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Co-ordinate control of synthesis of mitochondrial and non-mitochondrial hemoproteins: a binding site for the HAP1 (CYP1) protein in the UAS region of the yeast catalase T gene (*CTT1*)

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Control of expression of the *Saccharomyces cerevisiae* *CTT1* (catalase T) gene by the *HAP1* (*CYP1*) gene, a mediator of heme control of mitochondrial cytochromes, was studied. Expression of a *CTT1*–*lacZ* fusion in a *hap1* mutant showed that the *CTT1* promoter is under *HAP1* control. As demonstrated by a gel retardation assay, the HAP1 protein binds to a heme control region of the *CTT1* gene. This binding *in vitro* is stimulated by hemin. The HAP1-binding sequence was localized by using DNA fragments spanning different regions, by DNase I footprinting and by methylation interference of DNA–protein binding. The binding site was compared to the HAP1-binding sequences previously characterized in detail ($UAS1_{CYC1}$, UAS_{CYC7}). There is strikingly little similarity between the three sequences, which have only four of those 23 bp in common which are protected from DNase I digestion. However, the pattern of major and minor groove contacts in the complex is quite similar in all three cases. The results obtained show that there is true co-ordinate control of expression of mitochondrial cytochromes and at least some extra-mitochondrial hemoproteins. Heme acts as a metabolic signal in this co-ordination, which is mediated by the HAP1 protein.

Key words: catalase T/heme control/*HAP1*/*CYP1*

Introduction

In the yeast *Saccharomyces cerevisiae* the transcription of genes coding for a number of mitochondrial and non-mitochondrial hemoproteins is positively controlled by heme. The *CYC1* gene, which codes for the mitochondrial iso-1-cytochrome *c*, and the *CTT1* gene coding for the soluble catalase T have been studied most extensively. *Cis*-acting positive heme control regions have been identified in both cases (Guarente and Mason, 1983; Gudenus *et al.*, 1984; Spevak *et al.*, 1986). Both cytochrome *c* and catalase are involved in processes connected with the presence and utilization of molecular oxygen in living cells. Cytochrome *c* functions in mitochondrial respiration, a process involving transfer of four electrons to oxygen as terminal acceptor and its reduction to H_2O. In contrast, one- and two-electron transfer to O_2, which might also occur during respiration, leads to the formation of superoxide radicals and hydrogen peroxide. Catalase, which functions outside of mitochondria, is presumably involved in the detoxification of these products together with superoxide dismutase and peroxidases. Thus, a functional link might exist through mitochondrial cytochromes involved in electron transport to oxygen and extra-mitochondrial hemoproteins involved in detoxification of oxygen metabolites. Such a link could make co-ordinated control of synthesis of such proteins attractive from a teleological point of view. However, no direct evidence exists as yet for either a link in function or for true co-ordinated control of synthesis of such proteins by a regulatory protein acting on both classes of genes. Sequence similarity of the UAS_{CTT1} region and the core regions of UAS1 and UAS2 of the *CYC1* gene has been reported (Spevak *et al.*, 1986). However, no convincing evidence exists for a common function of these sequences in the three UAS regions. Nothing is known about pleiotropic regulatory mutations affecting the expression of yeast catalase and cytochrome *c* genes. Thus, before this study was carried out it seemed possible that co-ordination of synthesis of the two proteins was a very indirect process, having perhaps only heme as a common metabolic signal.

This study shows that *CTT1* gene expression is positively controlled by the product of the *CYP1* gene (Clavilier *et al.*, 1976), more recently also called *HAP1* (Guarente *et al.*, 1984; Verdiere *et al.*, 1986). This gene has been demonstrated to be a mediator of heme activation of transcription of genes coding for mitochondrial cytochromes (Pfeifer *et al.*, 1987a,b). It reports characterization of the HAP1 (CYP1) binding site within the *CTT1* upstream region by gel retardation (Garner and Revzin, 1981), DNase I footprinting (Galas and Schmitz, 1978) and methylation interference assays (Siebenlist and Gilbert, 1980). Comparison of this binding site with those of the *CYC1* and *CYC7* genes (Pfeifer *et al.*, 1987b) shows that there is virtually no sequence similarity between them. Nevertheless, they appear to have a very similar geometry.

