1}.

Cpf1p is a member of the bHLH group of transcription factors and binds as a homodimer to the octanucleotide sequence, 5′-RTCACRTG-3′, which is highly conserved in all centromeres of yeast chromosomes (14) and was defined as the centromere determining element I (CDE I). Mutations in CDE I motifs or deletion of the *CPF1* gene cause only a slight decrease in mitotic and meiotic stability of chromosomes (12,16).

In addition, CDE I-like elements occur in several promoters. Most genes encoding polypeptides involved in methionine biosynthesis (17) as well as some genes regulated by carbon source (18–20) contain Cpf1p-binding sites in their upstream

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Table 1. Carbon source dependence of *CYT1–lacZ* expression from single copy plasmids in wild-type (BWG1-7a) and *cpf1*-mutant (YSS90) yeast cells

Constructs [a]	ß-Galactosidase activity (U×O.D.$_{660}^{-1}$×ml·t^{-1}) [b]					
	BWG1-7a (wt)			YSS90 (*cpf1*$^-$)		
	Glc	Gal	Lac	Glc	Gal	Lac
YCpCL-S	5.8	24	66	17	59	73
YCpCL-P2	1.0	5.9	23	0.7	6.2	30
YCpCL-ΔP1H	0.2	0.3	1.4	0.1	0.2	1.0
YCpCL-UAS	4.6	12	62	14	36	70
YCpCL-Cpf$^-_{mut}$	12	20	95	12	35	54
YCpCL-Abf$^-_{mut}$	5.6	10	58	14	39	69

[a]B, *Bam*HI; H, *Hin*dIII; Hi1/2/3, *Hin*PI; P1/2, *Hpa*II; S, *Sau*3AI. Asterisks mark the sites of point mutations that destroy the elements recognized by Cpf1p or Abf1p, respectively (see Materials and Methods). Nucleotide positions in 5′-direction are given with respect to the A-residue of the 'ATG' translational start codon being +1.
[b]β-Galactosidase activities were measured after growth of the respective transformants on semi-synthetic media containing the carbohydrates listed in the Table.

regions. Disruption of the *CPF1* gene results in methionine auxotrophy, obviously due to the abolition of *MET16* expression (16,17). All other promoters show only minor disturbances in *cpf1*-mutants (10,20).

We show that the deletion of its binding site from UAS_{CYT1} as well as the mutation of the *CPF1* gene lead to increased adaptation rates to oxidative conditions after an anaerobic to aerobic shift and to higher steady state levels of *CYT1* expression. The data allow two possible interpretations. One assigns an adaptational function to the Cpf1 protein for fine tuning of UAS_{CYT1} activity to the needs of expression of the respiratory protein, especially under partially derepressing growth conditions. The alternative model attributes a more general role to Cpf1p in a different but also metabolically influenced, yet undefined process within the cell. The observed minor regulatory function of Cpf1p could then be caused by an indirect influence on the *CYT1* promoter. Both of these possibilities are discussed.

MATERIALS AND METHODS

Strains and growth conditions

Strain BWG1-7a (MAT**a**, *ade1-100, his4-519, leu2-3,112, ura3-52*) was used as wild-type strain (21); the mutants LGW1 (*hap2-1*) and LGW32 (*hap1-1*) are isogenic isolates thereof (22). YSS90 (MAT**a**, *ade2-101, leu2-1, lys2-801, ura3-52, cpf1::URA3*, Met$^-$) is a methionine-auxotrophic disruption strain of the *CPF1* locus (16). Yeast cells were grown on rich media (1% yeast extract) or semi-synthetic media (w/o Yeast Nitrogen Base; Difco) containing supplements according to the auxotrophic requirements of the respective strains with addition of (w/v) 5% glucose, 2% galactose or 2% L-lactate as carbon sources (23). Heme-dependent gene transcription was induced by deuteroporphyrin IX (10 µg/ml) or δ-aminolevulinic acid (25 µg/ml; both from Sigma, Deisenhofen) to the respective yeast cultures (24). Anaerobic yeast cultures were alternatively grown under an atmosphere of purified N_2 or CO_2 in the presence of 12 µg/ml ergosterol (Sigma, Deisenhofen) and 0.25% Tween 80 (BioRad, Munich) as described previously (15). For amplification of plasmids, *Escherichia coli* XL1-Blue cells (Stratagene) were transformed and grown according to standard procedures (25).

Plasmids and oligonucleotides

The yeast/*E.coli*-shuttle plasmid, YCp402 (26), served as a single copy vector in *S.cerevisiae* for insertion of a *CYT1–lacZ* fusion construct described in Oechsner *et al.* (15). The resulting plasmid, YCpCL-H, contained *CYT1* promoter sequences up to position –352. Additional promoter fragments of the *CYT1* gene were subcloned in pUC19 or pBR322 to obtain restriction ends compatible with insertion into the single *Hin*dIII site of the construct YCpCL-H (15). The resulting constructs were named according to the restriction sites at the 5′- and 3′-end of the respective promoter fragment or deletion (Δ) endpoints and can be derived from the schematic presentation of the *CYT1* promoter in Table 1. Two complementary oligonucleotides, 5′-GATCCGATATCACGTGACTAG-3′ and 5′-TCGACTAGTCACGTGATATCG-3′, containing the recognition sequence for Cpf1p-binding (underlined) were annealed, and ligated to pUC19 between the *Bam*HI and *Sal*I polylinker sites. The 150 bp *Pvu*II/*Hin*dIII fragment of this plasmid served to prove specificity of Cpf1p-binding in EMSA. For mutation of either the Abf1p or the Cpf1p binding sites the oligonucleotides 5′-GAATTCGGAGCGCCACGTGACTGAA**C**ATTTTTTTTT-CTCGG-3′ and 5′-GAATTCGGAGCGCC**T**CGTGACTGA-AG-3′ (mutated positions in bold-face and the binding element which is left intact underlined), respectively, were used in PCR together with a downstream primer to obtain DNA fragments which were inserted as upstream elements into plasmid YCpCL-H.

