
Demarcation of a sequence involved in mediating catabolite repression of the gene for the 11 kDa subunit VIII of ubiquinol-cytochrome *c* oxidoreductase in *Saccharomyces cerevisiae*

A.C.Maarse, M.de Haan, A.Bout and L.A.Grivell

Section for Molecular Biology, Department of Molecular Cell Biology, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

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ABSTRACT

A regulatory element has been identified in the promoter region of the gene encoding the 11 kDa subunit VIII of the ubiquinol-cytochrome *c* oxidoreductase in *Saccharomyces cerevisiae*. The element, which is approximately 40 bp long and situated 185 bp upstream of the initiator ATG, is essential for induction of gene expression during growth in the presence of non-fermentable carbon sources. This is shown by the regulated synthesis of β -galactosidase in yeast cells harbouring a CYC1-lacZ fusion gene, in which the CYC1 UAS's had been replaced by a 43 bp subunit VIII gene promoter fragment. In addition two DNA-binding activities, which may represent either separate factors or different forms of a single factor, have been detected. Both factors are abundant and they bind in a mutually exclusive fashion to a DNA sequence just upstream of the regulatory element. Although it is unlikely that these factors are directly involved in the response of the subunit VIII gene to catabolite repression, the position of their binding sites relative to the UAS and to the 3'-terminus of a gene located only 361 bp upstream suggest that they are important in modulating transcriptional activity of this region.

INTRODUCTION

The ubiquinol-cytochrome *c* oxidoreductase (bc₁-complex or complex III) in *Saccharomyces cerevisiae* consists of 9 to 10 different subunits (1,2), of which only cytochrome *b* is encoded by mitochondrial DNA (3). The genes coding for the other subunits are located in the nucleus on different chromosomes (4,5, P.J. Schoppink, personal communication). The synthesis of complex III is regulated in response to the need of the cell for mitochondrial function, which is determined by the available carbon source and levels of heme and oxygen. Regulation of expression of the genes encoding complex III subunits is mainly at the transcriptional level (6-8). In order to study the regulated expression of these genes, we have cloned several of them and determined their nucleotide sequences (8-11).

In this paper we describe the use of gene fusions with *E. coli* lacZ to establish the identity of a sequence element in the 5'-flank of the 11 kDa subunit VIII gene, which is essential for the induction of expression of this gene, in response to growth on a non-fermentable carbon source. We have in addition attempted to identify factors capable of mediating repression/induction by binding specifically to this site by use of a DNA-fragment retardation assay (12). These experiments have led to the detection of two specific DNA-protein complexes. A footprint analysis shows that the areas protected from DNaseI digestion in both

complexes are identical and located upstream of the regulatory element. Although available evidence suggests that these factors are themselves not directly responsible for mediating catabolite repression, the position of their binding sites relative to the UAS of the subunit VIII gene and 3' terminus of the URFx gene upstream, suggests that they may be involved in modulating transcriptional activity of this region. A more extensive analysis of these and other binding sites in the yeast genome (J.C.Dorsman et al., submitted) suggests a general role in both transcription and DNA replication and current efforts are directed at their further characterization.

MATERIALS AND METHODS

Strains and media

The yeast strain HR2 (α , leu2, his4, trp1::URA3) was used both as a host for expression of each fusion gene from the centromeric DNA constructs and for the isolation of yeast cell extracts used in DNA mobility shift experiments. Yeast cells were grown on medium containing 1% (w/v) yeast extract (Gibco), 1% (w/v) bactopectone (Difco) and 3% (w/v) of a particular carbon source as indicated in the legends.

Construction of subunit VIII-lacZ gene fusions

The 321 bp *Avall*-*Avall* fragment, indicated in figure 1a, was blunted with the Klenow fragment of *E. coli* DNA polymerase I and cloned into the *Sma*I site of pMCRF11 (generous gift from E.C.Friedberg, Stanford University, Stanford). From this clone a 284 bp *Hind*III-*Xho*I fragment was isolated and subcloned into pEMBL9 (13) digested with *Hind*III and *Bam*HI, regenerating the *Bam*HI site. This clone, p9-VIII-274 (fig. 1b), contains the first 7 coding nucleotides of the subunit VIII gene and 274 nucleotides of the contiguous 5'-flanking region. The 5'-flanking region was extended to 395, 487 and 883 nucleotides respectively by insertion of *Hind*III-*Hind*III fragments isolated from subunit VIII gene subclones in M13 mp8, described previously (8), into the *Hind*III site of p9-VIII-274. Plasmid p9-VIII-274 was linearized with *Hind*III whereas p9-VIII-395, p9-VIII-487 and p9-VIII-883 were linearized with *Pst*I. Each linearized plasmid was blunted with the Klenow fragment of *E. coli* DNA polymerase I and ligated with a *Bam*HI linker. After digestion with *Bam*HI, isolated *Bam*HI-*Bam*HI fragments were cloned into the unique *Bam*HI site of pJD1. Plasmid pJD1 (fig. 1d) is derived from the centromeric shuttle plasmid pLA433 (14) by replacing the 3.2 kb *Bam*HI-*Pst*I fragment indicated in figure 1c with the 5.5 kb *Bam*HI-*Pst*I fragment from pLG669-Z (15), containing *lacZ* gene sequences.

