Regulation of the Yeast CYTI Gene Encoding Cytochrome c\(_1\) by HAP1 and HAP2/3/4

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Mitochondrial biogenesis requires the coordinate induction of hundreds of genes that reside in the nucleus. We describe here a study of the regulation of the nuclear-encoded cytochrome c\(_1\) of the b-c\(_1\) complex. Unlike cytochrome c\(_1\), which is encoded by two genes, CYCI and CYC7, c\(_1\) is encoded by a single gene, CYTI. The regulatory region of the CYTI promoter contains binding sites for the HAP1 and HAP2/3/4 transactivators that regulate CYCI. The binding of HAP1 to the CYTI element was studied in detail and found to differ in two important respects from binding to the CYCI element. First, while CYCI contains two sites that bind HAP1 cooperatively, CYTI has a single high-affinity site. Second, while the CYTI site and the stronger HAP1-binding site of CYCI share a large block of homology, the HAP1 footprints at these sites are offset by several nucleotides. We discuss how these differences in HAP1 binding might relate to the difference in the biology of cytochrome c and cytochrome c\(_1\).

The mitochondrion of eukaryotic cells is the site of electron transport and energy production by oxidative phosphorylation. It is also the location of numerous biosynthetic and degradative pathways. While the organelle has its own genome which encodes about 10 proteins, the vast majority of the hundreds of proteins that reside in mitochondria are encoded in the nucleus (8). These include proteins that are necessary for macromolecular synthesis within the organelle, cytochromes and related proteins that carry out electron transport, and enzymes in anabolic and catabolic pathways that reside in mitochondria. All of these nuclear-encoded proteins must be imported into mitochondria after their synthesis in the cytoplasm.

The electron transport chain and related proteins are highly regulated in the yeast Saccharomyces cerevisiae. This organism will grow anaerobically, but under these conditions, the cytochrome cofactor, heme, is not synthesized (25). The activity of heme-requiring enzymes is muted under such conditions, resulting in several auxotrophies, such as for ergosterol, unsaturated fatty acids, and methionine. Further, the synthesis of the cytochromes themselves is repressed in anaerobically grown cells (13). When cells grow in the presence of oxygen, the level of synthesis of cytochromes depends on the carbon source in the medium. The level of synthesis of cytochromes is low when cells grow in glucose and high when they grow in a nonfermentable carbon source such as lactate (42).

Two trans-acting regulatory systems that govern cytochrome synthesis have been described. The first is a protein, HAP1 (CYPI), which is a transcriptional activator that responds to oxygen (12). The oxygen signal is registered by synthesis of heme (13), which activates the ability of HAP1 to bind to the upstream activation sequence (UAS) sites of particular cytochrome and related genes (30, 31, 41). The second system is composed of the HAP2/3/4 heteromeric complex that binds to CCAAT boxes of many genes involved in mitochondrial function, including cytochromes and heme biosynthetic enzymes (9, 29). The primary signal that regulates the activity of the HAP2/3/4 complex is the carbon source; the activity is low in glucose and high in lactate (12). The HAP2/3/4 complex has been conserved in eukaryotes that range from yeasts to mammals, although its cellular role has apparently been altered over evolution (1, 3, 28). The importance of the HAP complex to mitochondrial function in yeast cells is illustrated by the phenotype of hap2, or -3, or -4 mutants, an inability to grow on nonfermentable carbon sources. In contrast, hap1 mutants do grow on such carbon sources, because the genes activated by HAP1 can also be activated by the HAP2/3/4 system.

The regulatory circuitry of genes regulated by these HAP pathways has been partly delineated. These regulators were initially identified by their effects on CYCI (iso-1-cytochrome c). Both HAP1 and HAP2/3/4 bind to UASs in the CYCI promoter to activate transcription. A second gene, CYC7, encodes an isoform of cytochrome c, which is expressed at a low level even when cells grow under oxygen limitation (24, 35). While HAP1 activates CYC7 under aerobic growth, an independent, poorly characterized system keeps the gene on at a basal level under oxygen limitation (35). CYC7 is not regulated at all by HAP2/3/4. A situation similar to that of CYCI and CYC7 occurs in the case of the cytochrome c oxidase complex, which is encoded by several nuclear and mitochondrial loci. Subunit 5 of the complex is encoded by two nuclear genes, COX5A and COX5B (4, 5), which are expressed under aerobic growth and oxygen limitation, respectively (16). COX5A is regulated by both HAP1 and HAP2/3/4, while COX5B responds to HAP1 and not HAP2/3/4 (17, 40). It has been suggested that the anaerobic forms of these cytochromes may function more efficiently than their aerobic counterparts under conditions of oxygen limitation.