Preparation of nuclear extracts

For the preparation of nuclear protein extracts, cells were harvested from logarithmic cultures, washed in water, resuspended at 2 ml/g wet weight in 0.1 M Tris, 5 mM DTT (pH 9.4) and incubated at 30°C for 10 min. The pelleted cells were washed in 5 ml/g 1.1 M sorbitol, 20 mM KH_2PO_4, 2 mM EDTA (pH 7.4) and finally resuspended in an equal volume of the same buffer. Spheroplasts were generated by treatment with 1 mg/ml Zymolyase T20000 (Seikagaku Kogyo, Tokyo, Japan) at 30°C for 30–45 min. Two

washing steps in 1.25 M sorbitol, 20 mM KH_2PO_4, 2 mM EDTA (pH 7.4) removed the enzyme. The suspension of cell wall-depleted yeast at 15 ml/g in 0.6 M mannitol, 20 mM KH_2PO_4, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 50 U/ml trasylol and 0.6 µg/ml pepstatin (pH 7.0) was incubated on ice for 15–20 min. Then the spheroplasts were disrupted mechanically on ice. After sedimentation of cell debris and nuclei by centrifugation (Sorvall centrifuge, SS34 rotor) at 800 *g* for 5 min, the pellet was resuspended in the same buffer. Intact cells were removed at 30 *g* for 5 min. The nuclei were separated from the supernatant after another centrifugation at 500 *g* and nuclear proteins extracted as described by Pfeifer *et al.* (27), mainly through mechanical destruction of the nuclei in a Teflon-in-glass homogenizer in 2–5 ml of 200 mM Tris–HCl, 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 7 mM β-mercaptoethanol, 1 mM PMSF and 10% glycerol (pH 8.0). After precipitation with 1 vol of a saturated solution of $(NH_4)_2SO_4$ the proteins were dissolved in 0.2–0.5 ml of 20 mM HEPES, 5 mM EDTA, 7 mM β-mercaptoethanol, 1 mM PMSF and 10% glycerol (pH 8.0). Protein contents were determined according to the method described by Bradford (28).

Gel mobility shift assays and DNase I footprinting

DNA-binding assays in 20 µl of 10 mM HEPES/KOH (pH 8.0), 5 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol and 0.005% bromophenol blue (29) contained 3000–10000 c.p.m. (1–3 ng) of the radioactively end-labeled DNA fragment, 1–2 µg total *E.coli* DNA fragmented to an average length of 200 bp by digestion with DNase I and 10–20 µg nuclear proteins. Samples were incubated on ice for 15 min to allow binding of proteins to the DNA. The total volume was loaded on pre-run 4% polyacrylamide gels in 90 mM Tris, 90 mM borate, 2.5 mM EDTA (pH 7.5) and electrophoresed for 4–6 h at 120 V.

For DNase I footprinting four binding reactions were carried out in parallel using 40 µg protein per 20 µl each. After incubation on ice the samples received 2 µl of 10 mM $CaCl_2$ and 7.5 U DNase I (Boehringer, Mannheim). After 60 s the digestion was terminated by addition of 2 µl 0.2 M EDTA, extraction with phenol and precipitation with ethanol. Samples containing 10 000 c.p.m. were loaded on 6–8% denaturing polyacrylamide gels. Free DNA was treated in the same way except that nuclear proteins were omitted and digestion was carried out with 5 U DNase I. Sequencing reactions of a double-stranded plasmid (30) which contained an insertion of the fragment used for footprinting were performed in parallel. The respective primer was synthesized according to the sequence complementary to the insert and started with the nucleotide opposite to the radioactively labeled nucleotide at the 3′-end of the DNA fragment used in the binding assays.

Miscellaneous procedures

Ligation, transformation and restriction enzyme digests were performed according to standard procedures (25) or as recommended by the manufacturers. For Southern blotting, DNA was transferred osmotically from 1% agarose gels onto Biodyne A membrane (Pall, Dreieich) with 20× SSC at 4°C. Hybridization to randomly primed radioactive probes (Boehringer, Mannheim) was performed in the presence of 0.7% defatted milk powder in 6× SSC at 60°C overnight (31). Radioactive end-labeling of restriction fragments with single protruding 5′-ends was achieved through fill-in reaction with Klenow polymerase (25) and [α-^{32}P]dATP. β-galactosidase activities were determined after permeabilization of yeast cells with chloroform and sodium dodecyl sulfate (32).

RESULTS

The UAS region of the *CYT1* contains consensus sequences for the DNA binding of several constitutive and regulatory transcription factors

At the distal *CYT1*-upstream region, identified as the UAS (15), several protein-binding sites are concentrated within an interval of ~100 base pairs (positions –460 and –554; see the schematic drawing in Table 1). The factors which possibly interact with these sites are mainly regulators that modulate transcription in response to carbon source or oxygen (Adr1p, Hap2/3/4/5, Hap1p). It has been demonstrated that *CYT1* expression is, in fact, regulated by these parameters. An earlier report focused on the significance of the Hap1p- and Hap2/3/4/5-binding sites within the upstream region of *CYT1* (15). The presumptive Adr1p site immediately downstream of the Hap2/3/4/5 element is likely to be non-functional, since the distances to the next possible halfsites in 3′ direction (793 and 1640 bp, respectively) seem too long for an interaction between two bound Adr1p monomers. Also no evidence was found that the oligo(dT) tract (positions –530 to –538), discussed as a constitutive element in other promoters (33), contributes to the activation potential or regulation of the UAS_{CYT1}. At the 5′ boundary of UAS_{CYT1} overlapping consensus sequences for binding of Abf1p and Cpf1p are found. Both factors are members of the group of abundant DNA-binding nuclear proteins that participate in multiple nuclear processes (see Introduction; 34–36).