Results

Specific binding of proteins to upstream regions of yeast CTT1 and CYC1 genes

In the search for factors involved in heme control of the catalase T (*CTT1*) gene of the yeast *S.cerevisiae* and in co-ordination of control of this gene with heme control of other hemoprotein genes, crude extracts of strain D273-10B were tested for their ability to form complexes with a labelled fragment. According to deletion analysis (Spevak *et al.*, 1986), this fragment ($-522/-399_{CTT1}$) contains an element important for heme control of the gene. As demonstrated in Figure 1, panel A, extracts prepared from derepressed (glycerol-grown) cells were tested by the gel retardation method (Garner and Revzin, 1981). A prominent retarded band was observed, which can be competed by an excess of unlabelled *CTT1* upstream fragment. Competition for the factor binding to the *CTT1* fragment was also observed with unlabelled fragment $-380/-244_{CYC1}$ which contains the positive heme control (UAS) region of the *CYC1* gene coding for yeast iso-1-cytochrome *c*. In a reciprocal experiment with

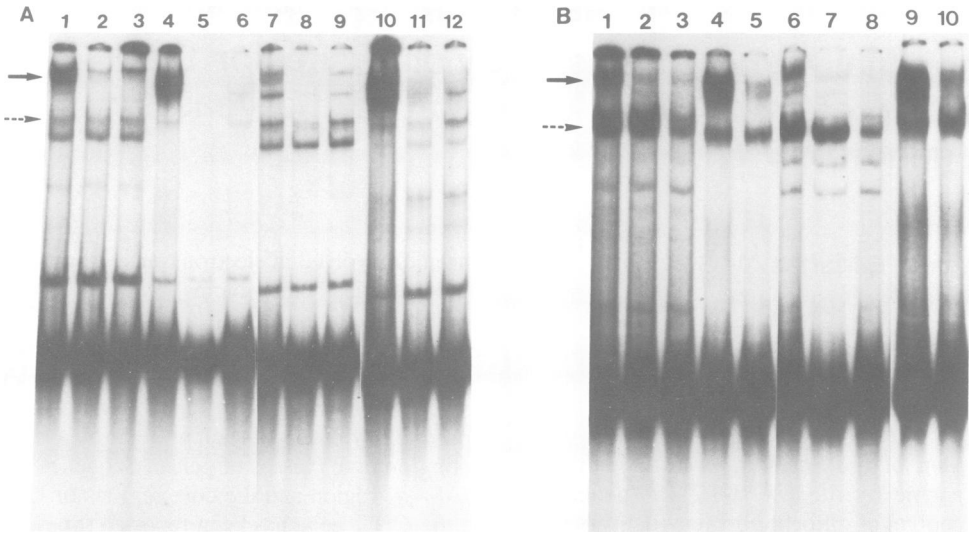

Fig. 1. Gel retardation assay of complex formation of *CTT1* and *CYC1* UAS fragments with proteins from yeast extracts. DNA fragments: (**A**) ^{32}P-end-labelled $-522/-399_{CTT1}$, (**B**) ^{32}P-end-labelled $-380/-244_{CYC1}$. (A) Extracts from strain D273-10B grown on: **lanes 1–3**, glycerol; **lanes 4–6**, 10% glucose; **lanes 7–9**, galactose; **lanes 10–12**, galactose, $-O_2$. Competitor DNAs: lanes 1, 4, 7 and 10, none; 2, 5, 8 and 11, $-522/-399_{CTT1}$; 3, 6, 9 and 12, $-380/-244_{CYC1}$. (B) Extracts from strain D273-10B grown on: **lanes 1–3**, glycerol; **lanes 4** and **5**, 10% glucose; **lanes 6–8**, galactose; **lanes 9** and **10**, galactose, $-O_2$. Competitor DNAs: lanes 1, 4, 6 and 9, none; 2, 5, 7 and 10, $-522/-399_{CTT1}$; 3 and 8, $-380/-244_{CYC1}$. Competitor DNAs were added in 30-fold excess, no hemin was added to incubation mixtures. Arrows: complex with factor binding to both *CTT1* and *CYC1* upstream regions (HAP1); discontinuous arrows, complexes with *CTT1* or *CYC1* upstream regions, respectively.

labelled fragment $-380/-244_{CYC1}$ a corresponding result was obtained; again a complex was observed that can be competed by an excess of both unlabelled fragments. Extracts from cells grown under various physiological conditions (10% glucose, galactose, absence of oxygen) were also tested. They all contain a factor binding to both *CTT1* and *CYC1* upstream fragments. Minor differences in migration of the complexes formed with different extracts might be due to modification of the factor or to binding of additional proteins.