Less is known about the regulation of the cytochrome b-c\(_1\) complex, which passes electrons to cytochrome c in the inner membrane of the mitochondria. This complex contains one mitochondrially encoded cytochrome, b (encoded by COB), and one nuclear-encoded cytochrome, c\(_1\) (encoded by CYTI) (26, 36). The complex also contains numerous other nuclear-encoded subunits, including the Rieske iron-sulfur protein (encoded by RIP1), and other subunits encoded by

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CORI and CORII involved in the assembly of the complex (2, 21, 22). In this report, we begin an analysis of the regulation of this complex by studying CYTI. A detailed picture of the UASs and trans-acting factors that govern CYTI expression is derived. The regulation of this gene is compared and contrasted with that of CYCI.

**MATERIALS AND METHODS**

**Yeast strains.** BWG1-7a is MATa ura3-52 leu2-3 leu2-12 his4-519 H.1-l (12). Strains hap1Δ::LEU2 (32), hap2Δ::LEU2 (27), and TPH1 (hem1::LEU2) (33) were isolated from the BWG1-7a (hemi::LEU2) strain BWG1-7a. Strain JS101 contains a lacZ fusion inserted into the CYTI locus of BWG1-7a; pJCS105, described below, was partially digested with EcoRI to direct integration at -120 upstream of the CYTI initiation codon. (All position numbers are relative to the start of translation.) This resulted in a fusion of all cognate upstream sequences as well as 90 bp of coding sequence 5' to the lacZ fusion. Strain JS105 is disrupted by LEU2 at the CYTI locus by transformation and recombination with a HindIII-BamHI fragment from pJCS108, described below.

**Media and assays.** Undefined rich medium for yeast cultures was 2% yeast extract–1% peptone (Difco) (YPEP) with 2% glucose (YPD) or lactate (YEP). Plates were made by pouring 2% glucose (YPD) or lactate (YEPL) into a 9-cm Petri dish (1 ml of 0.5 M sodium acetate–10 mM Tris (pH 8)–10 mM EDTA–0.5% sodium dodecyl sulfate (SDS) overnight; DNA was then ethanol precipitated. Alternately, gel slices were electroeluted for 50 min at 150 V into 3 M sodium acetate in an IBI electrophoresis chamber, run in a buffer made of 20 mM Tris (pH 8) 3 mM sodium chloride, and 0.2 mM EDTA. Extracts were prepared, bound to end-labeled DNA fragments, and electrophoresed through 4% acrylamide gels as described previously (30, 31). [35S]Met-labeled HAP1 was a gift from Karl Pfeifer.

**DNase I protection.** Labeled fragments were incubated with protein extract from BWG1-7a pSDSHAP1 and treated with DNase I as described previously (10). Sufficient HAP1 extract was used to bind DNA into a complex, as assessed by a gel shift assay.

**Ethylation interference footprinting.** DNA fragments were reacted with diethyl sulfate (DES) before being used in a binding reaction. The base specificity of this chemical is the same as for dimethyl sulfate (38), but ethyl groups are attached instead of methyl groups. Fragments in 0.1 ml of DES reaction buffer (500 mM sodium cacodylate, pH 8) were treated with 2 μl of DES for 1 h at 37°C, ethanol precipitated three times, dried, and resuspended in Tris-EDTA. The ethylated fragment was bound to protein extract and electrophoresed as described above to separate bound from free DNA. DNA was isolated from the gel by electrophoresis onto a sheet of NA45 DEAE-paper (Schleicher & Schuell) in 1× TBE at 4°C for 2 h at 0.5 A. Fragments were eluted from the paper in 1 M NaCl–0.1 M EDTA–29 mM Tris (pH 8) for 20 min at 65°C. The eluate was extracted twice with chloroform and ethanol precipitated. Fragments were resuspended in 43 μl of 20 mM sodium acetate–1 mM EDTA and cleaved at the ethylation sites at 90°C for 30 min by addition of 7.5 μl of 1 M NaOH. Samples were neutralized by adding 7.5 μl of 1 M HCl and 50 μl of 20 mM Tris, pH 7.5. DNA was precipitated with 4 μg of calf thymus DNA, 150 μl of 70% ethanol, and 1 ml of n-butanol. After resuspension in 150 μl of 1% SDS, DNA was precipitated again and washed with butanol, dried, and loaded on a sequencing gel.