In vitro binding of proteins to the UAS_{CYT1}

To examine whether the nuclear factors identified by sequence homology of their *cis*-acting sites, in fact, interact with the UAS_{CYT1}, we performed *in vitro* binding studies using a 140 bp *Sma*I–*Hin*dIII fragment excised from pUC19 with the inserted *Hin*PI fragment, Hi2Hi3 (Table 1; Fig. 2, below), which contains all sequences contributing to UAS_{CYT1} activity (15). Since analysis of the *CYT1* promoter has shown that expression depends on carbon source and oxygen, nuclear protein extracts were prepared from wild-type strains which were grown on different substrates under aerobic or anaerobic conditions. Figure 1 shows the protein–DNA interactions observed in EMSA after incubation of these extracts with the UAS-containing fragment mentioned above. Nuclear proteins from aerobically grown cells give rise to a total of three major protein–DNA associations (C_I, C_{II} and C_{III} in Fig. 1). The quantitative ratio between them varies and depends on the respective carbon source present in the culture medium: on fermentable sugars the complexes C_I and C_{II} prevail, whereas proteins from respiring cells produce mainly the complexes C_{II} and C_{III}. In contrast, nuclear factors derived from anaerobically grown cells cause only a single complex, C_{IV}, which exhibits significantly higher mobility and in some assays appears to consist of a doublet. The electrophoretic separation of a sample which contains *CYT1* promoter DNA incubated with a nuclear extract from yeast grown on galactose under reduced access of oxygen is presented in Figure 1, lane gal+/–O_2. Under these conditions all four protein–DNA interactions described above can be observed simultaneously. The slight differences to the comparable complexes of the other lanes are due to the fact that the results derived from two experiments have been compiled in

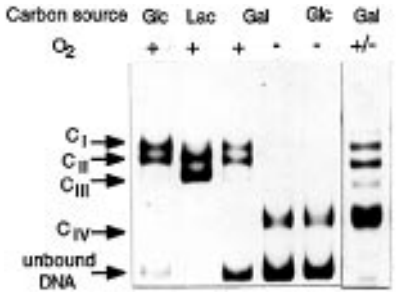

Figure 1. EMSA of the UAS_{CYT1} region bound by nuclear proteins extracted from wild-type yeast (BWG1-7a). Various carbon sources (Glc, glucose; Gal, galactose; Lac, lactate) and aerobic/anaerobic conditions (+/–O_2) during culturing are indicated. Protein–DNA complexes formed are designated C_I, C_{II}, C_{III} and C_{IV}, and the position of protein-free DNA is marked.

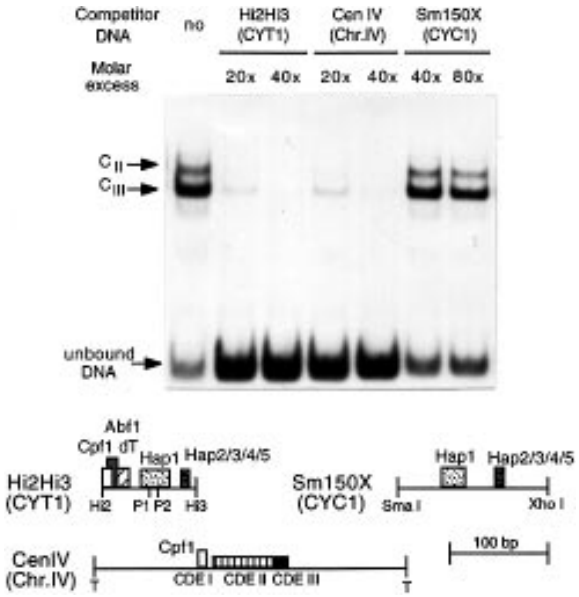

Figure 2. EMSA of protein–DNA interactions within UAS_{CYT1} without and with competition by homologous and heterologous DNA fragments. Proteins from lactate-grown wild-type yeast (BWG1-7a) were pre-incubated with unlabeled DNA fragments as indicated. Heterologous DNA (see bottom of the figure) was isolated as a 150 bp *Sma*I/*Xho*I fragment from the *CYC1* promoter (Sm150X) or as a 330 bp *Taq*I fragment from the centromere region of chromosome IV (CenIV). CDE I, II, III designate the tripartite consensus sequences of centromere determining elements in *S.cerevisiae*. The 105 bp *Hin*PI fragment (Hi2HI3) from the UAS of *CYT1* was used as the specific target for protein binding and competition.

this figure. This indicates that oxygen levels during the culturing of yeast specifically control the association of proteins with UAS_{CYT1} in a way characteristic of aerobiosis or anaerobiosis.

Competitive EMSA studies were performed to prove the possible participation of Hap1p-, Hap2p- or Cpf1p-binding sites in the observed complexes (Fig. 2). Protein–DNA interactions are not abolished when an unlabeled fragment (Sm150X) of the *CYC1* promoter, a gene which is regulated very similar to *CYT1*, is added to the binding assays to compete for the specific binding that might be caused by Hap1p or the Hap2 complex. Although the UAS region of *CYC1* as well as the UAS_{CYT1} contain Hap1p- and Hap2/3/4/5-binding sites (see lower part of Fig. 2), the *CYC1* fragment is not effective as a heterologous competitor. This

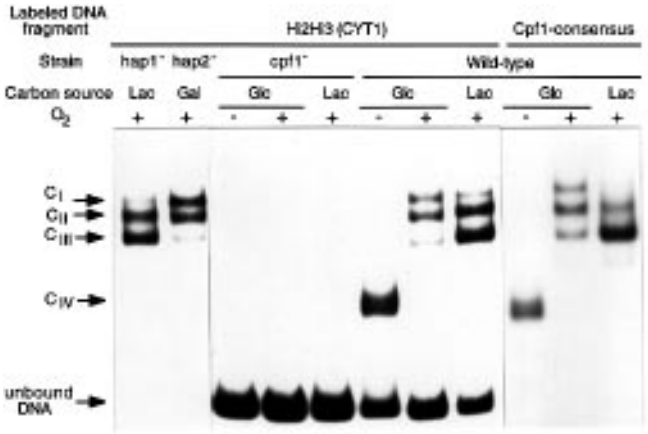

Figure 3. EMSA of radiolabeled DNA fragments containing the UAS region of *CYT1* (Hi2Hi3, see Fig. 2) or the cloned synthetic double-stranded oligonucleotide with the Cpf1p-binding site (Cpf1-consensus). Nuclear extracts were from wild-type (BWG1-7a) and mutant yeast strains (hap1$^-$, LGW32; hap2$^-$, LGW1; cpf1$^-$, YSS90) grown on various carbon sources under aerobic or anaerobic conditions. Protein–DNA complexes are designated according to Figure 1.

implies that neither Hap1p nor the Hap2 complex are involved in the formation of the observed protein–DNA associations. In contrast to the *CYC1* DNA fragment, addition of excess unlabeled fragment from the centromere region of chromosome IV (Cen IV in Fig. 2) abolishes all four protein–UAS_{CYT1} complexes simultaneously and therefore must be capable to serve as a target for those DNA-binding proteins. The candidate sequence common to both fragments, UAS_{CYT1} and Cen IV, is the consensus binding site of the centromere and promoter factor, Cpf1p, suggesting the participation of this protein in all four complexes.