Heme stimulation and HAP1 dependence of binding

Formation of the complex between fragment $-522/-399_{CTT1}$ and the factor binding to both *CTT1* and *CYC1* upstream regions can be stimulated by adding 40 μM hemin (Figure 2). The extent of stimulation observed is dependent on strains and growth conditions used and ranges from 5- to 50-fold. The specific *CTT1* complex detected after heme stimulation was again competed by an excess of the *CYC1*– UAS fragment. Since hemin-stimulated binding of the HAP1 protein to the UAS1 region of the *CYC1* gene was reported while this work was in progress (Pfeifer *et al.*, 1987a) it seemed possible that the factor binding to both *CTT1* and *CYC1* upstream regions was the *HAP1* gene product. Therefore, a *hap1* mutant lacking the protein binding to UAS1 of *CYC1* was tested for *CTT1* binding activity. In contrast to extracts from a corresponding wild-type strain, mutant extracts contain no detectable hemin-stimulated *CTT1*-binding factor (Figure 2). It appears extremely likely from these results and from those presented below that this factor is indeed the *HAP1* gene product.

HAP1 dependence of CTT1 gene expression

To test whether binding of the HAP1 protein to the *CTT1* upstream region is important for the regulation of the gene the *hap1* mutant and the corresponding wild-type strain were transformed with plasmids containing *CTT1–lacZ* fusion

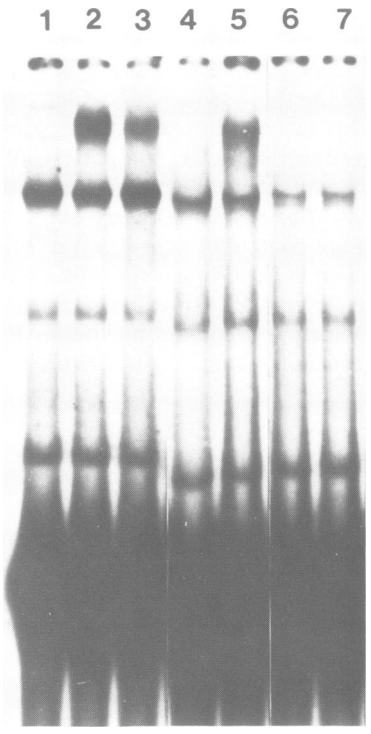

Fig. 2. Hemin stimulation of binding of factor to UAS_{CTT1} fragment and absence of binding factor from *hap1* mutant extract. Binding to labelled fragment $-522/-399_{CTT1}$ tested with extract from: **lanes 1–3**, strain BWG1-7A (wild-type) grown on 2% galactose; lane 1, no addition; 2, + 40 μM hemin; 3, + 40 μM hemin + unlabelled $-380/-244_{CYC1}$ (30-fold excess); **lanes 4** and **5**, strain BWG1-7A grown on 2% glucose; lane 4, no addition; 5, + 40 μM hemin; **lanes 6** and **7**, strain BWG1-7A-hap1-1 grown on 2% glucose; lane 6, no addition; 7, + 40 μM hemin.

genes (Spevak *et al.*, 1986). Transformants were grown under derepressing conditions and specific β-galactosidase

Table I. *HAP1* dependence of expression of a *CTT1*−*lacZ* fusion gene

Plasmid	β-Galactosidase activity (nmol/min/mg protein)	
	HAP1	*hap1*
pF14/1 (wild-type)	26.0	10.7
pF14/1 Δ−398/−226	639.7	23.4
pF14/1 Δ−518/−397	7.1	6.5

