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Regulation of mitochondrial biogenesis in *Saccharomyces cerevisiae*

Intricate interplay between general and specific transcription factors in the promoter of the *QCR8* gene

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Transcription of the *QCR8* gene, encoding subunit VIII of the *Saccharomyces cerevisiae* mitochondrial ubiquinol-cytochrome c oxidoreductase (QCR), is controlled by the carbon-source-dependent heme-activator protein complex HAP2/3/4 and the general transcriptional regulators autonomous replication-site-binding factor ABF1 and centromere-binding and promoter-binding factor CPFl. In this study, we investigate and dissect the relative contributions and mutual interactions of these regulators in transcriptional control. Transcription was analyzed both under steady-state conditions and during nutritional shifts, in *hapB* mutants and after site-specific mutagenesis of the various binding sites in the chromosomal context of the *QCR8* gene. We present evidence for both direct and indirect interactions between ABF1 and HAP2/3/4, and show that HAP2/3/4 is essential for a rapid transcriptional induction during transition from repressed to derepressed conditions. However, the activator is not the only determinant for carbon-source-dependent regulation, and we observe a functional difference between HAP2/3/4 and the HAP2/3 subcomplex. ABF1 is required for maintenance of basal repressed and derepressed transcription in the steady state of growth. The repressive action of the negative modulator CPFI during escape from glucose repression is overcome through the cooperative action of ABF1 and HAP2/3/4. The implications of the intricate interactions of these DNA-binding regulators for control of expression of mitochondrial protein genes are discussed.

**Keywords:** yeast; *Saccharomyces*; mitochondria; catabolerepression; transcription-factors.

In baker’s yeast *Saccharomyces cerevisiae*, the biogenesis of respiring mitochondria is controlled mainly by oxygen and the available carbon source. Regulation is exerted primarily at the level of transcription of nuclear genes encoding subunits of the respiratory chain complexes (De Winde and Grivell, 1993; Forsburg and Guarente, 1989a; Grivell, 1989). Biosynthesis of the enzyme complexes of oxidative phosphorylation offers a suitable model system to study regulatory mechanisms governing mitochondrial biogenesis. We have chosen to study the biosynthesis of the yeast mitochondrial ubiquinol-cytochrome c oxidoreductase (QCR) complex, that consists of one mitochondrial and nine nuclearly encoded subunits (Brandt et al., 1994; De Vries and Marres, 1987; De Winde and Grivell, 1993). Expression of all ten subunits is coordinately regulated (Van Loon et al., 1984).

Carbon-source-dependent transcriptional regulation of genes encoding many mitochondrial proteins is mediated through the heteromeric CCAAT-box-binding heme-activator complex HAP2/3/4 (Forsburg and Guarente, 1989b; Guarente et al., 1984; Olesen and Guarente, 1990). In the absence of one of the subunits of QCR, yeast cells are unable to grow on lactate as a sole non-fermentable carbon source, presumably because of under-expression of a number of genes necessary for respiratory function (Pinkham et al., 1987). Transcriptional activation by this complex during growth on a non-fermentable carbon source is assumed to depend on the availability of the activator subunit HAP4, as synthesis of only this subunit is regulated in response to the available carbon source (Forsburg and Guarente, 1989b).

All nuclear genes encoding QCR subunits contain consensus HAP2/3/4 target sites in their promoter regions (reviewed in De Winde and Grivell, 1993). A role for HAP2/3/4 in carbon-source-dependent transcriptional regulation has been demonstrated for the promoter regions of *CYT1*, encoding cytochrome c, (Oechsner et al., 1992; Schneider and Guarente, 1991) and for *QCR2* (Dorsman and Grivell, 1990), *QCR7* (De Winde, J., unpublished data) and *QCR8* (De Winde and Grivell, 1992; Maarse et al., 1988), encoding functionally important structural subunits of the QCR complex (Civellone et al., 1988; Schoppik, 1989).