In order to construct analogous fusion genes containing less than 274 nucleotides of 5'-flanking sequences, p9-VIII-274 was linearized with *Hind*III and treated with the exonuclease *Bal*31, in essence as described earlier (16). *Eco*RI-*Bal*31 fragments were subsequently cloned into M13 mp10 (17) digested with *Eco*RI and *Sma*I. After DNA sequence analysis, defined *Bam*HI-*Bam*HI fragments were cloned into pJD1 as described above.

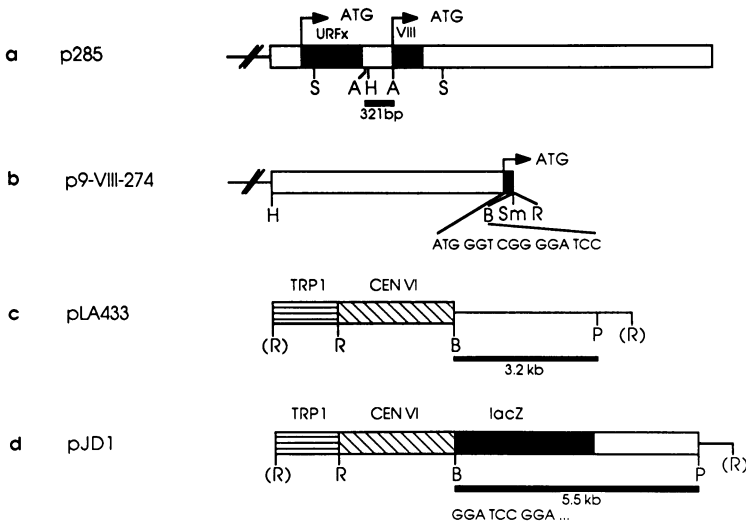


Figure 1. Plasmids used to construct subunit VIII-lacZ gene fusions. **a.** Plasmid p285 carries yeast DNA sequences (boxed area) containing two reading frames (filled boxes), one coding for subunit VIII of ubiquinol-cytochrome c oxidoreductase (VIII), the other coding for an unknown protein (URFx). **b.** 5'-Flanking region of the subunit VIII gene subcloned into pEMBL9; the initiator ATG codon is indicated. **c.** Plasmid pLA433 contains centromere sequences derived from chromosome VI (CEN VI) and the TRP1 marker (TRP1) of *S. cerevisiae*. The thin line indicates pBR322 sequences. **d.** Plasmid pJD1 is derived from pLA433 by replacing the 3.2 kb BamHI-PstI fragment indicated in **c** with a 5.5 kb BamHI-PstI fragment from pLG669-Z, containing lacZ sequences (filled box). A=Aval; B=BamHI; H=HindIII; P=PstI; R=EcoRI; (R)=EcoRI site destroyed during construction; S=Sall; Sm=SmaI.

Construction of hybrid promoter lacZ fusions

A 241 bp BamHI-BamHI fragment was isolated from a subclone in M13 mp10 (fig. 2a), generated with Bal31 as described above, containing 224 nucleotides of 5'-flanking sequences of the subunit VIII gene. A Sau3A digest of this fragment was cloned into the BamHI site of M13 mp18 (18) and the clone mp 18-UAS_{VIII} (fig. 2b), containing subunit VIII gene promoter sequences running from position -224 to -185, was selected by DNA sequence analysis. The transcription initiator site as well as the translation initiator ATG were isolated from p9-cyc1-I (fig. 2c), constructed by cloning of a 388 bp BamHI-SmaI fragment from pLG669-Z into the BamHI-HindIII sites of pEMBL9. A 264 bp CYC1 promoter fragment was isolated after digestion of this subclone with XhoI, treatment with the Klenow fragment of *E. coli* DNA polymerase I and subsequent digestion with EcoRI. This CYC1 promoter fragment was cloned both into M13 mp18 and mp18-UAS_{VIII}, each vector digested with EcoRI and SmaI. A CYC1 promoter fragment, including the CYC1 UAS's, was cloned in an analogous fashion as a 414 bp EcoRI-HindIII (blunt) fragment from p9-cyc1-I into M13 mp18. In order to fuse the promoter fragments present in mp18-UAS_{VIII}-