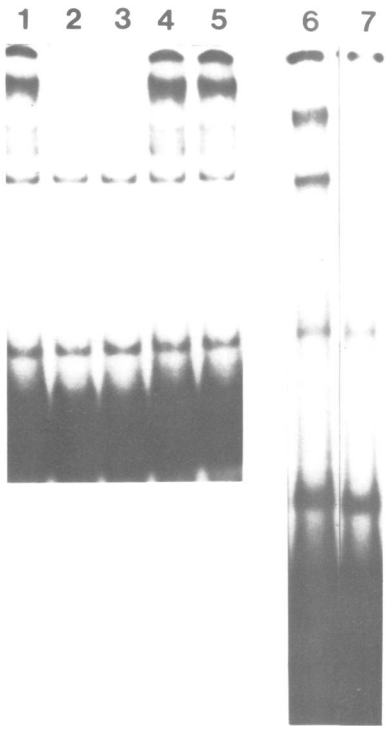

Fig. 3. Competition of unlabelled *CTT1* upstream fragments with ^{32}P-labelled fragment $-522/-399_{CTT1}$. Extracts were from strain D273-10B grown on galactose. Competitors: **lanes 1** and **6**, none; **lane 2**, $-522/-421_{CTT1}$; **lane 3**, $-522/-455_{CTT1}$; **lane 4**, $-454/-399_{CTT1}$; **lane 5**, $-522/-472_{CTT1}$; **lane 7**, synthetic oligonucleotide ($-480/-451_{CTT1}$). Competitors were added in 50-fold excess.

activities of crude extracts were assayed (Table I). β-Galactosidase is 2.4-fold higher in the wild-type strain than in the *hap1* mutant. DNA−RNA (Northern) hybridization showed that differences between wild-type and mutant cells in levels of mRNA transcribed from the chromosomal *CTT1* gene are no greater than those estimated from β-galactosidase activities of cells transformed with the multicopy *CTT1*−*lacZ* fusion plasmid. However, the difference between wild-type and mutant is enhanced to 27-fold when a region of the *CTT1* promoter is deleted (plasmid pF14/1 Δ−398/−226) (Spevak *et al.*, 1986). When the HAP1-binding region is deleted (pF14/1 Δ−518/−397) β-galactosidase activity drops 3.7-fold compared to pF14/1, and *HAP1* dependence is lost almost entirely.

The results presented above allow the conclusion that the HAP1 protein mediates positive heme-inducible transcriptional control not only of the *CYC1* but also of the *CTT1* gene. However, the RC2 protein (Arcangioli and Lescure, 1986), which binds to the HAP1 recognition sequence of $UAS1_{CYC1}$ (Pfeifer *et al.*, 1987a), does not bind to the corresponding region of the *CTT1* gene: the RC2 complex migrates ahead of the HAP1 complex and can be competed

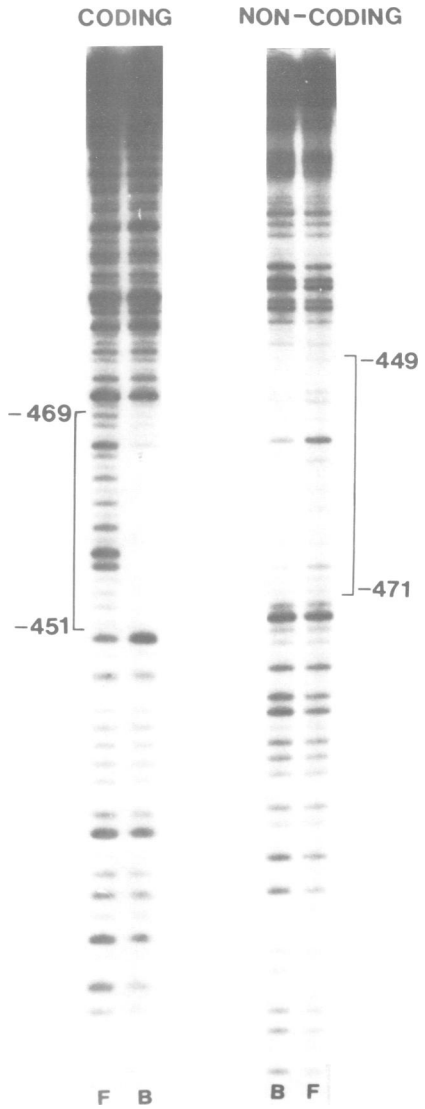

Fig. 4. DNase I footprint analysis of *HAP1*−UAS_{CTT1} complex. Incubations were carried out with extracts from strain D273-10B grown on galactose, $-O_2$. Fragment $-522/-420_{CTT1}$ (created by a *SalI* linker insertion next to position −420), 3′-labelled at the *SalI* site, was used for analysis of the coding strand, fragment $-522/-399_{CTT1}$, 3′-labelled at the *Eco*RI site for the non-coding strand. F, free DNA; B, DNA bound to HAP1 protein.

with an excess of UAS_{CYC1} (Figure 1, panel B, lanes 3 and 8), but not with the UAS_{CTT1} fragment (lanes 2, 5, 7 and 10). Correspondingly, we also observe a complex specifically formed with the UAS_{CTT1} fragment (Figure 1, panel A). A complex migrating somewhat faster than the HAP1 complex can be competed with the *CTT1* upstream fragment (lanes 2, 8 and 11), but not with the *CYC1* fragment (lanes 3, 9 and 12).