**Binding rates.** The on-minus-off rate was measured by
TABLE 1. Activity and regulation of the CYTI-lacZ fusion*

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>HAP1 HAP2</th>
<th>hap1 HAP2</th>
<th>HAP1 hap2</th>
<th>heml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Lactate</td>
<td>Glucose</td>
<td>Lactate</td>
</tr>
<tr>
<td>CYTI</td>
<td>21</td>
<td>142</td>
<td>2</td>
<td>78</td>
</tr>
<tr>
<td>CYCI</td>
<td>164</td>
<td>1,024</td>
<td>6</td>
<td>192</td>
</tr>
</tbody>
</table>

* β-Galactosidase was assayed in strain BWG1-7a, or mutant derivatives, bearing pJCS109 (CYTI-lacZ) or pLG312 (CYCI-lacZ) (33). The mutant strains bearing the hap1-1, hap2-1, or heml-1 mutation have been described elsewhere (12, 35). Strains were grown in glucose or lactate. The heml strain was grown in glucose in the presence of low (0.5 μg/ml) or high (50 μg/ml) concentrations of β-ALA. The HAP1 hap2 strain was grown only in glucose because the hap2 mutation prevents growth in nonfermentable carbon sources. ND, not determined.

mixing crude extracts with fragment and then loading the gel at various times after mixing. This is not a strict on rate, since dissociation of the complex is possible during the reaction.

The off rate was measured by allowing binding of crude extract and fragment to proceed to equilibrium (30 min) and then adding unlabeled competitor (0.5 μg of XhoI-digested pLGA312 at time zero, an amount previously determined to be enough to eliminate binding to the labeled fragment.) The gel was loaded at various times after addition of the competitor.

RESULTS

Cloning a genomic CYTI fragment with extensive 5’ DNA. The yeast CYTI clone described by Sadler et al. (36) contained only 352 nucleotides of 5’ flanking DNA. To isolate the gene with a more extensive upstream region, we generated a strain in which a CYTI clone could be selected, by using the clone of Sadler et al. to disrupt the CYTI coding sequence. An internal 148-base BstEII fragment was replaced with the LEU2 gene to generate pJCS108, and a DNA fragment encompassing this replacement was used to transform BWG1-7a to Leu+. Disruption of CYTI was confirmed by Southern blotting (data not shown). The disrupted strain could not grow on glycerol, nor could revertants that would grow on glycerol be isolated, even after ethyl methanesulfonate mutagenesis. This finding indicates that CYTI is the only gene in S. cerevisiae encoding cytochrome c1. The CYTI clone was isolated in this strain from a 2-μm library by selection for growth on glycerol.

Construction of a CYTI-lacZ reporter. To examine the regulation of the CYTI nuclear gene of S. cerevisiae, we initially used the clone of Sadler et al. (36) to construct a fusion with 352 nucleotides of CYTI 5’ DNA (pJCS105). However, when this plasmid was transformed into S. cere-
visiae, no β-galactosidase activity was detected, suggesting that key sequences in the CYTI promoter were missing. Using the genomic clone that we isolated, a CYTI-lacZ fusion containing about 1,800 bases of CYTI 5′ flanking DNA was constructed in a vector containing an autonomously replicating sequence and a centromere (pJCS109).

The regulation of the reporter vector, pJCS109, was studied under various conditions and in different mutant strains (Table 1). Several important aspects of regulation were noted. First, like CYCI, CYTI was regulated both by carbon source and by heme. Activity was very low under heme limitation, intermediate in glucose in the presence of heme, and fully induced in lactate. The full range of regulation was about 70-fold. Second, mutations in either HAP1 or HAP2 reduced expression, indicating that CYTI, like CYCI, responds to both the HAP1 and HAP2/3/4 systems.