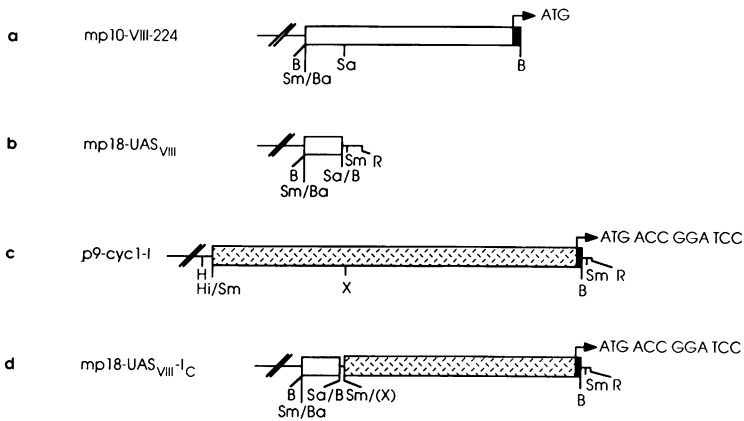


Figure 2. Construction of a subunit VIII-CYC1 hybrid promoter. The 43 bp BamHI- Sau3A fragment, present in the BamHI-BamHI fragment indicated in **a** by the boxed area, was subcloned into mp18 giving mp18-UAS_{VIII} (**b**). Transcriptional and translational initiator sites were introduced on an EcoRI-XhoI(blunt) fragment from p9-cyc1-I (**c**) into the EcoRI-SmaI sites of mp18-UAS_{VIII}, giving mp18-UAS_{VIII}-1C (**d**). B=BamHI; Ba=Bal31; H=HindIII; Hi=HindII; R=EcoRI; Sa=Sau3A; Sm=SmaI; X=XhoI; (X)=blunted XhoI.

1C (fig. 2d), mp18-1C and mp18-UAS_C-1C to lacZ, the corresponding BamHI-BamHI fragments were cloned into the unique BamHI site of pJD1. The correct orientation was verified by restriction enzyme digestion and ds DNA sequence analysis.

β-Galactosidase assay

Yeast cells were isolated from 10 ml cultures at an optical density of about 3 (glucose grown cells) or about 1 (cells grown on either glycerol or raffinose). Cells were washed once with 1 ml cold bidest, resuspended in 0.5 ml breaking buffer (100 mM Tris-HCl, 20% (w/v) glycerol, 1 mM DTT, pH 8) and disrupted at 4°C by agitation for 15 min with a vortex mixer at maximum speed with about half a volume of glass beads (0.45 mm) in the presence of 1 mM PMSF. The suspension was diluted with 0.5 ml breaking buffer + 1 mM PMSF and cleared from cell debris by centrifugation for 15 min at 15000g and 4°C. β-Galactosidase activity was measured using ONPG as a chromogenic substrate. Aliquots of 0.1-0.5 ml of supernatant were diluted to 1 ml with breaking buffer and incubated for about 3 hrs in the presence of 0.2 ml ONPG (4 mg/ml in breaking buffer) at 28°C. The activity is expressed as the number of nmoles o-nitrophenol produced x min⁻¹x (mg protein)⁻¹, using a molar extinction coefficient for o-nitrophenol of 4.5 x 10³ cm⁻¹M⁻¹ at 420 nm (19). Plasmid loss was determined by plating aliquots of the 10 ml cultures on non-selective medium and replica-plating the colonies on selective medium.

DNA binding assay

Yeast cell extracts used in DNA binding assays were isolated as described by Arcangioli and Lescure (20). In a typical DNA binding assay 1 µg protein was incubated with 1 fmol of a 3'-end-

labeled 284 bp HindIII-BamHI fragment in the presence of a 30-fold molar excess of aspecific competitor DNA (pEMBL9 digested with AluI) for about 15 min at 30°C in 20 mM Hepes pH 7.8, 100 mM NaCl, 5 mM MgCl₂, 2 mM EDTA, 7 mM β-mercaptoethanol, 1 mM PMSF and 10% (w/v) glycerol in a volume of 20 μl. The sample was loaded immediately on a 4% polyacrylamide gel (length x thickness = 135mm x 1.5mm) in TBE buffer (TBE: 90 mM Tris, 90 mM H₃BO₃, 2.5 mM EDTA, pH=8.3) and electrophoresed at 4°C for 5 min at 180 V followed by about 3.5 hrs at 130 V. After electrophoresis the gel was treated for 10 min in 10% (v/v) acetic acid, 10% (v/v) methanol, dried and autoradiographed. The competitor DNA p9-pho5, was constructed by cloning a 622 bp BamHI-Sall fragment containing 5'-flanking sequences of the yeast PHO5 gene (21) into pEMBL9 digested with BamHI and Sall.

From fig.5D we can roughly estimate the minimum abundance of the DNA-binding activities per cell, assuming a 100% efficient extraction from the yeast cells, and assuming 1 molecule factor being bound per complex with an infinite binding constant. Incubation of about 1 fmol DNA fragment with about 2 μg protein results in a retardation of 0.5 fmol fragment, equally distributed over C1 and C2. Therefore a minimum amount of 0.25 fmol of each DNA-binding activity is present in 2 μg protein. About 20 mg protein was isolated from a 250 ml culture at an OD_{600nm}=3, roughly corresponding to 1.5.10¹⁰ cells. The minimum amount of each DNA-binding activity is therefore at least 100 molecules per cell.