Localization of the HAP1 binding site(s)

Localization of the HAP1 binding site(s) within fragment $-522/-399_{CTT1}$ was carried out by competition with a set of smaller fragments, by DNase I footprinting (Galas and Schmitz, 1978) and by methylation interference analysis (Siebenlist and Gilbert, 1980; Hendrickson and Schleif, 1985). Results of competition analysis are summarized in Figure 3. They show that a functional binding region is present in a synthetic oligonucleotide covering the upstream

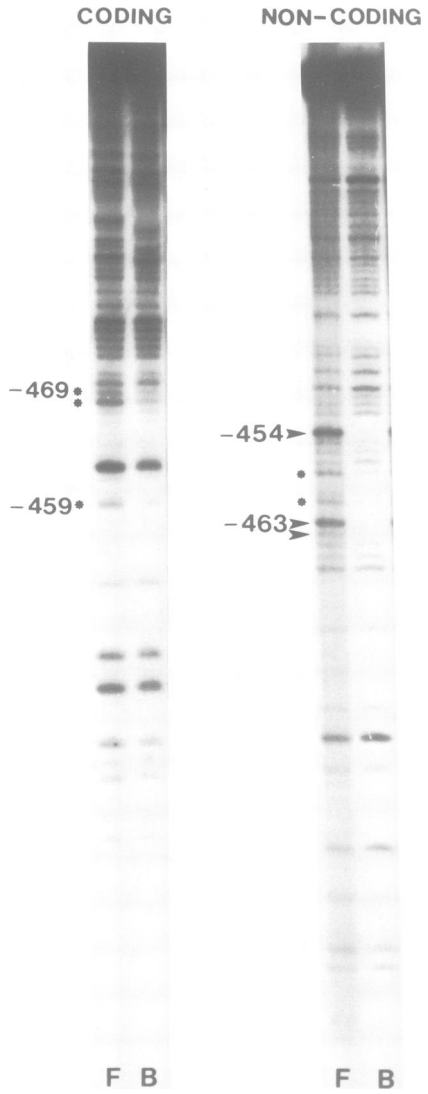

Fig. 5. Methylation interference analysis of *HAP1*−UAS_{CTT1} complex. Incubations were carried out with extracts from strain D273-10B grown on galactose, $-O_2$. Fragment $-522/-420_{CTT1}$, 3′-labelled at the *SalI* site, was used for analysis of the coding strand, fragment $-522/-399_{CTT1}$, 3′-labelled at the *Eco*RI site, for the non-coding strand. F, free DNA; B, DNA bound to HAP1 protein. Arrowheads: guanine contacts, asterisks: adenine contacts.

sequence from bp −480 to −451, and that no binding site can be detected by the competition assay between bp −522 and −472 and between −454 and −398, respectively.

The DNA region protected in the HAP1-complex was determined by DNase I footprinting. The coding and the non-coding strand of double-stranded fragments containing the binding region were end-labelled in separate experiments, labelled fragments were incubated with extract containing HAP1 protein and treated with DNase I. The DNA−protein complex was then separated from the free DNA by polyacrylamide gel electrophoresis, and DNA from both fractions was analyzed on a sequencing gel. As demonstrated in Figure 4, a 19 bp region (−469 to −451) is protected from DNase I digestion in the coding strand, whereas a 23 bp segment (−471 to −449) shows reduced DNase I sensitivity in the non-coding strand.

Methylation interference analysis was used to probe the binding region for protein−base contacts in the complex.