The promoter regions of all QCR genes also contain binding sites for the general transcriptional regulators autonomous replication-site-binding factor (ABF1) and centromere-binding and promoter-binding factor (CPFl) (reviewed in Diffley, 1992 and Mellor, 1993), resulting in various combinations of cis-acting regulatory elements (De Winde and Grivell, 1993; Dorsman et al., 1988). Recently, we have demonstrated a functional role for ABF1 and CPFI, binding to overlapping sites in the QCR8 promoter (De Winde and Grivell, 1992). ABF1 is required for optimal QCR8 transcription under repressing growth conditions and for efficient derepression upon escape from glucose repression. CPFI appeared to function as a negative modulator of the induction response. The mechanism of transcriptional control and of
Table 1. Yeast strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL1</td>
<td>MATa his3-11,15 leu2-3, 112 ura3-251, 328, 372</td>
<td>Van Loon et al., 1984</td>
</tr>
<tr>
<td>DLS80</td>
<td>Same as DL1 but with qcr8A::LEU2</td>
<td>De Winde and Grivell, 1992</td>
</tr>
<tr>
<td>DLU80</td>
<td>Same as DL1 but with qcr8A::URA3</td>
<td>this study</td>
</tr>
<tr>
<td>DLH811</td>
<td>Same as DL1 but with qcr8-11</td>
<td>De Winde and Grivell, 1992</td>
</tr>
<tr>
<td>DLH821</td>
<td>Same as DL1 but with qcr8-21</td>
<td>this study</td>
</tr>
<tr>
<td>DLH880</td>
<td>Same as DL1 but with qcr8-80</td>
<td>this study</td>
</tr>
<tr>
<td>DLH881</td>
<td>Same as DL1 but with qcr8-81</td>
<td>this study</td>
</tr>
<tr>
<td>DLH882</td>
<td>Same as DL1 but with qcr8-82</td>
<td>this study</td>
</tr>
<tr>
<td>BWGl-7A</td>
<td>MATa ade1-100 his3-119 leu2-3, 112 ura3-52</td>
<td>Guarente et al., 1984</td>
</tr>
<tr>
<td>LGW32</td>
<td>Same a BWGl-7A but with hapl-1</td>
<td>Guarente et al., 1984</td>
</tr>
<tr>
<td>JO1-1A</td>
<td>Same a BWGl-7A but with hap2A</td>
<td>Olesen and Guarente, 1990</td>
</tr>
<tr>
<td>SHY40</td>
<td>Same a BWGl-7A but with hap4A::HIS4</td>
<td>Olesen and Guarente, 1990</td>
</tr>
<tr>
<td>SLY401</td>
<td>Same a BWGl-7A but with but with hap4A::LEU2</td>
<td>Forsburg and Guarente, 1989b</td>
</tr>
<tr>
<td>TKY20</td>
<td>MATa ade1 leu2-3, 112 ura3-52 hapl-1::LEU2</td>
<td>Keng, 1992</td>
</tr>
</tbody>
</table>

the interaction between the regulators was, however, not clear. By creating a nucleosome-free window in the QCR8 promoter, ABF1 may interact directly with HAP2/3/4, ensuring accessibility under various growth conditions (De Winde et al., 1993). In addition, ABF1 and HAP2/3/4 may synergistically activate QCR8 transcription, possibly in cooperation with other factors.

We wished to investigate the role of the HAP2/3/4 complex in more detail, and to further dissect the relative contributions and mutual interactions of HAP2/3/4, ABF1 and CPF1 in transcriptional regulation of the QCR8 gene. To this end, we analyzed regulation of QCR8 transcription in yeast mutants, either lacking subunits of the HAP2/3/4 complex or containing a mutagenized CCAAT box in the chromosomal QCR8 promoter, alone or in combination with mutagenized ABF1-binding or CPF1-binding sites. Results present evidence for both direct and indirect interactions between ABF1 and HAP2/3/4, and show that HAP2/3/4 is essential for rapid transcriptional induction during escape from glucose repression. HAP2/3/4 is not the only determinant for carbon-source-dependent regulation, but the activator is required to obtain fully derepressed transcription. ABF1 is required for maintenance of basal and derepressed transcription in steady-state growth. CPF1 functions as a negative modulator of the induction response, whose action is overcome by cooperation between ABF1 and HAP2/3/4. We discuss the implications of these intricate interactions for regulation of expression of mitochondrial protein genes.

**MATERIALS AND METHODS**

**Strains and media.** Yeast strains used in this study are listed (Table 1). A new QCR8-disruption mutant, replacing QCR8 with URA3, was constructed by transforming a 3.8-kbp KpnI–EcoRI fragment from disruption construct pURA38 to yeast strain DL1, and selecting for uracil prototrophy. Yeast cells were grown in 1% yeast extract, 2% Bacto-Peptone, containing either 4% glucose (YPD), 2% (mass/vol.) galactose (YPGal), or 2% (mass/vol.) ethanol and 2% (mass/vol.) glycerol (YPEG). Selective media contained 0.67% yeast nitrogen base without amino acids (Difco) and either 4% glucose, or 0.05% yeast extract, 0.04% glucose, 2% (mass/vol.) ethanol and 2% glycerol, and were supplemented with the appropriate amino acids and nucleotides. Solid media contained 2% Bacto Agar, 1% yeast extract, 2% Bacto-Peptone and 4% glucose (YPDagar), or 2% (mass/vol.) ethanol and 2% (mass/vol.) glycerol (YPEGagar), or 2% galactose (YPGalagar), or contained 2% Bacto Agar, 0.5% yeast extract, 8 mM MgSO4, 45 mM (NH4)2HPO4, 1.5% lactic acid, 2% sodium lactate, pH 4.5, and 0.1% glucose (YPLacagar).