DNAseI protection experiment

About 40 μg protein from cells grown on medium supplemented with glucose, about 20 fmol of the 284 bp HindIII-BamHI fragment, 3'-end-labeled at the HindIII site, and about 400 fmol pEMBL9 digested with AluI were incubated in a volume of 60 μl for 10 min at 30°C. Following the incubation, 3 μl 10 mM CaCl₂ was added and the sample was split into 3 equal aliquots. After addition of 5 μl 100 mM EDTA to one aliquot it was put on ice. The other two aliquots were first treated for respectively 1 and 2 min with 50 ng DNAseI (Worthington) at 30°C before 5 μl 100 mM EDTA was added and they were put on ice also. DNA-protein complexes and free DNA were separated as described above, excised from the gel after one night autoradiography and eluted overnight at 37°C into 10 mM Tris pH 7.8, 1 mM EDTA, 0.2% (w/v) SDS, 0.3 M NaCl and 1 μg/ml sheared E. coli DNA (carrier). DNA fragments were precipitated with ethanol and analysed on an 8% polyacrylamide gel containing 7 M urea and TBE buffer. The gel was dried and autoradiographed.

Miscellaneous

Published procedures were used for: manipulation of DNA (22); determination of protein concentrations (23); ss DNA sequence analysis (24); ds DNA sequence analysis (25). Restriction enzymes, unless otherwise stated, and the large fragment of E. coli DNA polymerase I (Klenow fragment) were purchased from Boehringer. PstI, XhoI, XhoII, exonuclease Bal31 and T4 DNA ligase were obtained from Biolabs, whereas the BamHI linker was obtained from Amersham.

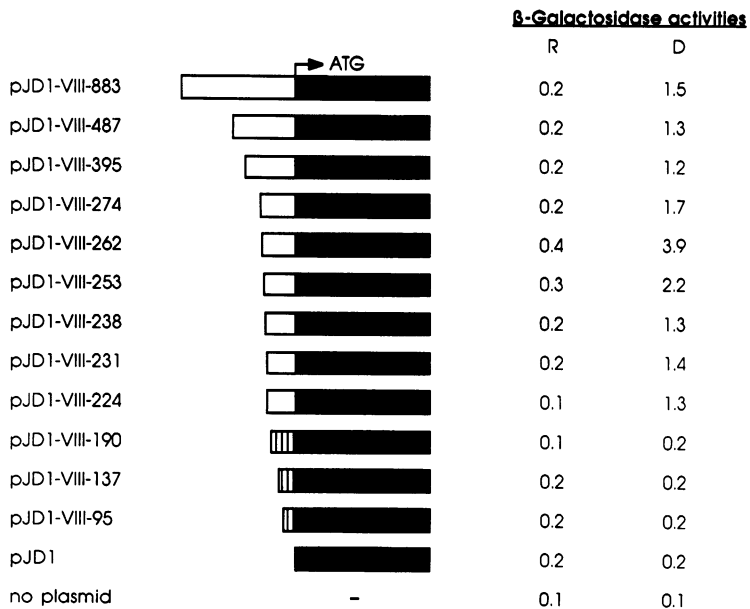


Figure 3. *Regulated β -galactosidase synthesis in yeast cells carrying subunit VIII-lacZ gene fusions.* Constructs containing from 883 bp (pJD1-VIII-883) to 95 bp (pJD1-VIII-95) of 5'-flanking sequences of the subunit VIII gene fused to lacZ, as well as the vector without subunit VIII gene sequences (pJD1) were transformed into the yeast strain HR2. Transformants and HR2 were grown on glucose (R) or glycerol (D) and β -galactosidase activities were determined in cell lysates as described in Materials and Methods. Each number indicates the average activity of at least two independent experiments, whereas in one experiment each activity was determined in duplicate. Activities are expressed as the number of nmoles *o*-nitrophenol produced per minute per mg protein. Filled boxes symbolize the lacZ reading frames, whereas open and striped boxes indicate 5'-flanking regions of the subunit VIII gene, being capable, respectively incapable of directing a regulated synthesis of β -galactosidase.