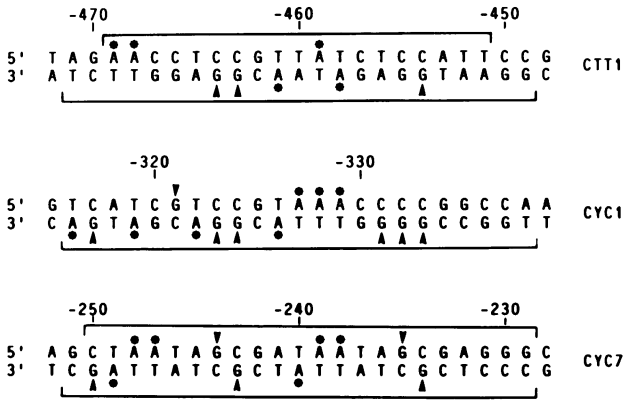

Fig. 6. Summary of DNase I footprint and methylation interference analysis of *HAP1*−UAS_{CTT1} complex and comparison with HAP1 complexes at $UAS1_{CYC1}$ and UAS_{CYC7} (Pfeifer *et al.*, 1987b). Brackets indicate sequences protected from DNase I by HAP1. Guanine contacts of protein are marked by arrowheads, adenine contacts by asterisks. The orientation of $UAS1_{CYC1}$ has been inverted to obtain an optimal alignment with the other two sequences.

The end-labelled DNA fragments used in the DNase I footprint experiments were pretreated with dimethyl sulfate, incubated with extract, and free DNA and DNA−protein complexes were again separated electrophoretically. N-7 methylated guanine and N-3 methylated adenine residues were then detected by alkaline cleavage of DNA strands (Hendrickson and Schleif, 1985) and analysis of cleavage products on sequencing gels. Weakening of bands representing cleavage sites in bound compared to free DNA indicates major groove contacts in the case of guanine and minor groove contacts in the case of adenine residues. As demonstrated in Figure 5, three adenine contacts were detected in the coding strand and three guanine and two adenine contacts were observed in the non-coding strand within the binding region determined by DNase I footprinting.

In Figure 6 the HAP1 protein-binding sequence of the *CTT1* upstream region is aligned with the corresponding regions of the *CYC1* and *CYC7* genes (Pfeifer *et al.*, 1987b) to obtain an optimal fit of DNase I footprints and methylation interference-derived contacts in the three sequences. The overall sequence similarity between the three binding sites aligned in this manner is surprisingly low. Whereas there is a significant similarity between the *CTT1* and *CYC1* binding sites in a core region (9 of 12 bp) no similarity exists in the residual 11 bp of the region defined by DNase I footprinting. Furthermore, and even more strikingly, both *CTT1* and *CYC1* sequences have very little similarity to the HAP1 binding site of the *CYC7* gene (6 of 23 and 7 of 23 bp, respectively; in both cases 5 of 12 bp in the core region are identical). Only 4 of the 23 bp are identical in all three sequences, all of them located in the core region.

In contrast to the DNA sequence, the geometry of the three HAP1 complexes appears to be quite similar. DNase I footprints extend over 23 bp in all three cases, and alignment of footprints results in a remarkable similarity of methylation interference-derived contacts. Although experiments on the *CTT1* gene and on the two cytochrome *c* genes have been carried out in different laboratories under at least somewhat different experimental conditions seven of the eight guanine and adenine contacts of the *CTT1* sequence are present at equivalent positions of the *CYC1* or the *CYC7* sequence. Three of 4 bp identical in the three sequences are

contact residues in all cases. All three binding regions show major groove and minor groove contacts alternating such that the HAP1 protein, which is in contact with two full helical turns, binds at least predominantly from one side of the helix. In contrast to the *CYC7* and *CYC1* binding sites where one and two DNA–protein contacts were detected at the opposite side of the double helix (Pfeifer *et al.*, 1987b) all methylation interference-derived contacts at the UAS region of the *CTT1* gene are accessible from one side of the double helix.

Discussion

Like some other hemoprotein genes of the yeast *S.cerevisiae* the *CTT1* gene is under positive control by heme. Hemoproteins have a broad spectrum of catalytic functions like oxygen binding, electron transport, peroxidation or oxygenation. They are located in different subcellular compartments like mitochondria, peroxisomes, the endoplasmic reticulum or the cytoplasm. Therefore, it is by no means obvious that their formation is co-ordinately controlled. Thus, any true co-ordination of control between hemoproteins of different classes would indicate a functional link between these proteins.