**Escherichia coli** strains used for DNA manipulations were JF1754 (lac, gal, metB, leuB, hisB, hsdR), BMH 71–18 mutS (thi, supE, A[lac-proAB], mau5::Tn10, [F’, proAB, lacI2ZM]) and JM109 (endA, recA, gyrA, thi, hsdR, relA, supE, A[lac-proAB], [F’ traD, proAB, lacI2ZM]). Bacterial cultures were grown in 1.6% Bacto-Tryptone, 1% yeast extract, 0.5% NaCl, supplemented with 100 μg/ml ampicillin or 15 μg/ml tetracycline when necessary.

**Construction of plasmids.** 2.8-kb KpnI–EcoRI fragments, containing the 3′-half of FPS1 and QCR8, either with wild-type or mutated promoters, were isolated from pJH1, pJH1-10 (qcr8-HII) and pH1-20 (qcr8-21) (De Winde and Grivell, 1992) and inserted into the KpnI and EcoRI sites of pSelect™.1 (Promega Corp.). The resulting plasmids are named pSQ8, pSQ811 and pSQ821. Site-directed mutagenesis resulted in additionally mutated QCR8 promoters on pASQ880, pASQ881 and pASQ882, which were recovered on 2.8-kb BamHI–EcoRI fragments. These fragments were inserted into the BamHI and EcoRI sites of YCp50 (Rose et al., 1987), YCplac33 and YEpplac195 (Gietz and Sugino, 1988), or used to insert promoter mutations into the chromosomal context of QCR8.

The QCR8-disruption construct pJURA38 was obtained by inserting a 1.85-kb Smal–SalI fragment from YCp50 (Rose et al., 1987) containing the URA3 gene, into the HindIII and SalI sites of pH1 (De Winde and Grivell, 1992) after blotting the HindIII site with DNA polymerase Klenow fragment.

**Construction of QCR8 promoter mutants.** Site-directed mutagenesis was performed using the Altered Sites in vitro Mutagenesis System (Promega Corp.), with minor modifications. A single-stranded phosphorylated oligonucleotide containing the qcr8-80 mutation (Fig. 1) was annealed to single-stranded pSQ8, pSQ811 and pSQ821 templates. After second-strand synthesis with T4 DNA polymerase and T4 DNA ligase and successive transformation into BMH71-18 mutS and JM109 or JF1754, colonies were tested for the presence of the desired QCR8 promoter mutants by digestion of isolated double-stranded plasmids with Ncol, followed by sequence analysis. Thus, qcr8-80, qcr8-81 and qcr8-82 mutations (Fig. 1) were obtained on pASQ880, pASQ881 and pASQ882, respectively.

**QCR8 promoter mutations.** Promoter mutations were introduced into the chromosomal context of the QCR8 locus by direct gene substitution in qcr8-disruption strains (Table 1). pASQ880, pASQ881 and pASQ882 were digested with BamHI and EcoRI, and 10 μg portions were used to transform DLS80 and DLU80 (Klebe et al.,
Fig. 1. Organization of wild-type and mutant QCR8 promoter regions used in this study. See also De Winde and Grivell, (1992) and Fig. 2. Primary sequence organization of the QCR8 promoter (non-coding strand). Distances relative to the ATG codon are indicated. Underneath the schematic promoter region, the target sequences for ABFl, CPFl and HAP2/3/4 are highlighted. Mutated sequence elements in each of the alleles qcr8-11, qcr8-21 (De Winde and Grivell, 1992), qcr8-80, qcr8-81 and qcr8-82 are indicated separately, as is the NcoI restriction site that is the result of the mutation in the HAP2/3/4-binding site.

1983). After transformation, cells were allowed to recover for 4 hours in YPD at 30°C and plated on YPEGagar. Restoration of the QCR8 locus, as indicated by growth on a non-fermentable carbon source, was subsequently confirmed by Southern-blot hybridization (data not shown).

RNA isolation and Northern-blot analysis. Total yeast RNA was isolated and analyzed as described previously (De Winde and Grivell, 1992) with minor modifications. Total yeast RNA was separated on a horizontal 1.0% agarose gel in 0.25X (22.5 mM Tris/HCl, 22.5 mM boric acid, pH 8.3, 0.63 mM EDTA) without ethidium bromide at 20 µg/lane. The hybridization temperature was maintained at 38°C, and filter washing was extended with incubation in 0.1 0.15 M NaCl, 15 mM sodium citrate, pH 7.0 (NaCl/Cit), 0.1% SDS at room temperature. Following autoradiography, probed filters were washed for new probing by incubation in 0.1 0.15% SDS, 1 mM EDTA, 50 mM Tris/HCl, pH 7.5, at 55°C for several hours, then washed in 0.5×NaCl/Cit containing 0.1% SDS at room temperature.