RESULTS

5'-Boundary of a sequence element involved in catabolite repression

In order to identify *cis*-acting regulatory elements in the promoter region of the 11 kDa subunit VIII gene, different parts of the region were fused to the *E. coli* lacZ gene in a yeast centromere-containing plasmid. The promoter fragments all have the same 3'-boundary namely nucleotide +7 relative to the ATG start codon of the subunit VIII gene. The 5'-boundary was varied from position -883 to -95, using fragments created by treatment with the exonuclease Bal31. Each construct was transformed into the yeast strain HR2 and β -galactosidase activities were measured in cell lysates after growth on a medium supplemented with either glucose or a non-fermentable carbon source. The results shown in figure 3 clearly demonstrate that the constructs with 224 or more nucleotides of the subunit VIII gene promoter fused to lacZ still

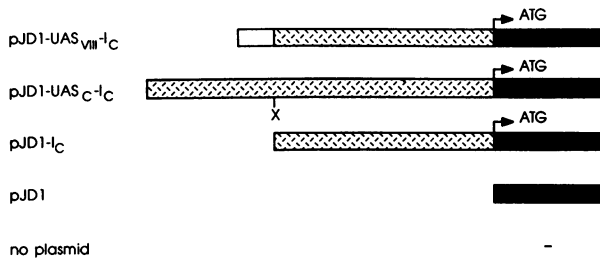
		<i>β</i>-Galactosidase activities	
		R	D
pJD1-UAS ^{VIII} -l _C	0.3	8.9	
pJD1-UAS _C -l _C	9.8	35.2	
pJD1-l _C	0.2	0.1	
pJD1	0.2	0.2	
no plasmid	-	0.1	

Figure 4. Regulated *β*-galactosidase synthesis in yeast carrying a subunit VIII-CYC1-lacZ fusion gene. The plasmids indicated were transformed into the yeast strain HR2 and *β*-galactosidase activities were determined in cell lysates after growth on glucose (R) or raffinose (D). Boxes filled with small stripes symbolize CYC1 promoter sequences. Further details are as described in the legend to figure 3.

allow induction of *β*-galactosidase activity in a lysate from cells grown on a medium supplemented with a non-fermentable carbon source, whereas the construct with only 190 nucleotides of the subunit VIII gene promoter did not. We conclude that 224 nucleotides directly upstream of the ATG start codon are both necessary and sufficient for a regulated expression. The clone with 262 nucleotides of the promoter region displays a reproducibly higher expression than either longer or shorter constructs. Although vector sequence effects cannot entirely be ruled out, this activity may indicate that additional sequences (and factors) contribute to the final level of expression of the subunit VIII gene.

3'-Boundary of the cis-acting element

As described in the previous section, sequences located between position -224 and -190 relative to the ATG initiator codon of the subunit VIII gene are necessary for a regulated expression. In order to establish whether sequences downstream of position -190 are also necessary for a regulated response to carbon source, use was made of a CYC1-lacZ fusion gene, kindly provided by L. Guarente. The UAS's of CYC1 in this construct were substituted by a 43 bp DNA fragment containing promoter sequences of the subunit VIII gene running from position -224 to -185 relative to the ATG start codon (fig. 2). The expression of this fusion gene, present on a centromere-containing plasmid, was induced in yeast cells grown on a medium supplemented with a non-fermentable carbon source and compared to its expression in cells grown in the presence of glucose. Constructs with and without the CYC1 UAS's were also tested for synthesis of *β*-galactosidase, after growth in the presence of either glucose or a non-fermentable carbon source. The activities found are shown in figure 4 and these show that the subunit VIII gene promoter fragment is capable of activating transcription, albeit at a lower level than the original CYC1 upstream region does. We conclude that the sequence lying between positions -224 and -185 relative to the ATG start codon indeed behaves as a

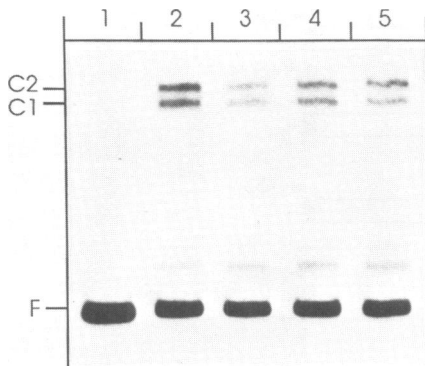


Figure 6. Comparison of lysates from cells grown on media supplemented with either glucose or glycerol in a DNA-binding assay. Samples were analyzed on a 4% polyacrylamide gel after incubation of the labeled fragment shown in fig. 5A without any protein (lane 1), with protein from glucose grown cells (lane 2), with protein from glycerol grown cells (lane 4) and with both types of protein (lane 5). In addition equal amounts of the samples analyzed in lanes 2 and 4 were mixed and subsequently analyzed in lane 3. Each lane, apart from lane 1, contains 1.6 μ g protein. Both complexes, C1 and C2, as well as free DNA are indicated.

typical upstream activating sequence (UAS) and that sequences downstream of -185 are not strictly necessary for this activity.