Similar conclusions came from gel shift experiments using various *CYT1* promoter DNA fragments (not shown). All of the protein–UAS_{CYT1} associations observed mapped to the short 50 bp interval between the *Hin*PI site (Hi2, position –556) and the *Hpa*II site, P1 (position –506). For several reasons (see Discussion) these assays were not suitable to reveal interactions of Hap2/3/4/5 and Hap1p with their cognate DNA-binding sites, possibly due to the heteromeric nature of the Hap2 (8) and Hap1p complexes (2) and their low cellular concentration. Both activators have been shown to effect *CYT1* expression through their binding sites within UAS_{CYT1} (15). But as can be seen in gel shift assays with nuclear extracts from the mutant strains, LGW1 and LGW32 which are defective in either *HAP1* or *HAP2*, respectively, the band shift patterns remain unchanged (Fig. 3, lanes hap1$^-$ and hap2$^-$). Thus, neither Hap2p nor Hap1p contribute to the formation or seem to be involved in the regulation of the carbon source- and oxygen-dependent protein–DNA interactions described here.

For further binding studies a 150 bp DNA fragment was prepared with the insertion of the double-stranded DNA oligonucleotide which exclusively contains the Cpf1p consensus (see Materials and Methods). To compete for vector sequences, the 140 bp *Pvu*II/*Hin*dIII fragment out of pUC19 without insertion was added to these binding reactions. Figure 3 shows that the differential bandshift pattern with the specific DNA, Cpf1-consensus, is qualitatively identical to the one obtained with the fragment comprising the complete UAS_{CYT1} region. In binding assays carried out with nuclear extracts from the *cpf1*-mutant

strain, YSS90, no protein–DNA interactions were detectable (Fig. 3, lanes cpf1⁻). These results point to the exclusive participation of Cpf1p and its cognate binding element within UAS_{CYT1} in the oxygen- and carbon source-dependent formation of all the protein–DNA complexes observed.

DNase I protection patterns within UAS_{CYT1}

To analyze the quality of Cpf1p/DNA contacts at the sequence level in the UAS region of *CYT1* we performed DNase I protection assays (see Materials and Methods). After binding of nuclear proteins to UAS_{CYT1}-containing DNA fragments mild DNase I digestion revealed nucleotide positions exhibiting either reduced or increased susceptability to the enzymatic attack compared to free DNA (Fig. 4). The differences are the result of steric protection or increased exposition of single sugar–phosphate bonds due to conformational changes of the DNA after the association with the respective DNA-binding factors. The region inaccessible to DNase I after Cpf1p-binding has asymmetric character. Although the palindromic structure of the recognition sequence and the dimeric association of Cpf1p have been confirmed (16), qualitative differences of the contacts between each Cpf1p subunit of the homo-dimer and the respective half-site of the bound sequence have been reported also for other promoters (37,38). But more importantly, Figure 4 demonstrates that, although the pattern of protected bases around the site for Cpf1p binding is not altered, the incidence of hypersensitive sugar phosphate bonds in the 5′-flank of the footprint differs in dependence on whether proteins from aerobically (x) or anaerobically (u) grown yeast were used in these assays. The data indicate that, apart from changes in the bandshift pattern, also the quality of DNA contacts made by Cpf1p and/or the DNA conformation in the immediate vicinity of the bound dimer are affected by the oxygen tension.

Generation times of yeast cells in different carbon sources depend on a functional *CPF1* gene

Cpf1p-binding sites are found in the promoters of a number of genes encoding proteins required under glucose-free conditions (see Discussion). We performed long term shift experiments to test the possibility that Cpf1p might have an activating potential or a regulatory function in the expression of *CYT1* and other respiratory genes that are essential for the adaptation to or the maintenance of the oxidative metabolism. In this case, deletion of *CPF1* should result in altered levels of the respective gene products and a slower growth, particularly on non-fermentable carbon sources. For this purpose, wild-type and *cpf1*-mutant yeast cells were kept in logarithmic growth phase on fermentable (glucose) and non-fermentable (lactate) carbon sources. For several days the cultures were successively re-inoculated with defined cell numbers into fresh media containing the same or alternating nutrients. At the end of the experiment, the generation times over the total growth period were determined.

As can be seen from Table 2, fermentative metabolism (glucose) seems not to be influenced by the *cpf1*-mutation: the generation times of both, mutant and wild-type, yeast are identical. The non-fermentative culture of YSS90 on lactate, indeed, shows a slight reduction in growth rate. Compared to the wild-type yeast strain, generation times in alternating carbohydrates also are prolonged in the *cpf1*-mutant by nearly 20%. These changes in growth of YSS90, observed especially under non-fermentative conditions are moderate, but may hint to a possible marginal role