Probe fragments used in this study included an 840-bp HindIII–SalI fragment containing QCR8 (De Winde and Grivell, 1992), a 425-bp HindIII–PstI fragment containing QCR7 (Schoppink et al., 1988), a 1.6-kb HindIII–HindIII fragment containing a histone H4 gene (De Winde, 1992), and a 1.6-kb BamHI–KpnI fragment containing the yeast actin gene (Ng and Abelson, 1980).

Medium-shift experiments. Shifts from glucose to a non-fermentable carbon source were performed essentially as described previously (De Winde and Grivell, 1992). Yeast cells were grown in YPD (4% glucose) to early log-phase (absorbance at 600 nm, 2.0–2.5, using a CamSpec spectrophotometer) at 29°C and, after harvesting and washing, resuspended in warmed YPEG at the zero time point. Samples (usually 20 ml) were taken at various time points as indicated, and immediately cooled to 0°C. Cells were recovered by centrifugation, quick frozen in liquid nitrogen, and stored at −20°C.

Gel-retardation assay. Preparation of total cell lysates and retardation assays were performed as previously described (De Winde and Grivell, 1992; Dorsman et al., 1988).

RESULTS

Role of the HAP2/3/4 activator complex in derepression of QCR8. In S. cerevisiae, derepression of many genes encoding mitochondrial proteins is dependent on a functional HAP2/3/4 activator complex (Forsburg and Guarente, 1989; Guarente et al., 1984; Olesen and Guarente, 1990). For QCR8, the HAP2/3/4 complex has also been implicated in basal and carbon-source-dependent activation of transcription, as QCR8 transcription is decreased in hap2-1 and hap3-1 mutants under repressed and derepressed conditions (De Winde and Grivell, 1992). However, examination of QCR8 and actin mRNA levels in hap2A, hap3A and hap4A disruption mutants indicates that, in the absence of HAP4, transcription of QCR8 is still slightly induced under derepressing conditions (Fig. 2, lane 5 compared to lane 10), whereas in the absence of HAP2 or HAP3, QCR8 expression no longer responds to a change of carbon source (Fig. 2, lanes 3 and 4 compared to lanes 8 and 9).
results are in line with results from analysis of steady-state moter. is essential for rapid and efficient induction of only the activator subunit HAP4, DNA-binding subunits HAP2 or HAP3, transcription is low, ir-
slightly induced upon escape from glucose repression. In the absence of hap2d
pression of medium to medium containing a non-fermentable carbon source, states that respiration is not completely abolished. Consequently,
beit very slowly (unpublished data; see Discussion). This indi-
these mutants can be transferred from glucose-containing me-
sequence of the medium shift, as indicated by a sudden decrease
and before they are subsequently blocked in G1 phase as a con-
expected to completely abolish the HAP2/3/4 function on the
qcr8-80
promoter (Forsburg and Guarente, 1988). This difficulty in visualizing
in vitro, presumably due to the low concentration and low affinity of the activator complex in total yeast cell lysates (Mulder et al., 1994a). This difficulty in visualizing in vitro DNA binding of HAP2/3/4 has also been reported by others (Schneider and Guar-
te, 1991; Trawick et al., 1992). As a consequence, gel-shift analysis of qcr8-80 shows the same pattern as wild-type QCR8 (De Winde and Grivell, 1992; Dorsman and Grivell, 1990; Guarente et al., 1984), often in combination with HAP2/3/4 target sites in artificial promoter-reporter fusions (Forsburg and Guarente, 1988; Keng and Guarente, 1987; Maarse et al., 1988; Oechsner et al., 1992; Trawick et al., 1992). This approach suffers from the serious drawback that such mutants display a pleiotropic pet-
type phenotype that obscures primary effects of the mutations and thus hampers studies of the exact function of the activator complex.

In order to circumvent this limitation, we have mutated the recognition element for the heteromeric activator in the promoter of QCR8. This target site has been established to be involved in HAP2/3/4-mediated carbon-source-dependent transcriptional regulation of QCR8 (De Winde and Grivell, 1992; Maarse et al., 1988). The mutation, resulting in the qcr8-80 allele (Fig. 1) is expected to completely abolish the HAP2/3/4 function on the QCR8 promoter (Forsburg and Guarente, 1988). The effect of the qcr8-80 mutation cannot be assayed directly, as we have not been able to show DNA binding of the HAP2/3/4 complex in vitro, presumably due to the low concentration and low affinity of the activator complex in total yeast cell lysates (Mulder et al., 1994a). This difficulty in visualizing in vitro DNA binding of HAP2/3/4 has also been reported by others (Schneider and Guar-
te, 1991; Trawick et al., 1992). As a consequence, gel-shift analysis of qcr8-80 shows the same pattern as wild-type QCR8 (De Winde and Grivell, 1992; Dorsman and Grivell, 1988); only bind-
mg of the general regulators ABFI and CPFI can be detected
(Fig. 4, lane 2). When introduced on multi-copy as well as sin-
gle-copy shuttle vectors, qcr8-80 restores growth of the qcr8-
disruption strain DLL80 on media containing non-fermentable carbon sources. The efficacy of the qcr8-80 mutation is, how-
ever, confirmed by the severely reduced QCR8 mRNA levels in these transformants (data not shown). Reduced synthesis of QCR8 is known not to cause a proportional decrease in the growth rate, because in wild-type yeast, the QCR complex is synthesized in excess and hence exerts only minor control on cell growth (De Winde and Grivell, 1992; Schooppink, 1989).