Proteins binding to the 5'-flank of the subunit VIII gene

We attempted to identify proteins capable of specifically binding to the UAS region of the 11 kDa subunit VIII gene defined above, by making use of a DNA fragment mobility-shift technique devised by Garner and Revzin (12). On incubation of a labeled fragment of 284 bp, containing 274 bp of 5'-flanking sequences (fig. 5A) with a crude lysate of glucose-grown cells, we were able to detect two prominent retarded bands, designated C1 and C2. Formation of the complexes was strongly reduced when unlabeled fragment was present in a molar excess of about 30 (fig. 5B, lane 13), whereas addition of promoter sequences of the yeast acid phosphatase gene or pEMBL9 sequences, also in a 30-fold molar excess (fig. 5B lanes 19 and 3), failed to reduce complex formation to any significant extent. Only when the amount of pEMBL9 competitor DNA was increased to a 60- or 90-fold molar excess, did reduced complex formation become apparent. Formation of complex was completely abolished on inclusion of 0.1 % (w/v) SDS in incubations (fig. 5C, lane 1). Incubation with increasing amounts of protein resulted in an equally increased formation of both complexes, indicative of the mutually exclusive binding of two or more proteins to the DNA fragment (fig. 5D). From the amounts of complex formed, we estimate that the factors bound in C1 and C2 are abundant, being present at a level of at least 100 molecules per cell (see Materials and Methods). This level is approximately the same in lysates from cells grown on medium supplemented with either glucose or glycerol, as judged by the comparable formation of C1 and C2 (fig. 6). An analysis of labeled fragment, incubated with protein from both lysates, added either separately (lanes 2 and 4) or simultaneously (lane 5), shows the same pattern of retarded bands on a native polyacrylamide gel. As is shown in figure 7, the factors present in C1 and C2 bind somewhere between position -253 and -238 relative to the initiator ATG (lanes 6 and 8). To identify the binding sites more precisely, an indirect footprint analysis was performed as

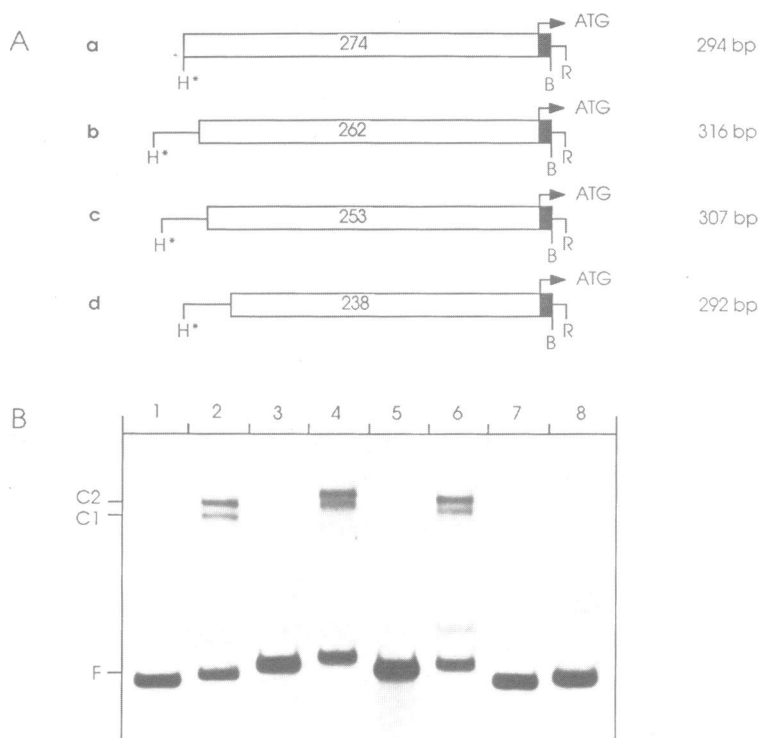


Figure 7. DNA-binding assay with subunit VIII gene promoter fragments containing 274, 262, 253 and 238 bp of 5'-flanking sequences respectively. Panel A depicts the fragments used, all 3'-end-labeled at the HindIII site (H*). Fragment lengths are indicated in the right column. B=BamHI; R=EcoRI. Panel B. Gel retardation analysis of fragments shown in panel A after incubation without any protein (lanes 1,3,5,7) and with about 4 μ g protein isolated from cells grown on medium supplemented with 3% (w/v) glucose (lanes 2,4,6,8). Lanes 1,2: fragment a; lanes 3,4: fragment b; lanes 5,6: fragment c; lanes 7,8: fragment d. Complexes (C1 and C2) and free DNA (F) are indicated.

described in Materials and Methods, using the labeled fragment shown in figure 5A. After DNaseI treatment free DNA (F) and DNA-protein complexes (C1 and C2) were separated on a native polyacrylamide gel (fig. 8A), isolated from this gel and rerun on a denaturing polyacrylamide gel (fig. 8B). It is apparent from this gel that the proteins in C1 and C2 protect the same DNA region (compare lanes 2 and 3 with lane 1). By lining up the DNaseI pattern with a known sequence ladder the protected area could be identified as shown in figure 8C. Deletion analysis reveals that the DNA-binding activities, responsible for the formation of C1 and C2, are not obligatory for induction of expression. A construct containing only 238 bp of the 5'-flank, which includes the cis-acting element defined above, but lacks the factor binding sites (fig.7 lane 8), shows an apparently normal regulated expression (fig. 3).