Mapping of the HAP1- and HAP2/3/4-responsive elements of CYTI. The sequence of CYTI 5′ flanking DNA was determined to about -800 from the ATG (Fig. 1). Inspection of this sequence revealed strong similarity to both the HAP1 site in CYCI, UAS1B (to be described in detail later), and the HAP2/3/4 consensus sequence (TNA/GTTGG) between -464 and -512 (each underlined in Fig. 1). The 5′ ends of CYTI mRNA were mapped by extension of a primer which binds to lacZ (Fig. 2). Strains from which RNA was obtained contained either no reporter plasmid (lane 1), the CYTI-lacZ fusion in pJCS105 inserted into the chromosome (strain JS101) (lanes 2 and 3), or the multicopy plasmid pJCS110 (a 5′ deletion of pJCS109 ending at -736) (lanes 4 and 5). Because pJCS110 contains a small insertion of DNA linker nucleotides at the CYTI-lacZ junction relative to pJCS105, the five prominent extension products are shifted slightly to a slower mobility in the gel. These products correspond to initiation sites at -140, -182, -268, -284, and -297 (indicated by triangles above these sites in Fig. 1) relative to the CYTI ATG.

Deletions were constructed to determine the possible significance of the HAP1 and HAP2/3/4 binding-site homologies mentioned above (Fig. 3). A 5′ deletion to -510 did not affect activity in glucose or lactate. Deletion to -492, which removed the HAP1 binding-site homology, greatly reduced the activity in glucose but only mildly lowered the induced activity in lactate. This is expected for loss of HAP1 responsiveness, because HAP1 is much more active than HAP2/3/4 when cells grow in glucose. Deletion of the HAP1 site in CYCI, UAS1, also has a large effect on activity of the promoter in glucose (12). A further deletion to -464, which removed the HAP2/3/4 homology, abolished activity in lactate as well as glucose. A corresponding deletion of both sites in CYCI has similar effects on activity. An internal deletion removing the HAP2/3/4 but not the HAP1 homology gave a low intermediate level of activity in glucose and lactate. This intermediate level of activity required HAP1 because it was greatly reduced in a hap1 mutant strain but was unaffected in a hap2 mutant (data not shown). These findings provide evidence that the HAP1 and HAP2/3/4 homologies are both functional and respond to the two regulators in vivo.

The HAP1 element was studied in detail and is described below. The HAP2/3/4 element was not investigated in detail, in part because of the difficulty in obtaining in vitro binding to sequences that do not contain precisely the canonical CCAAT. It is extremely likely, however, that HAP2/3/4 functions at the -492 to -464 element. First, the region contains the sequence TTGGTTGGTGG, which matches perfectly the UAS2 of CYCI, a known HAP2/3/4 element (7). Second, transcription of CYTI is greatly reduced in the hap2 mutant strain (Table 1).

Attempts to study the response of this element to HAP2/3/4 in vivo was complicated by the strong apparent synergy between the HAP1 and HAP2/3/4 homologies in the CYTI promoter. Thus, deleting either the HAP1 homology (del -492) or the HAP2/3/4 homology (del -478 to -368) greatly reduced expression. This level of synergy is somewhat greater than for UAS1 and UAS2 in CYCI. Nevertheless, we were able to show that the low level of activity in del -492 was reduced further in a hap2 strain (from 0.4 to 0.1 U of activity). In summary, our in vivo analysis suggests that the CYTI promoter bears functional HAP1 and HAP2/3/4 sites that are arranged in a manner similar to that of CYCI.

Binding of HAP1 to the CYTI UAS. We wished to obtain evidence that HAP1 itself bound to the HAP1-responsive element of CYTI. Accordingly, we carried out gel shift analysis of a 77-bp fragment encompassing bases -528 to -455 in CYTI 5′ DNA. A protein bound to this probe that was absent in a strain with a hap1 disruption (Fig. 4, lane 1) and increased in concentration in strains that expressed increased amounts of HAP1. As found for UAS1 or CYCI, a strain bearing HAP1 on a high-copy-number plasmid (lane 3) displayed a moderate increase in the levels of the complex compared with a wild-type strain (lane 2), and a strain with HAP1 under GAL control (pSD5HAP1) showed a large increase (lane 4). This protein-DNA complex required heme
FIG. 3. 5' deletions in the CYT1 promoter. Deletions were constructed in the CYT1-lacZ vector as described in Materials and Methods. The deletion endpoints are indicated, as are the HAP1 and HAP2/3/4 consensus sequences (boxes). The deletion constructs were assayed for β-galactosidase activity in BWG1-7a grown in glucose or lactate.
for its formation (data not shown), a requirement also seen for HAP1 binding to UAS1 or CYC7.