For further analysis, the qcr8-80 allele was introduced into the chromosomal QCR8 locus via direct gene substitution in DLL80. The resulting strain, DLLH880 (Table 1), has no obvious phenotype on glucose-containing media, but exhibits signifi-
cant impaired growth on galactose and on non-fermentable carbon sources (Fig. 5). This is consistent with a significant decrease of the steady-state QCR8 mRNA level in this mutant when grown on a non-fermentable carbon source (Fig. 6, lane 7). Strain DLH811, in which the ABF1 binding site in the QCR8 promoter has been mutated, does not show an obvious growth defect, even though on non-fermentable carbon sources, QCR8 mRNA levels are only about 60% of wild-type levels (Fig. 5; De Winde and Grivell, 1992). In DLH880, transcription of QCR7, encoding another subunit of the core complex which is initially formed during assembly of the QCR enzyme (see Discussion), is not affected (Fig. 6, lane 7). Hence, significantly reduced synthesis of QCR8 mRNA, caused by the absence of HAP2/3/4 from the QCR8 promoter, severely limits growth on a non-fermentable carbon source. QCR8 mRNA levels in glucose-grown mutant cells are not reduced compared to those of wild-type cells (Fig. 6, lane 1 compared to lane 3). The latter contrasts results from hapA mutants described above, which are probably explicable in terms of their pleiotropic character (see Discussion). In the absence of HAP2/3/4 the QCR8 promoter still responds to glucose derepression, albeit to a lesser extent (Fig. 6, compare lanes 3 and 7). Hence, we conclude that the HAP2/3/4 activator complex is required for full-level transcriptional induction of QCR8. However, HAP2/3/4 is not the only determinant for carbon-source-dependent regulation of the QCR8 promoter.

Interplay between ABF1, CPF1 and HAP2/3/4 on the QCR8 promoter. Transcriptional regulation of the QCR8 gene has previously been shown to be dependent on the action of the specific activator HAP2/3/4 and the general transcription factors ABF1 and CPF1 (De Winde and Grivell, 1992). The nature of the interactions between these DNA-binding proteins was, however, not examined in any detail. To further dissect the relative contributions of HAP2/3/4, ABF1 and CPF1 to the transcription regulatory mechanism, and to obtain insight into mutual interactions at the QCR8 promoter, we combined QCR8 promoter mutations which abolish binding of either ABF1 or CPF1 (De Winde and Grivell, 1992) with the qcr8-80 mutation described above, that prevents HAP2/3/4 action (Fig. 1). Gel-shift analysis of qcr8-81 (non-ABF1 binding) and qcr8-82 (non-CPF1 binding) shows binding of CPF1 and ABF1, respectively (Fig. 4, lanes 4 and 6). Like qcr8-80, when introduced on multi-copy and single-copy shuttle vectors, qcr8-81 and qcr8-82 complement the growth defect of qcr8-disruption strain DLL80 on a non-fermentable carbon source (data not shown).

The qcr8-81 and qcr8-82 alleles were introduced into the chromosomal QCR8 locus, via direct gene substitution in DLL80 and DLL80 (Table 1). As described above for DLH880, both strains display significantly impaired growth on galactose and on non-fermentable carbon sources (Fig. 5). Growth-curve analysis indicates that, on a non-fermentable carbon source, the growth defect is most severe for DLH881 (data not shown), a result in line with a severe decrease in the steady-state QCR8 mRNA level in this mutant (Fig. 6, lane 8). The QCR8 mRNA level in DLH882 (Fig. 6, lane 9), grown on a non-fermentable carbon source, is slightly lower than that in DLH880 (Fig. 6, lane 7) and hence significantly reduced compared to the wild-type level. In contrast to DLH880, absence of ABF1 from the QCR8 promoter in DLH881 causes a reduced mRNA level during growth on glucose compared to than in the wild-type cells (Fig. 6, lane 4), again indicating the importance of ABF1 for basal level expression of QCR8. The QCR8 mRNA level in glucose-grown DLH882 is slightly elevated compared to the wild-type level (Fig. 6, lane 5). Prevention of binding of only CPF1 to the QCR8 promoter does not cause this effect (De Winde and Grivell, 1992). Thus, the absence of the HAP2/3/4 activator may enhance the previously described moderate repressive effect of CPF1. The steady-state mRNA levels for both DLH881 and DLH882 are comparable in glucose-grown and ethanol/glycerol-grown cells (Fig. 6, lanes 4 and 5 compared to lanes 8 and 9), indicating the apparent absence of derepression of QCR8 transcription. In DLH882, ABF1 causes reasonable transcriptional activation on both glucose and ethanol/glycerol, but the slight derepression remaining in DLH880 (see above) is lost.