The results obtained in this investigation show that transcription of the gene coding for an extra-mitochondrial hemoprotein, the non-peroxisomal, presumably cytoplasmic, catalase T, is controlled by the *HAP1* gene product. The HAP1 protein has been found to regulate transcription of several genes coding for mitochondrial cytochromes. Evidence obtained in this investigation that it also controls formation of an extra-mitochondrial catalase indicates that HAP1 has a more general function as a regulator of synthesis of hemoproteins and as a co-ordinator of the activity of genes coding for proteins involved in various aspects of oxygen metabolism in different subcellular compartments. The details of a function of a cytoplasmic catalase in connection with mitochondrial electron transfer to oxygen are not clear yet, but yeast would be an ideal system for studying this function. In any case, the HAP1 protein appears to act by mediating the effect of a low mol. wt compound, hemin, which functions as a metabolic signal, to a number of promoters. This conclusion is substantiated by our findings that, as in the case of the *CYC1* gene, the HAP1 protein binds to a *CTT1* upstream region previously found by *in vitro* mutagenesis to be involved in heme control, and that this binding is stimulated by hemin *in vitro*. So hemin, which is produced in the mitochondria and requires oxygen for its synthesis, activates the HAP1 protein, which functions in the nucleus. The HAP1 protein in turn activates a number of genes and triggers the formation of proteins necessary in connection with oxygen utilization.

Although the details of the mechanism of activation of transcription by the HAP1 protein are presently unknown, comparison of the three well-characterized binding sites upstream of the *CYC1*, *CYC7* and *CTT1* genes allows a number of interesting insights. When Pfeifer *et al.* (1987b) reported their results of a comparison of the HAP1 binding sites of the *CYC1* and *CYC7* genes they noted a surprising lack of similarity of the two binding sequences and a much higher degree of similarity of the geometry of the two complexes. Including a third site creates a firmer basis for an attempt to identify the features in the DNA sequence or structure specifically recognized by the HAP1 protein. Compared to the two sites of the cytochrome *c* genes, the HAP1 site of the *CTT1* gene is again strikingly different. Significant sequence similarity is only present within a 12 bp core region of the *CYC1* and *CTT1* sites. The 4 bp common to all three sites are located within this core region. According to methylation interference analysis three of them are critical protein–DNA contacts at all three binding sites. One might conclude from this analysis that the HAP1 binding site of the *CTT1* gene is only a variant of the *CYC1* site; however, the situation is really more complex. The latter site is recognized by a second protein, RC2 (Arcangioli and Lescure, 1985), which binds to it with a geometry surprisingly similar to that of the *HAP1*–UAS1 complex (Pfeifer *et al.*, 1987a). However, RC2 does not bind to the *CTT1* site. Therefore, in contrast to HAP1, RC2 is likely to have no general function in hemoprotein gene activation. This obviously does not exclude a *CYC1* gene-specific function. In fact, the second factor binding to the *CTT1* heme control region observed in this study appears to be gene specific as well. Its binding site, which is presently characterized in detail, at least overlaps the HAP1 binding site. Its function might be analogous to that of RC2. Alternatively, this protein might be similar, but not identical to the region A factor described by Pfeifer *et al.* (1987a), which binds to $UAS1_{CYC1}$ next to the HAP1 protein.

How can HAP1 specifically bind to three DNA sequences which share only 4 of 23 bp? Our results confirm the conclusion of Pfeifer *et al.* (1987b) that different affinities of the protein to the sites do not provide an adequate explanation. According to our results and to those of Pfeifer *et al.* the *in vitro* affinities of the three sites seem to be fairly similar. The data from both studies offer several alternative conclusions. (i) The same binding site of the HAP1 protein specifically interacts with the different sequences. The virtually identical size of the three DNase I footprints, the similarities in the pattern of major groove and minor groove contacts and the fact that three of four bases conserved in all three sequences are protein contacts in all cases make this at least an attractive hypothesis. (ii) The HAP1 protein possesses two or three different binding sites which interact with the three sequences. Allosteric effects or some overlap between the sites would have to explain the competition between the three DNA segments observed in the two investigations. (iii) Since binding experiments with purified HAP1 protein (or HAP1 protein produced in a heterologous system) have not been carried out yet, the possibility that HAP1 does not directly bind to the indicated sequences cannot be rigorously excluded. HAP1 action could theoretically be exerted by virtue of gene-specific factors. Whereas alternatives (ii) and (iii) presently cannot be excluded there is little if any evidence in favour of them. For a completely satisfactory answer to the question raised a combination of *in vitro* mutagenesis of the three binding sites and of the *HAP1* gene and a purification of the HAP1 protein is necessary.