Although these data made it extremely likely that this complex represented HAP1 binding to the CYT1 site, we confirmed this conclusion by synthesizing the protein in vitro labeled with [35S]Met. As shown in Fig. 5, the DNA-binding domain of HAP1 (residues 1 to 245) synthesized in vitro bound to probes containing CYT1 (as well as UAS1 or CYC7) but not UAS2 of CYC1. A band seen in all lanes corresponds to HAP1 (residues 1 to 245), while only those lanes containing fragments to which HAP1 binds showed the additional HAPI-DNA complex, indicated by the arrow. We conclude from this binding analysis that CYT1 contains a single HAPI-binding site which mediates activation by the protein in vivo.

The HAP1-binding site in CYT1 was pinpointed by DNase I and ethylation interference analysis (Fig. 6). In the DNase I experiment (lanes 1 to 5), we detected protection by an extract that contained sufficient HAP1 to bind to all of the probe. The protected sequences are indicated with a bracket. In the ethylation interference experiment (lanes 6 to 10), the DNA was pretreated with DES as described in Materials and Methods and then subjected to gel shift after incubation with an extract containing HAP1. The HAP1-bound and free DNA was excised and cleaved prior to analysis on a sequencing gel (Fig. 6). Bases which when ethylated interfere with binding are indicated by arrows.

A summary of the footprinting data along with a comparison of similar data for UAS1 (30, 31) is shown in Fig. 7. Best alignment of the sequences reveals a 15-nucleotide block in which 12 bases match. Amazingly, this alignment offsets the DNase and alkylation interference footprints of the two sites by several bases, a point to which we return in Discussion. The CYT1 site also contains a strong dyad symmetry (indicated by inverted arrows in Fig. 7) that is not nearly as evident in the UAS1 site (30, 31). This dyad predicts that HAPI binds to its target sites as a dimer.

Affinity of HAPI for the CYT1 site versus UAS1 of CYC1.

The UAS1 of CYC1 contains two sites, the A and B site, to which HAPI binds cooperatively (19, 30). UAS1B is a much stronger binding site than UAS1A, and it binds HAPI with an affinity comparable to that for the single HAPI-binding site of CYC7 (31). To compare the relative affinities of HAPI for UAS1 versus CYT1, we determined on and off rates of the protein for UAS1B and the CYT1 site (Fig. 8). While on times were rapid and comparable for the two sites, the off time was three- to fivefold longer for the CYT1 site. These results suggest that HAPI has a higher affinity for the CYT1 site than for UAS1B. This higher affinity could give rise to a higher activity in vivo. The intact UAS1 may compensate for this difference by containing the second HAPI-binding site, UAS1A.

FIG. 4. Binding of HAPI in yeast extracts to the CYT1 site by gel shift. Extracts were prepared from BWG1-7a bearing a hapl null mutation (hapl1::LEU2), a single copy of HAP1 (hap1::LEU2), a 2μm multicopy HAP1 plasmid (pHAPI1) (30), or a plasmid with HAPI under control of the GAL promoter (pSD5HAPI1) (18). The probe was a Smal-TaqI fragment of the del 202 plasmid from −528 to −455 in CYT1 upstream DNA. The binding mix contained 0.5 μg of salmon sperm DNA, 15 μg of heme per ml, and 0.5 ng of labeled CYT1 fragment. The complex with the mobility characteristic of a HAPI-DNA complex is indicated by the arrow.

FIG. 5. Binding of HAPI synthesized in vitro to the CYT1 site. A [35S]Met-labeled HAPI fragment (residues 1 to 245) was synthesized in vitro in a rabbit reticulocyte system and used in a binding reaction with 5 ng of unlabeled DNA. The binding mix contained 0.3 μg of salmon sperm DNA and 4 μl of translation extract. The DNA fragments used are a 160-base Smal-BamHI fragment from del 202 (−528 to −368) containing the CYT1 UAS1 and UAS2, a 90-base Smal-XhoI fragment containing CYCI UAS1, a 125-base Smal-XhoI fragment from del 205 containing the CYT1 UAS2, and an 80-base BglII-XhoI fragment containing the CYC7 HAPI site. The band present in all lanes corresponds to labeled HAPI fragment. The HAPI-DNA complexes are indicated by the arrow.