From the findings described above, we conclude, in line with previous results, that ABF1 serves an important role in regulation of QCR8 transcription under both repressing and derepressing growth conditions. Results again point to a moderate repressive effect of CPF1. Nevertheless, in the absence of HAP2/3/4, the combined action of ABF1 and CPF1 renders the QCR8 promoter susceptible to carbon-source regulation, indicating that HAP2/3/4 is not the only determinant for this control. The additional absence of either ABF1 or CPF1 causes constitutive steady-state transcription on glucose and a non-fermentable car-
DLH881, with mutated ABFI and HAP2/3/4 target sites in the to the shift-mediated induction response, level during steady-state growth on a non-fermentable carbon tants DLH881 and DLH882 were analyzed in parallel. In exposed to aid visualization of relatively low hybridized to probes specific for actin mRNA and

Fig. 7. Derepression of QCR8 transcription is absent from promoter mutants lacking a HAP2/3/4 target site. Medium shift from YPD to YPEG was performed for wild-type DL1 and mutant strains DLH880, DLH881 and DLH882. Total RNA from subsequent time samples was hybridized to probes specific for actin mRNA and QCR8 mRNA. Autoradiographs for the three QCR8 promoter mutants have been overexposed to aid visualization of relatively low QCR8 mRNA levels. Refer to Fig. 6 for proper comparison of steady-state mRNA levels during logarithmic growth on YPD or YPEG.

bon source, however transcriptional activation through ABF1 is far more efficient.

HAP2/3/4 is essential for short-term induction of QCR8 transcription upon escape from glucose repression. To assess the kinetics of the transcriptional response of a mutant QCR8 promoter lacking the HAP2/3/4 target site, DLH880 cells were shifted from glucose-based medium to medium containing ethanol/glycerol as a non-fermentable carbon source, and QCR8 mRNA levels from successive time samples were investigated by Northern-blot analysis. Wild-type DL1 cells under these conditions show a rapid and strong biphasic increase of mRNA from genes encoding respiratory chain proteins (see also Fig. 3, and De Winde and Grivell, 1992). Surprisingly, in DLH880, QCR8 mRNA drops to very low levels around 8 hours following the shift (Fig. 7). The mRNA level increases again after 14–16 hours (data not shown), reaching the moderately derepressed level during steady-state growth on a non-fermentable carbon source (Fig. 6). Interestingly, DLH880 shows a severely prolonged lag-phase of growth compared to wild-type DL1 when transferred from glucose to ethanol/glycerol (data not shown). Apparently, retarded synthesis of QCR8 mRNA inhibits proper adaptation to non-fermentable growth medium. Thus, HAP2/3/4 is not only required for full-level derepression, but the activator is essential for a rapid short-term induction response upon escape from glucose repression.

To further analyze the relative contributions of the specific activator HAP2/3/4 and the general regulators ABF1 and CPFl to the shift-mediated induction response, QCR8 promoter mutants DLH881 and DLH882 were analyzed in parallel. In DLH881, with mutated ABF1 and HAP2/3/4 target sites in the QCR8 promoter, the QCR8 mRNA level starts to decrease sooner than in DLH880 (Fig. 7). The level is almost undetectable 8 hours after the shift. Like DLH880, this mutant shows a severely prolonged lag-phase of growth (data not shown). After 24 hours, QCR8 mRNA has again reached a well-detectable level which, from steady-state Northern-blot analysis, was interpreted as not being induced. However, QCR8 transcription is obviously resumed after an initial phase of repression. In contrast, transcription from the QCR8 promoter with mutated CPFl and HAP2/3/4-binding sites in DLH882 remains constitutive upon a shift to a non-fermentable carbon source. Thus, the absence of CPFl and HAP2/3/4 causes loss of carbon-source-dependent regulation from the QCR8 promoter. ABF1 mediates constitutive transcription from this promoter, resulting in relatively wild-type QCR8 mRNA levels when grown on glucose, and significantly reduced levels during growth on ethanol/glycerol, as observed by steady-state analysis (Fig. 6). Binding of CPFl to the QCR8 promoter, alone (DLH881) or in putative competition with ABF1 (DLH880), causes severe inhibition of transcription upon escape from glucose repression, as previously described for a combination of CPFl and HAP2/3/4 (DLH811).

From the results presented above, we conclude that, in conjunction with the wild-type QCR8 promoter, HAP2/3/4 is absolutely essential for a rapid and efficient short-term induction response during escape from glucose repression. In addition, the activator complex is required to obtain fully derepressed transcription levels, but it is not the only determinant for carbon-source-dependent transcriptional regulation. ABF1 is required for normal basal-level transcription on glucose, and enhances derepressed transcription during the steady-state of growth. CPFl functions as a repressor, modulating the derepression response. Strong repression of the induction response by CPFl is overcome through the cooperative action of ABF1 and HAP2/3/4.