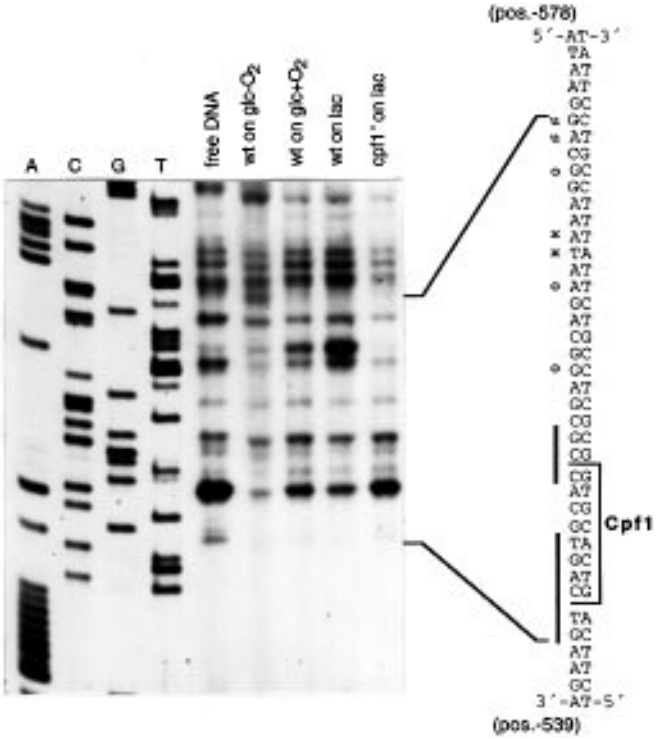

Figure 4. Protection of the Cpf1p recognition element and neighboring sequences within UAS_{CYT1} against digestion by DNase I. Nuclear extracts were prepared from wild-type (wt, BWG1-7a) and *cpf1*-mutant (cpf1⁻, YSS90) yeast after growth under the conditions indicated. The *Sma*I/*Hin*dIII fragment, radioactively 3′-end-labeled at the *Hin*dIII site, was derived from the insert of the plasmid pUC-S310T, the sequencing of which is shown on the left side of the figure. Free or protein-bound DNA was incubated for 1 min with 5 or 7.5 U DNase I, respectively. The region of interest is displayed at the right side of the figure with the Cpf1p binding site indicated by a bracket. Protected bases are highlighted by bars or 'o'; hypersensitive positions are emphasized by 'x' and 'u' (see text).

of Cpf1p in expression of genes coding for respiratory enzymes, whereas fermentative metabolism is not affected.

*CYT1–lac*Z expression in the *cpf1*-mutant, YSS90

To detect a possible regulatory role that Cpf1p might have in the transcription of *CYT1*, a set of fusions of *CYT1* upstream sequences to the *lac*Z gene of *E.coli* on single copy vectors were introduced into wild-type and *cpf1*-mutant yeast, and the β-galactosidase activities were monitored (Table 1).

Table 2. Generation times of wild-type (YPH266) and *cpf1*-mutant (YSS90) yeast during growth in media with constant or alternating (glucose↔lactate) fermentable and non-fermentable carbon sources

Strain	Generation times (h) in complete medium		
	Glucose	Lactate	Glucose↔Lactate
YPH266 (wt)	1.9	4.4	2.1
YSS90 (*cpf1*⁻)	1.9	4.7	2.5

Transformants which contain the plasmid YCpCL-ΔP1H carrying only the Cpf1p-, Abf1p-binding sites and the oligo(dT) region—with the sites for Hap1p and Hap2/3/4/5 binding deleted—exhibit no activation potential at all. None of these elements possesses UAS function on its own in this context. Albeit Abf1p-dependent activation of neighbouring oligo(dT)

tracts has been shown recently (11), this constellation is not efficient in the UAS_{CYT1} (Table 1, construct CL-ΔP1H).

The construct, CL-UAS, which has sequences upstream of the Cpf1p binding site deleted but leaves the UAS_{CYT1} region intact, provides nearly the same expression as the 5′-extended construct, CL-S. The additional DNA in the latter construct seems not to be necessary for *CYT1* expression. A point mutation which exclusively destroys the potential recognition sequence of Abf1p (CL-Abf_{mut}) has no effect on transcription. Therefore, Abf1p is not involved in *CYT1* expression under the conditions tested. In contrast, the mutation of the Cpf1p binding site has comparable consequences as observed when the intact constructs, CL-UAS and CL-S, are expressed in the *cpf1*-mutant strain: transcription is slightly elevated during growth on derepressing carbon sources and is 2–4-fold higher on glucose. This can only be explained by the lack of Cpf1p binding to the UAS region of the *CYT1* promoter in those cases. This means that Cpf1p has a repressing impact on UAS_{CYT1} activity particularly under fermentative growth conditions.

Construct CL-P2 which lacks the recognition sequences for both, Cpf1p and Hap1p, but contains the Hap2/3/4/5-binding site, shows a level of β-galactosidase activity which is independent on whether the Cpf1 protein is present in the cells or absent. As expected, Cpf1p has no influence when sequences upstream of the *Hpa*II restriction site (position –495), including its own recognition element, are missing. When the upstream region extends in 5′ direction to allow binding of Cpf1p to its own recognition sequence (CL-UAS and CL-S in Table 1), the derepressing activity of Hap2/3/4/5 is reduced strongly under the respective growth conditions.

To investigate further a possible effect of Cpf1p on the heme-dependent regulation of *CYT1* transcription mediated by the Hap1 protein, expression from *CYT1–lac*Z constructs was examined under aerobic and anaerobic as well as under heme-deficient and heme-induced conditions (Table 3). The latter was achieved by the addition of deuteroporphyrin IX (D9).

Table 3. Comparison of β-galactosidase expression from *CYT1–lac*Z constructs in wild-type (BWG1-7a) and *cpf1*-mutant (YSS90) yeast strains under various growth conditions[a]

	β-Galactosidase activity ($U \times OD_{660}^{-1} \times ml^{-1}$)					
	Glucose			Galactose		
Strain	–	+D9	$+O_2$	–	+D9	$+O_2$
BWG1-7a (wt)						
+ YCpCL-S	0.4	1.3	3.4	7.6	29	26
+ YCpCL-ΔP1P2	0.8	1.2	0.9	2.7	3.5	6.0
YSS90 (*cpf1*$^-$)						
+ YCpCL-S	0.7	8.3	13	28	58	46
+ YCpCL-ΔP1P2	1.1	1.6	1.8	30	25	44

[a]The respective plasmid-borne constructs are shown in Table 1. D9, deuteroporphyrin IX.

Cpf1p has no influence when the intact UAS_{CYT1} (CL-S in Table 3) is fully repressed during anaerobic growth on glucose. Under these conditions none of the activating factors, Hap1p or Hap2/3/4/5, is functional. After addition of the heme analogue, deuteroporphyrin IX, which fails to complement heme deficiency but substitutes for heme as a transcriptional co-activator, Hap1p is able to confer heme-dependent induction on *CYT1* transcription. The 4–6-fold higher expression in the *cpf1*-mutant under anaerobic, heme-induced conditions confirms that in wild-type cells Cpf1p has a repressing effect on the function of Hap1p. This influence is missing when the Hap1p-binding site has been deleted (Table 3, construct CL-ΔP1P2). In this construct Cpf1p has no influence during growth on glucose under heme-induction or aerobiosis, since the Hap2/3/4/5 complex is non-functional under these conditions and the binding site for Hap1p action is lacking.