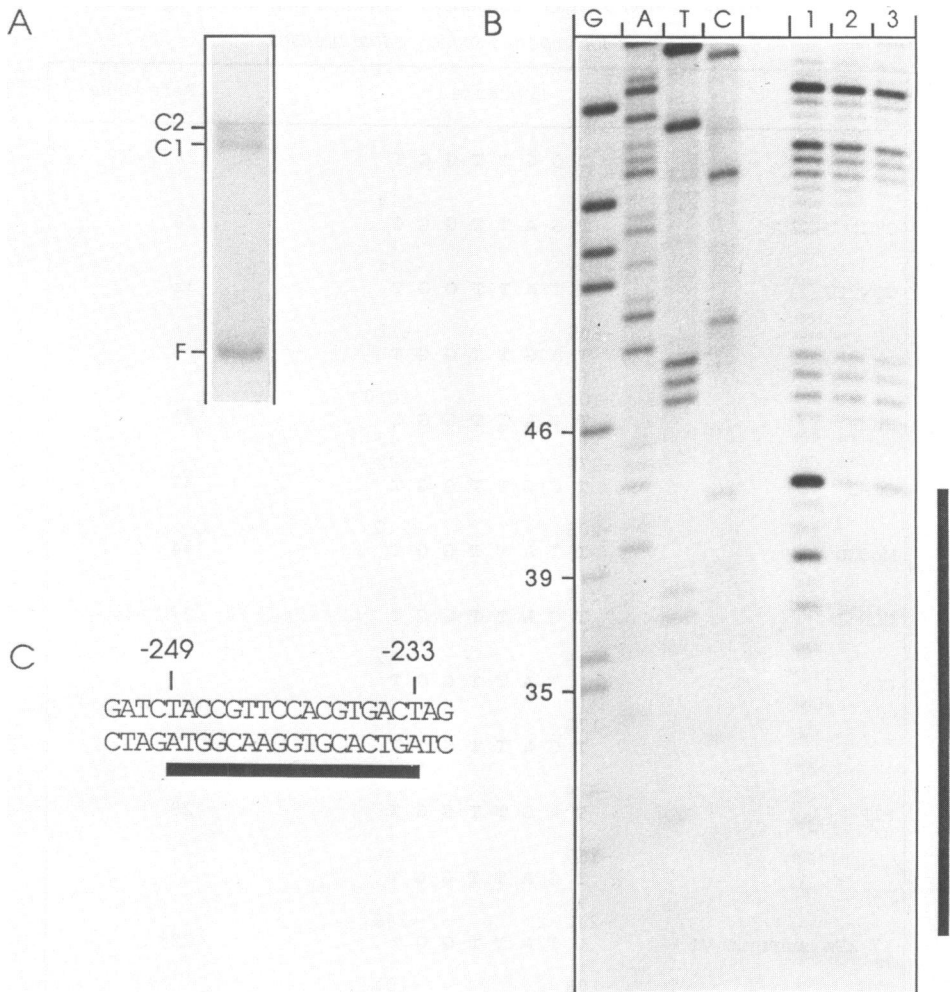


Figure 8. DNaseI footprint analysis of complexes C1 and C2. Panel A. Separation of DNA-protein complexes (C1 and C2) and free DNA (F) after DNaseI treatment (1 min). Panel B. Separated bands, shown in panel A, were isolated and rerun on a denaturing polyacrylamide gel. Lane 1: F; lane 2: C1; lane 3: C2; GATC: sequence ladder as size marker. The protected DNA region is indicated by the black bar. Panel C. Nucleotide sequence of the protected DNA region in the lower strand is underlined with a thick black bar. Positions are relative to the ATG initiator codon.

DISCUSSION

We have previously characterized genes coding for four of the imported subunits of the ubiquinol-cytochrome c oxidoreductase in yeast (8-11). Expression of these genes is regu-

Table 1. Comparison of TNPuTTGGT consensus sequence with 5'-flanking regions of genes for imported mitochondrial proteins

Gene	Sequence	Reference
CYC1	T G G T T G G T	26
	-281 -274	
CYC1-UAS2UP1	T G A T T G G T	26
	-281 -274	
COX IV	T T A T T G G T	32
	-605 -612	
	T A G T T G G T	
	-1017 -1010	
HEM1	T C A T T G G T	32
	-379 -372	
CCPO	T C G T T G G T	33
	-203 -210	
MnSOD	T T A T T G G T	34
	-214 -221	
COR I	T C A T T G G T	27
	-345 -352	
COR II	C T A T T G G T	9
	-179 -186	
	T C A T T G T T	
	-424 -417	
RIP	T A G T T G G T	28
	-153 -146	
	T G A T T G G T	
	-241 -248	
17 kDa subunit VI	G T A T T G G T	29*
	-108 -101	
	C C A T T G G T	
	-235 -242	
14 kDa subunit VII	T G A T T G G T	11
	-170 -177	
11 kDa subunit VIII	T C A T T G G C	8
	-205 -212	
CONSENSUS	T N P u T T G G T	26

Positions are indicated with respect to the initiator ATG cocons. Bold-faced characters indicate identities. *The corresponding coordinates are (1463,1470) and (1336,1329).