DISCUSSION

We present in this report a detailed analysis of the roles played by the specific activator complex HAP2/3/4 and the general regulators ABF1 and CPFl in transcriptional regulation of QCR8, which encodes subunit VIII of the yeast mitochondrial respiratory chain complex III or the bc1-complex. As indicated previously (De Winde and Grivell, 1992; reviewed by Felsenfeld, 1992), transcriptional regulatory mechanisms are intimately bound with processes governing chromatin structure and assembly. This specifically involves ‘general regulators of chromosome function’ (Diffl ey, 1992; Doorenbosch et al., 1992) like ABF1 and CPFl. We, therefore, chose to study transcription regulatory mechanisms in the native chromosomal context. In addition, as the vast majority of transcription regulation studies addresses the function of regulatory proteins during the steady state of growth, we integrated steady-state and medium-shift analyses, which allowed us to discriminate between long-term and short-term effects of the transcriptional regulators under study.

Derepression of many genes encoding mitochondrial proteins is known to be dependent on a functional HAP2/3/4 complex (Forsburg and Guarente, 1989b; Guarente et al., 1984; Olesen and Guarente, 1990). Mutants lacking functional HAP2, HAP3 or HAP4 proteins are unable to grow on lactate as a sole non-fermentable carbon source. This pleiotropic growth defect is likely to be caused by underexpression of a number of gene...
products involved in mitochondrial respiration (Pinkham et al., 1987), but the physiological basis for this defect is not clear. We and others have observed that hap mutants do grow on media containing ethanol and/or glycerol, albeit very slowly (De Winde, J. H., unpublished data; Daignan-Fornier et al., 1994; Mattoon et al., 1990). Mattoon et al. (1990) have indicated that slow growth on a non-fermentable carbon source cannot be due solely to a partially decreased synthesis of all cytochromes. Here, we show that disrupting the CCAAT target site for HAP2/3/4 in the QCR8 promoter results in a significant decrease in transcription during growth on ethanol/glycerol, and a concomitant growth defect on non-fermentable carbon sources and galactose. Severely reduced expression of QCR8 is likely to retard formation of the initial core complex consisting of QCR7p, QCR8p and cytochrome b, whose formation is an absolute prerequisite for subsequent assembly of the mature bc, complex (Grivellone et al., 1988; Schoppink, 1989). Thus, preventing HAP2/3/4-mediated regulation of expression of only one important subunit of a respiratory chain complex already limits growth under non-fermentative conditions.

QCR8 mRNA levels in glucose-grown hapΔ mutants are reduced compared to wild-type levels. From comparable results, we previously suggested that HAP2/3/4 is involved in basal transcription under glucose-repressed conditions. However, mutagenesis of the target CCAAT box in the chromosomal context, and hence inhibition of HAP2/3/4-dependent regulation at the QCR8 promoter alone, does not result in decreased transcription on glucose. The former result is directly attributable to the pleiotropic phenotype of the hapΔ mutants. We, therefore, now conclude that HAP2/3/4 is not involved in basal transcription of QCR8. Basal transcription is, however, dependent on the general regulator ABFI.

Examination of QCR8 transcription in hap2Δ, hap3Δ and hap4Δ mutants presents evidence for a functional difference between the DNA-binding subunits HAP2 and HAP3, and the activator subunit HAP4 of the heteromeric activator complex. In the absence of HAP2 or HAP3, QCR8 is constitutively transcribed. In contrast, in the absence of HAP4 but in the presence of the two DNA-binding subunits of the complex, a minor level of long-term derepression is maintained on a non-fermentable carbon source. This suggests that HAP4 is required for rapid and efficient induction of transcription in response to the increasing need for respiratory-chain constituents; the DNA-binding subcomplex HAP2/3 appears to be required for a measure of carbon-source-dependent regulation. Indeed, recent results indicate that the HAP2/3 subcomplex is able to bind the target CCAAT box irrespective of the presence of HAP4 (Olesen and Guarente, 1990; Xing et al., 1993; Xing et al., 1994). Support for a regulatory role of the HAP2/3 subcomplex comes from the observation that transcriptional control of the HAR1/SDH3 gene, encoding cytochrome b, of the mitochondrial succinate dehydrogenase, is stimulated by HAP2 and HAP3 in the absence of HAP4 (Daignan-Fornier et al., 1994b). Thus, HAP2/3 is likely to interact with other regulators at the QCR8 promoter to establish a level of "basal regulation".