Comparable influence of Cpf1p can be observed on the action of Hap2/3/4/5. During anaerobic growth on partly derepressing but fermentable carbon sources (galactose) this multi-subunit complex stimulates UAS activity ~20-fold above the level found on glucose. In the *cpf1*-mutant the derepression effect mediated through Hap2/3/4/5 is nearly 40-fold (Table 3, CL-S on Gal-O_2).

Addition of deuteroporphyrin IX or oxygen to cells growing anaerobically on galactose induce the UAS_{CYT1} to the derepressed state (Table 3, construct CL-S on Gal+O_2/D9). Both the Hap2 complex and Hap1p are active under these conditions, but Cpf1p has little effect on expression.

In summary, under conditions leading to half-induced expression of *CYT1*, e.g. under aerobic growth on glucose or anaerobic growth on derepressing carbon source when either Hap1p or the Hap2 complex is active, respectively, UAS_{CYT1} only reaches partial activity. This is not only due to the dependence on the single activator but also to a negative impact of Cpf1p on the activating potential of this effector. Whether this influence is direct, through Cpf1p acting as a UAS-regulating factor, or simply by interference with the activating potential of Hap1p or Hap2/3/4/5 via an indirect mechanism in which Cpf1p is involved, cannot be decided at present.

Thus, the results obtained with *CYT1–lac*Z constructs indicate a possible interaction between Cpf1p and Hap1p, the factor responsible for aerobic activation of *CYT1* transcription. To strengthen this conclusion, analyses of transformants grown under conditions that alter the influence of the Hap1 protein were carried out. Anaerobic cultures were shifted to aerobic conditions and β-galactosidase expression driven by the *CYT1* promoter was assayed at successive time intervals to monitor adaptation rates. Figure 5 shows that adaptation of *CYT1* expression to aerobic conditions is a slow process taking ~6 h in wild-type yeast cells. In the *cpf1*-mutant this process occurs much faster and, despite of activities 3–4-fold above the wild-type level, the steady state characteristic of aerobic expression in this strain was already reached within 2 h after the shift to aerobiosis. Thus, Cpf1p seems to have a strong negative influence on the Hap1p-mediated derepression of UAS activity in the *CYT1* upstream region, confirming the results from the previous chapter. Apparently, both the velocity of the adaptational process and the steady state of oxygen dependent expression of *CYT1* are impeded by Cpf1p.

Similar assays performed to observe the adaptational process of *CYT1* expression to changing nutrients did not show any differences in the time course in wild-type or *cpf1*-mutant yeast (not shown).

Placing its binding site between adjacent promoter elements enhances the negative influence of Cpf1p

All recognition elements for DNA binding proteins within UAS_{CYT1} are located on 105 bp between two *Hin*PI restriction sites (Hi2Hi3, position –555 to –450; see Table 1). In order to test whether Cpf1p and its binding site have a positional effect, the UAS_{CYT1}-bearing *Hin*PI DNA fragment, Hi2Hi3, was fused in

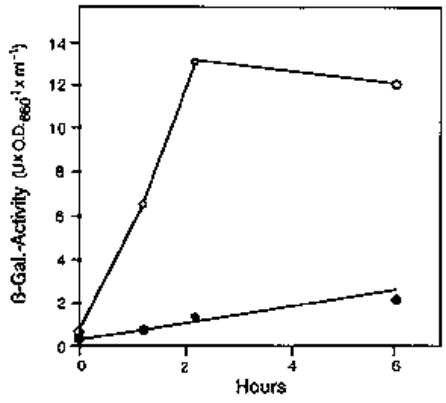

Figure 5. Influence of Cpf1p on *CYT1* expression during adaptation to aerobic growth conditions. Glucose-grown transformants of wild-type (●, BWG1-7a) and *cpf1*-mutant (○, YSS90) yeast cells containing the plasmid YCpCL-S were assayed for β-galactosidase expression from the *CYT1–lac*Z gene after shift to aerobiosis (time point zero).

Table 4. Influence of Cpf1p on expression of β-galactosidase from *CYT1–lac*Z constructs carrying UAS_{CYT1} in alternative orientations

Construct	β-Galactosidase activity (U×O.D.$_{660}^{-1}$×ml^{-1})					
	BWG1-7a (wt)			YSS90 (*cpf1*$^-$)		
	Glc	Gal	Lac	Glc	Gal	Lac
Cpf1 Hap1 Hap2/3/4/5 Hi2 Hi3 UAS H CYT1/lacZ YCpCL-Hi2Hi3	2.8	12	25	4.4	17	45
UAS H CYT1/lacZ YCpCL-Hi3Hi2	1.4	5.7	11	8.4	24	61

both orientations directly to the basal promoter in front of the *CYT1*/*lac*Z hybrid gene (Table 4).

The naturally oriented UAS had only 50% activity in wild-type yeast under all conditions tested (compare Tables 1 and 4, constructs CL-S and CL-Hi2Hi3 in BWG1-7a), yet the regulation potential remained unchanged. In the *cpf1*-mutant again an elevated expression is observed, but not as obvious as in the construct with the complete upstream region (compare Tables 1 and 4, constructs CL-S and CL-Hi2Hi3 in strain YSS90). Inversion of UAS_{CYT1} caused an additional decrease of UAS activity to 25% (Table 4, CL-Hi3Hi2) in wild-type transformants. In contrast, β-galactosidase expression from this construct is clearly enhanced in the *cpf1*-mutant. Obviously, the inverted arrangement of protein-binding sites improves *CYT1* expression. But, after binding of Cpf1p to its recognition site in this construct, proximal to the *CYT1* basal promoter (Table 4, CL-Hi3Hi2, *CPF1* wild-type background), transmission of derepressing interactions between the activators Hap1p and Hap2/3/4/5 and the basal promoter of *CYT1* may be severely disturbed. In the absence of the Cpf1 protein its binding site is non-functional, allowing higher UAS activity (Table 4, CL-Hi3Hi2, *cpf1*-mutant). Hap1p, the main activator during aerobic growth on glucose, and Hap2/3/4/5, predominantly responsible for elevated expression under derepressing conditions, are impeded in their action by Cpf1p to a comparable degree (50%).