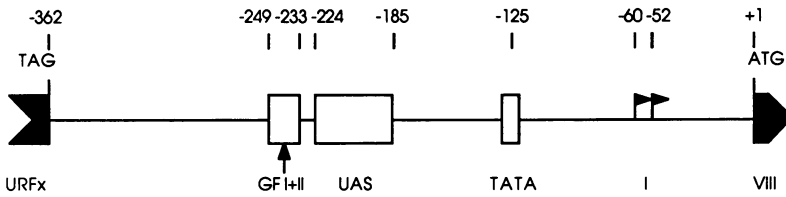


Figure 9. Schematic drawing of the 5'-flanking region of the 11 kDa subunit VIII gene. Transcription initiator sites (I) are indicated with arrow heads. In addition a TATA box, the UAS and the DNA region protected either by GF I or by GF II, are shown. At the very 5'-end, the 3'-end of URFx is depicted.

lated at the level of transcription in parallel both with each other and with that of genes for other mitochondrial proteins. In the case of the gene encoding the 11 kDa subunit VIII, our previous studies have revealed that the gene is preceded at a distance of only 361 bp by a gene encoding an as yet unidentified protein (URFx). Sequences necessary for expression regulated in response to carbon source are located within an area lying downstream of position -883 with respect to the initiator ATG of subunit VIII (8). A better demarcation of these sequences has been possible, by measuring the expression of the E.coli lacZ gene, directed by different subunit VIII gene promoter fragments. As shown in fig.9, these measurements reveal that the area between positions -224 and -185 relative to the ATG start codon is sufficient to promote regulated transcription, a property characteristic of other upstream activating sequences in yeast. Expression of lacZ under control of the UAS of the subunit VIII gene is lower than that given by the UAS elements of the CYC1 gene itself, which is in line with the relative levels of expression of these two genes in the cell.

Comparison of the sequence of this 40 bp segment with the 5'-flanks of other genes of complex III and other similarly regulated imported mitochondrial proteins has failed to reveal extensive similarity. There is however a sequence, centred around position -208, that displays a correspondence in 6 out of 8 positions to the element TNPuTTGGT/ACCAAPyN'A, identified by Olesen et al. in the 5'-flanks of genes responding to regulation by the products of the HAP2 and HAP3 loci (26). As table 1 shows, at least one copy of a similar element is present in the 5'-flanks of the genes for the 44 kDa CORI protein (27), the 40 kDa CORII protein (9), the Rieske iron-sulphur protein (28), the 17 kDa subunit VI (=3'-flank of PHO4 gene, 29) and the 14 kDa subunit VII (11). The physiological significance of this element has yet to be established, however.

In an attempt to identify factors capable of binding specifically to the promoter region of the subunit VIII gene, the DNA mobility shift assay introduced by Garner and Revzin (12) has been used. The technique has resulted in the detection of two DNA-protein complexes, which according to a subsequent, more detailed analysis (J.C.Dorsman et al., submitted) appear to be formed by the binding of at least two different factors (designated GFI and GFII), in a mutually

exclusive fashion to the same site. A DNaseI footprint analysis indeed confirms that the protected DNA segments in both C1 and C2 are identical and shows that they are located about 40 bp upstream of the UAS (fig.9). Examination of the protected region reveals it to contain the palindromic sequence motif CACGTG, an element recognized by the centromere binding protein CP1 (30) and subsequent retardation-competition experiments (J.C.Dorsman et al., submitted) make it likely that GFI is either identical to, or closely related to CP1.

Since deletion analysis using lacZ fusions shows that sequences upstream of -224 are apparently dispensable for regulated expression from a centromeric plasmid, neither GFI nor GFII seems to be directly responsible for the induction of expression of the subunit VIII gene during growth on non-fermentable carbon sources. Whether this also holds for the subunit VIII gene in a chromosomal context is at present unknown. So far we do not know the function of either factor, whose binding sites have turned out to include ARS (GFI) and CEN (GFII) sequences.

As yet, no factor binding to the regulatory region of the subunit VIII gene has been characterized. This is likely to be due to a low concentration and/or a relatively low affinity for the target sequence. Difficulties in detecting such regulatory interactions are not uncommon, as exemplified by the interactions of GCN4 and HAP2/HAP3 with their respective UAS's (31,26). Experiments to identify such regulatory factors using short promoter fragments and more highly purified protein fractions are now in progress.

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