DISCUSSION

In this report, we describe an analysis of the transcriptional regulation of the yeast nuclear gene, CYT1, encoding cytochrome c, of the b-c complex. In several respects, the regulation of this gene mirrors that of the CYC1 gene encoding the major isofrom of cytochrome c. Both genes are regulated by the two signals of heme and carbon source. Activity is low or missing in the absence of heme, intermediate in glucose in the presence of heme, and fully induced in lactate. In the case of CYT1, this regulation spans a 70-fold range.

The organization of the CYT1 promoter is also similar to that of the CYC1 promoter. In both cases, transcription is driven by a UAS region that contains binding sites for the two activators, HAPI and HAP2/3/4. The UAS region in CYT1 lies between −510 and −464 from the ATG. Tran-
scripts initiate at five predominant sites that span a 160-nucleotide region, between -300 and -140. Both the HAPI site and the HAP2/3/4 site were deemed functional by analyzing deletion-bearing constructs in wild-type and hap mutant strains. As for CYCI, the HAPI site is promoter distal from the HAP2/3/4 site. Since HAP2/3/4 sites are asymmetrical, they may be assigned an orientation relative to the promoter. The orientation at CYTI is the same as that of CYCI.

However, there are several important differences between the CYCI and CYTI UAS regions. First, the CYCI UAS contains two adjacent HAPI-binding sites, which bind the protein cooperatively (19, 30, 31), while CYTI has a single site to which HAPI binds with a higher affinity than to either

UAS1 site. We believe that this difference may reflect the biological context of the expression of these two genes, a point on which we elaborate below. A second difference, also discussed below, is that the HAPI DNase I footprints on the CYTI site and on the stronger CYCI site are offset with respect to the DNA homology element. A third difference between the CYTI and CYCI UAS regions is that the distance between the HAPI footprint and the HAP2/3/4 UAS box in CYTI (20 bases) is closer than in CYCI (42 bases). This closer spacing may facilitate synergy between HAPI and HAP2/3/4 at CYTI, but this point has not yet been firmly established.

A major consequence of the two cooperative sites in UAS1 is that the HAPI activation profile of CYCI should be sigmoidal. In fact, the activation curve of CYCI-lacZ as a function of concentration of the heme supplement 6-ALA is sigmoidal (15). Thus, the activity of UAS1 will drop off sharply under conditions of reduced HAPI activity (due to reduced synthesis of heme when cells grow under oxygen limitation). In contrast, the single UAS site in CYTI should provide that gene with a more linear reduction in activity as HAPI activity is lowered. Why should CYCI and CYTI respond differently under conditions of oxygen limitation? One salient difference between the biology of cytochromes c and c\textsubscript{1} is that there exist two forms of cytochrome c, encoded by CYCI and CYC7. Under conditions of oxygen limitation, cells preferentially express the CYC7 form of cytochrome c (24). We speculate that a precipitous decline in UAS1 activity as oxygen levels fall ensures that CYC7 will direct the bulk of cytochrome c synthesis under oxygen

![Figure 6](http://mcb.asm.org/)

**FIG. 6.** DNase and ethylation interference footprinting of the HAPI site in CYTI. (A) A fragment in which the bottom strand of CYTI DNA was labeled was prepared by digesting the del 202 plasmid (Fig. 3) with AccI (which cleaves in the URA3 gene), filling in with DNA polymerase and α\textsuperscript{32}P-labeled nucleotides, and cleaving with BamH1. The fragment bearing CYTI sequences from -528 to -368 was gel isolated for DNase I protection. Protein extract from BWG1-7a bearing the GAL promoter-HAPI plasmid was added to levels that complexed all of the labeled fragment. Heme (15 \(\mu\)g/ml) and salmon sperm DNA (0.5 \(\mu\)g/ml) were also added. DNase treatment was as described in Materials and Methods. The samples were run on standard sequencing gels. Lanes: 1, size marker; 2, free DNA, 10 ng of DNase; 3, free DNA, 1 ng of DNase; 4, extract plus DNA, 120 ng of DNase; 5, extract plus DNA, 60 ng DNase. (B and C) For ethylation interference, the fragment described above (bottom strand) as well as a fragment labeled at a Taq site at -456 in CYTI (top strand) were used. The latter fragment was subcut at the AccI site as described above. The fragment contains CYTI sequences from -528 to -456. DNA fragments were modified with DES and then combined with extract as described above. Bound (B) and free DNA (F) were separated by gel shift, isolated separately, and treated with sodium hydroxide to cleave at modified bases. The samples were run on sequencing gels, as shown. Positions in CYTI DNA that interfere with binding are missing in the bound lanes, compared with the free lanes, and are indicated by arrows. Lanes: 6 and 8, bound DNA; 7, 9, and 10, free DNA.