Cpf1p does not influence the copy number of centromere-containing vectors in yeast transformants

It has been shown that the loss of Cpf1p-dependent centromere function results in ~10-fold elevation of chromosomal non-disjunction during mitotic and meiotic cell division in yeast (12,16). The same should be true for centromere-containing single copy plasmids, when transfected into *cpf1*-mutants. Then, selective growth conditions for the maintenance of these plasmids in transformants over long periods might enrich those mutant cells that contain additional copies of the plasmid as a consequence of this maldistribution. To exclude that the elevated β-galactosidase expression observed in the *cpf1*-mutant (see Tables 1, 3 and 4) is due to such a mechanism, wild-type yeast and the *cpf1*-mutant strain, YSS90, with and without the plasmid YCpCL-S were kept in logarithmic, aerobic glucose cultures (selective, semi-synthetic medium) for several days. One aliquot was examined for β-galactosidase activity, and from the other total DNA was prepared. Figure 6A shows autoradiographs of two Southern blots of these electrophoretically separated DNAs after hybridization to one of two probes, comparable in length and specific radioactive labeling. The 346 bp *Hin*dIII/*Bam*HI fragment was specific for the pBR322 portion of the plasmid YCpCL-S; the 348 bp *Hin*dII fragment obtained from the *CYT1* reading frame, not contained in the *CYT1–lac*Z fusion, served as a label for the genomic *CYT1* gene as a standard for single copy. As expected, in untransformed cells no vector signal is found. The intensities of the plasmid bands seem comparable in both transformants, indicating equal copy numbers in the wild-type and the strain lacking Cpf1p. Hybridization to the *CYT1* specific probe, gives an additional signal in the genomic DNA of the untransformed wild-type. The utilization of identical amounts of probes in both hybridizations reveals similar intensities of vector- and *CYT1*-specific signals—an indication of the maintenance of the plasmid as a single copy in both transformants. In contrast, Figure 6B demonstrates that expression of the Cyt1p–β-gal chimaeric protein is 4-fold increased in the *cpf1*-mutant background. Obviously the activity and thereby the expression of the *CYT1–lac*Z fusion in this strain is not paralleled by an increase in copy number of the carrier plasmid. Expression data shown in Tables 1, 3 and 4 also prove that there is no general increase of enzyme activity in *cpf1*$^-$ transformants, as would be expected for all vectors in the case of centromeric dysfunction in this strain. But, only single copy plasmids carrying *CYT1* promoter sequences upstream of position –495 show high enzyme levels even under partially induced conditions in the absence of Cpf1p. Therefore, the influence of Cpf1p on *CYT1* expression must be due to the respective promoter region and rather results from an inhibitory action exerted by Cpf1p.

DISCUSSION

Aerobiosis- and carbon source-dependent mechanisms jointly act on the promoter of the cytochrome c_1 gene to adapt expression to the needs of the cellular metabolism. The *CYT1* gene has been shown to be regulated by Hap1p mediating the response of transcription to the oxygen tension in the environment and by the Hap2/3/4/5 complex which stimulates mRNA synthesis when glucose is exhausted (15).

Besides the binding sites for these main regulators, an oligo(dT) tract is present in UAS_{CYT1} between positions –530 and –538. Such elements are discussed as possible constitutive effector sites with activating function (39) or as repressing elements after the DNA-binding of the Dat1 protein (33).

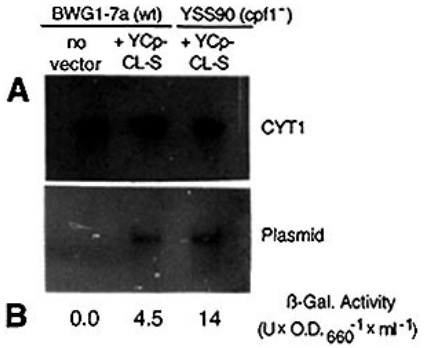

Figure 6. Plasmid-driven expression from a *CYT1–lacZ* hybrid gene and the copy number of the respective plasmid in dependence on the presence or absence of the CDE I-binding factor Cpf1p. Cells were grown in media containing 5% glucose. Preparation of total DNA and measurement of β-galactosidase activity were performed in parallel. (**A**) Southern blots of genomic DNA isolated from the strains indicated after hybridization to *CYT1*- and plasmid-derived probes (see text). (**B**) β-galactosidase activities obtained from the same cultures to monitor *CYT1* promoter-driven expression of the hybrid gene encoded by the plasmid (compare Table 1).

Recently it has been shown that (dA:dT) stretches rather appear to have auxiliary or intrinsic activating potential due to the exclusion of nucleosomes, without the need for the oligo(dT)-specific binding of a nuclear factor (39). In addition, consensus sequences for the multi-functional Abf1- and Cpf1-proteins are found at the 5′ border of UAS_{CYT1}, with the 5′ half-site of the palindromic Cpf1p element simultaneously representing the conserved 3′-end of an Abf1p recognition sequence. But, none of these (potential) protein binding sites, including the oligo(dT) tract, seems to play an important role in the expression of *CYT1*. A construct carrying these elements upstream of the *CYT1–lacZ* fusion gene (with the binding sites for both Hap-activators removed) is unable to stimulate transcription above the basal level under all conditions tested. On the other hand, the complete deletion of this region only slightly reduces UAS activity and does not disturb regulation of *CYT1* expression.

Interestingly, the UAS regions of other genes encoding respiratory proteins in yeast also contain overlapping elements for Cpf1p and Abf1p (18–20). In the case of the *QCR8* promoter a competitive association with the DNA of both factors was proposed, whereby Abf1p contributes an activating function and Cpf1p is the regulator for adaptation of gene expression to cell growth or cell division (40). This situation may not apply to the *CYT1* promoter. Cpf1p actually binds to its target *in vitro* and presumably also *in vivo*, as indicated by functional assays. But, the repressing effect of Cpf1p observed in *CYT1–lacZ* expression studies cannot be explained by a competition for binding with the activator Abf1p. We could show in this work that the Abf1p element is not functional and a protein–DNA interaction was not detected—even in a *cpf1*-mutant background.