In QCR8 promoter mutant DLH880, in which the HAP2/3 target site has been disrupted, the rapid induction response upon a shift from glucose to a non-fermentable carbon source is completely absent and the initial response appears to be that of transcriptional repression. The initial repression is mediated through CPF1, since concomitant mutagenesis of the CPF1-binding site relieves repression and results in ABFI-mediated constitutive transcription. We previously reported this repressive effect of CPF1 during escape from glucose repression in the absence of ABF1 from the QCR8 promoter and showed that alleviation of CPF1-mediated repression requires passage through the S phase of the cell cycle (De Winde and Grivell, 1992). The current findings go further and indicate that, at the wild-type QCR8 promoter, ABF1 and HAP2/3/4 cooperate to overcome the repressive effect of CPF1, thus ensuring rapid and efficient transcriptional induction independently of DNA replication and chromatin rearrangement. Further evidence for a direct interaction between ABF1 and HAP2/3 comes from the finding that, by shortening the distance between the binding sites for both activators in the QCR8 promoter, the efficiency of derepression is dramatically decreased (De Winde and Grivell, 1993; De Winde, J. H., unpublished data). In the absence of HAP2/3/4, ABF1 and CPF1 together ensure a minor, long-term transcriptional derepression of QCR8, which is likely to be regulated in response to changes in the growth rate and chromatin structure, exerted through, amongst others, ABF1 (Silve et al., 1992; De Winde et al., 1993). ABF1 alone maintains constitutive transcription from the remaining promoter. With CPF1 binding alone to the QCR8 promoter, initial repression is enhanced and transcription appears to be resumed to a minor extent, stressing the importance of ABF1 for maintenance of appreciable transcription levels.

As indicated above, upon a shift to a non-fermentable carbon source, QCR8 mRNA levels remain constitutive in hap2Δ cells lacking one of the DNA-binding subunits of the HAP2/3/4 complex. In contrast, disruption of the target CCAAT box in the QCR8 promoter, and, hence, prevention of DNA binding of the whole complex, causes initial repression mediated by CPF1. As stated above, ABF1 and HAP2/3/4 interact on the wild-type promoter to alleviate the repressive effect of CPF1. It is tempting to speculate that, in hap2Δ cells, ABF1 interacts with a DNA-binding factor at the CCAAT box to overcome CPF1-mediated repression. A putative candidate for this factor is HAP3. Recently, we have identified a novel Zn-finger motif present in the HAP3 proteins from the yeasts Kluyveromyces lactis and Saccharomyces cerevisiae, and in HAP3 homologues from various eukaryotes (Mulder et al., 1994a). This finding prompted us to speculate that all HAP3-like proteins may bind DNA directly, even though, as yet, there is no direct experimental evidence for binding of HAP3 or HAP2 independent of the other factor.

ABF1 and HAP2/3/4 are involved in transcriptional regulation of various genes encoding mitochondrial proteins (Bowman et al., 1992; Dorosman and Grivell, 1990; Dorosman et al., 1988; Trawick et al., 1992; reviewed in De Winde and Grivell, 1993). Based on the results presented in this and previous reports (De Winde and Grivell, 1992; De Winde et al., 1993) we present the following model for the interplay of these DNA-binding regulators. The HAP2/3/4 complex is absolutely essential for a rapid transcriptional induction upon transition from repressed to derepressed growth conditions. Additionally, the activator is required for fully derepressed transcription levels. ABF1 enhances HAP2/3/4-dependent rapid derepression, presumably through direct interaction with the heteromeric activator. In addition, ABF1 ensures basal and induced transcription levels during the steady-state of growth on repressing and derepressing media, respectively, by maintaining a nucleosome-free window in the QCR8 promoter region under various growth conditions (De Winde et al., 1993). By maintaining basal level transcription, ABF1 prevents total catabolite repression of mitochondrial biogenesis, which would ultimately lead to loss of mitochondrial DNA. At QCR8, CPF1 acts as a repressor, functioning in modulating the derepression response. As ABF1 and CPF1 bind to the QCR8 promoter in a mutually exclusive fashion, we previously speculated on the possibility of temporal displacement of ABF1 by CPF1 during the initial phase of derepression, thus enabling CPF1 to participate in the regulatory mechanism. In DLH880, lacking HAP2/3/4 from the QCR8 promoter, CPF1 obviously
exerts its repressive effect in response to a medium shift, indicating that, indeed, CPFl temporarily replaces ABFl.

Interestingly, the unusual topology of overlapping binding sites for ABFl and CPFl is also found in the promoter regions of two other genes encoding subunits of the QCR complex (Fig. 9). In the promoters of QCR10, encoding 8,5-kDa subunit 10 (Brandt et al., 1994), and of CYTI1, encoding cytochrome c, (Oechsner et al., 1992; Schneider and Guarente, 1991), a potential low-affinity ABFl target site overlaps a site completely deleted from deletion analysis, is exerted by ABFl, and not by CPFl as suggested by the authors (Oechsner et al., 1992). Further analysis is required to investigate the combined action of ABFl and CPFl in regulating biogenesis of the QCR complex.

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