![Figure 7](http://mcb.asm.org/)

**FIG. 7.** Comparison of HAPI sites of CYTI and CYCI. The DNase I footprints of the HAPI-binding sites of CYTI and CYCI (UAS1B) are shown. Bases that are contact sites by ethylation (CYTI) or methylation (CYCI) interference are in bold. The inverted repeat sequence in the CYTI site is indicated by the arrows. The best alignment of the sequences yields a 15-nucleotide region (pluses and minuses) in which 12 bases are identical (pluses). Note that the footprints are offset by several nucleotides.

<table>
<thead>
<tr>
<th>CYTI</th>
<th>UAS1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCCGCCCTATTGCCGCCG</td>
<td>TGGCCGCCGTTACGCGAGTA</td>
</tr>
<tr>
<td>CGCCGCCCTAAAGCGCCCGG</td>
<td>ACCGGGGCCAAAATGCGCTCCTACT</td>
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</tbody>
</table>

GGCCGCCCTATTGCCGCCG
CGCCGCCCTAAAGCGCCCGG

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**GGCCGCCCTATTGCCGCCG**

**CGCCGCCCTAAAGCGCCCGG**

**TGGCCGCCGTTACGCGAGTA**

**ACC GG GG CC AAA ATGCGCTCCTACT**
limitation. For its part, CYC7 has a single HAPI site and an additional site which ensures that the gene is not turned off under anaerobic conditions (35). Our finding that a CYTl gene disruption is a tight mutation which does not revert is consistent with the view that CYTl is the only gene encoding cytochrome c₁ in S. cerevisiae. The linear reduction in CYTl expression and cytochrome c₁ synthesis as oxygen levels fall would match the changes in levels of total cytochrome c driven by CYCI and CYC7 combined.

A comparison of the HAPI-binding site at CYTI and the strong site at UAS1 of CYC1, UAS1B, reveals an unusual feature. The best alignment of these two sequences shows a 12-of-15-base match which is within the DNase I footprints of both sites (Fig. 7). Surprisingly, although the footprints overlap along most of their lengths, including the homology block, they are offset by several nucleotides at the ends. The contacts as determined by methylation interference at UAS1B and ethylation interference at CYTI show some similarities but also many differences. Most striking is the fact that UAS1B contains several contacts that lie at the end of the DNase I footprint but are outside of the corresponding region in the DNase I footprint at CYTI. These protection profiles suggest that HAPI can bind to these related sequence elements in qualitatively different ways. The differences in the nature of the protein-DNA interactions at the two sites may account for the observed difference in HAPI binding affinity for CYTI versus CYCI.

This kind of flexibility in the HAPI DNA-binding domain was suggested from several earlier observations. First, the CYC7 site, to which HAPI binds with high affinity, bears minimal sequence similarity to UAS1B or CYTI (31). Second, the response of the CYC7 and UAS1 elements to HAPI differs quantitatively in vivo. UAS1 is more active than CYC7 (11, 18). Third, the effects of internal deletions in HAPI are opposite at UAS1B and CYC7 (19). These deletions increase activity at UAS1 and decrease activity at CYC7. To address these observations, we have proposed models that invoke different conformations in the DNA-binding domain of HAPI when the protein is bound at the dissimilar UAS1 and CYC7 sequences (18). Such differences would impose an additional layer of regulation to adjust the activity level of HAPI at different UAS elements.

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REFERENCES


FIG. 8. On and off rates for HAPI binding to CYTI and CYC1.

Fragments used were the Sma-Taq fragment from CYTI del 202 (−528 to −456) and the Xhol-Hinfl fragment from pLG-312 ΔAX (13) containing UAS1B. On (A) and off (B) rates were determined as described in Materials and Methods. Heme (15 μg/ml) and salmon sperm DNA (0.5 μg) were included in the binding reactions. The intensity of the bands on the autoradiograms derived from gel shift assays was quantitated by densitometry. Symbols: □, CYC1 UAS1; ○, CYTI UAS1.