In EMSA the occurrence of the various forms of Cpf1p–UAS_{CYT1} complexes in dependence on carbon source and oxygen may be attributable to different versions of Cpf1p. In the literature, Cpf1 proteins produced *in vivo* and *in vitro* can be found which differ in their molecular masses: 16 kDa (41), 58 kDa (42), 37, 60 and 64 kDa (16), respectively. This correlates with the appearance of multiple protein–DNA complexes in gel shift experiments. Affinity purified forms of Cpf1p (37 and 64 kDa) were demonstrated to dimerize in all three possible combinations which can be distinguished in EMSA after association with DNA (16). These complexes resemble those of Cpf1p with UAS_{CYT1} DNA, C_I, C_{II} and C_{III}. One possible interpretation of the data presented here is that under aerobic conditions two different versions (A and B) of Cpf1p coexist, which aggregate to give rise to the homodimeric DNA associations C_I (AA) and C_{III} (BB) and to the heterodimeric complex C_{II} (AB). The Cpf1p form A present in C_I prevails in glucose medium so that no homodimeric C_{III} is observed, whereas form B abounds in lactate medium with the consequence that no C_I is seen. Under anaerobic conditions a third version (C) of Cpf1p must be postulated interacting as a homodimer (CC) and making up complex C_{IV}.

Unspecific, possibly autocatalytic, proteolytic processing has been proposed to be the mechanism responsible for the appearance of truncated forms of Cpf1p (16,38,43). The following issues derived from our work argue against a fortuitous degradation of the protein: (i) nuclear extracts have been prepared in the presence of protease inhibitors so that the various versions of Cpf1p must have formed during growth prior to cell breakage. (ii) Comparable results were obtained with extracts from a strain (ABYS) deficient in proteinases A and B and carboxypeptidases Y and S (not shown). (iii) The occurrence of certain complexes reproducibly depends on carbon source and oxygen present during culturing of the cells and is independent of the strain which was used for preparation of the extracts. (iv) All four Cpf1p–DNA complexes can be observed simultaneously with extracts from hypoxic, partially derepressed cells. These observations point to a specific processing and/or modification of the Cpf1 protein, depending on nutritional and oxygen conditions during growth of the cells, leading to different forms of Cpf1p within the nucleus. Various Cpf1p–DNA complexes have also been observed by others (16,42,43) and were discussed controversially. In those cases, different metabolic states of the cells (derepressed late versus repressed log phase glucose cultures) may have led to the discrepancies. On the other hand, total cell extracts were used by other authors in their binding assays. This includes the possibility that predominantly cytoplasmic forms of Cpf1p were present in their DNA binding assays. The use of nuclear extracts in our experiments should lead to the enrichment of the functional nuclear forms of Cpf1p.

We analysed the highly regulated *CYT1* promoter and had to envisage different states of UAS activity in dependence on the crucial parameters, carbon source and oxygen. The finding of differential protein binding in the UAS region of *CYT1* was not unexpected, but that the Cpf1p–DNA complexes are affected by environmental conditions is unprecedented and surprising because Cpf1p is considered to be a constitutively DNA-binding protein. Recently, in the case of the constitutively DNA-binding factor, Abf1p, carbon source-dependent phosphorylation has been described to result in differential band shift patterns (44). Experimental evidence suggests that the differences obtained with Cpf1p are not primarily caused by phosphorylation and most likely are due to specific proteolytic processing (data not shown).

In contrast to Abf1p, which is involved in DNA replication (34) and transcriptional activation (35,36), no clear function can be attributed to Cpf1p. It binds to the centromere regions of all yeast chromosomes, where the binding site for Cpf1p is highly conserved and was defined as the ‘centromere determining element I’ (CDE I). To exert centromeric function, the DNA-binding domain of Cpf1p is important. However, Cpf1p is dispensable. Its absence just leads to a slightly higher error rate of chromosome segregation, due to chromosomal non-disjunction

during mitotic and meiotic processes (12,16). The same mechanism may be responsible for the loss of centromere-containing single copy plasmids from *cpf1*-mutant yeast during non-selective growth observed by others (16,42). This is not the case with the constructs in our investigation. Apparently, Cpf1p is not the decisive factor in the control of fidelity of chromosome segregation.

In promoters, the role of Cpf1p is even more puzzling. Without exception all genes encoding enzymes involved in methionine biosynthesis contain at least one CDE I-like sequence motif in their 5′ regions (17). Deletion of *CPF1* leads to methionine auxotrophy showing that for transcriptional activation of these genes Cpf1p is indispensable (43). The mechanism underlying these effects still remains obscure. A second set of genes with recognition sites for Cpf1p in their promoter regions are expressed under glucose-free and/or aerobic conditions, e.g. *GAL2*, *CBP2*, *QCR8* and *RIP* (18–20). In these cases the effect of Cpf1p on transcription has been described controversially from slightly activating to repressing. Cpf1p is not essential for expression of these genes, since loss of Cpf1p function does not result in Gal$^-$ or respiratory deficient phenotypes. We could show that, compared to the wild-type situation, generation times of *cpf1*-mutant yeast are slightly prolonged (10–20%) during oxidative and alternating growth on fermentable and non-fermentable carbon sources. In contrast to observations made by other investigators (16,42), this is not a general consequence of the *cpf1* mutation, since we found no reduction of growth rate in glucose-fermenting log-phase yeast cultures. Therefore, Cpf1p seems to exert some positive function during the adaptation to and/or in oxidative metabolism. Supposed that Cpf1p has a truly transcriptional function within the promoters of the above-mentioned genes, then it should act as an activator under conditions of glucose depletion. In contrast, the results obtained from *CYT1–lacZ* expression studies propose a negative regulatory function of Cpf1p in the *CYT1* promoter. In these experiments Cpf1p impedes the action of Hap1p and Hap2/3/4/5 and thereby retards aerobic and oxidative adaptations as well as processes leading to the derepressed state of promoter activity. Under growth conditions which are only half-optimal for oxidative metabolism, full induction of a respiratory gene like *CYT1* is not required. The data point to an additional fine-tuning control modus where Cpf1p is involved as a negative effector to reduce the potential of neighboring activators, i.e. of Hap1p or the Hap2/3/4/5 complex. As a consequence Cpf1p causes the UAS_{CYT1} to reach only partial activity under semi-induced conditions, i.e. when either Hap1p or the Hap2- complex is active—but not when both are active or inactive.

The appearance of carbon source- and oxygen-dependent interactions of Cpf1p with its binding site could be interpreted as an indication of a specific function of Cpf1p in the UAS_{CYT1}. Whether Cpf1p has a primary role in the modulation of *CYT1* gene expression or possibly is involved in a completely different process which may be controlled by parameters that affect cell metabolism and mitotic activity and has only indirect implications on transcription, cannot be decided at present.

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