Materials and methods

Yeast strains and growth conditions

The *S.cerevisiae* strains D273-10B (α, ATCC 24657), BWG1-7A (*a leu2-2, leu2-112, his4-519, ade1-100, ura3-52*) and BWG1-7A–hap1-1 (*a leu2-2, leu2-112, his4-519, ade1-100, ura3-52, hap1-1*) were used in this study. The latter two strains were kindly provided by L.Guarente, MIT, Cambridge, USA. Strains were grown on YP medium (1% yeast extract, 2%

bacto-peptone) with 2% glucose, 10% glucose, 2% galactose or 2% glycerol as carbon source. In the case of anaerobic cultures (Zimniak *et al.*, 1976), galactose medium supplemented with Tween 80 (0.26 g/l) and ergosterol (12 mg/l) was used. Transformants were grown on synthetic complete medium (Sherman *et al.*, 1986) without leucine.

Cell extracts

Cell extracts for DNA binding experiments were prepared as described by Arcangioli and Lescure (1985). Cells were harvested at an A_{600} of 2.0, washed with buffer A (200 mM Tris−HCl, pH 8.0, 400 mM ammonium sulfate, 10 mM $MgCl_2$, 1 mM EDTA, 7 mM β-mercaptoethanol, 10% glycerol) and resuspended in an equal volume of buffer A containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were broken with glass beads (0.45−0.5 mm diameter) by vigorous agitation on a Vortex mixer (10 × 1 min with 1 min intervals for cooling on ice). All further steps were carried out at 4°C unless otherwise indicated. After breakage of cells an additional volume of buffer A was added, and after 10 min the homogenate was centrifuged at 1500 *g* for 3 min. The resulting supernatant fraction was subjected to centrifugation at 100 000 *g* for 1 h. To the thus cleared lysate an equal volume of saturated ammonium sulfate in buffer A with 1 mM PMSF was added. After shaking for 30 min the sample was centrifuged at 16 000 *g* for 30 min. The protein pellet was dissolved in 1 ml buffer B (20 mM Hepes, pH 7.8, 5 mM EDTA, 7 mM β-mercaptoethanol, 10% glycerol, 1 mM PMSF). Alternatively, for small scale extract preparation, the ultracentrifugation step was omitted. The final protein concentration of the extracts was ~10 μg/μl.

Plasmids and DNA fragments

CTT1 fragments were derived from a plasmid containing the *CTT1* upstream region in vector pUC13, from *SalI* linker insertion derivatives of this plasmid and from deletion mutants of plasmid pF14/1 (Spevak *et al.*, 1986). A synthetic double-stranded oligonucleotide [bp −480 to −451 (base pairs are numbered defining the A of the start ATG as +1 in all cases) of the *CTT1* upstream region plus *Eco*RI sticky ends] was prepared by hybridization of two single-stranded oligonucleotides supplied by R.Hauptmann, Ernst Boehringer-Institut für Arzneimittelforschung, Vienna. DNA fragments were end-labelled with ^{32}P by filling in ends with the Klenow fragment of *Escherichia coli* DNA polymerase I.

Gel retardation assay

The gel retardation assays were carried out essentially according to Arcangioli and Lescure (1985). Incubation mixtures (20 μl) contained linear DNA fragment (~10 fmol) end-labelled with ^{32}P, 1.0−2.0 μg sonicated herring sperm DNA (average length 500−1000 bp), 5 mM $MgCl_2$, 50 mM NaCl, 10 mM Hepes, pH 7.8, 0.5 mM EDTA, 5% glycerol, 0.01% bromophenol blue and 0.5−2.5 μg protein. Binding reactions were carried out at 20°C for 10−15 min prior to loading onto a 6% polyacrylamide gel [90 mM Tris, 90 mM borate, 2.5 mM EDTA, pH 8.0 (TBE)]. In certain experiments hemin (2 mg hemin in 100 μl 95% ethanol plus 2 volumes 500 mM Hepes, pH 9.0, dissolved by agitation on a Vortex mixer, diluted with 30 volumes 10 mM Hepes, pH 9.0, and finally with an equal volume of 87% glycerol) was added to a final concentration of 40 μM. In control experiments, an identical amount of solvent without hemin was added. Electrophoresis was carried out at 10 V/cm for 2−4 h. Gels were treated with 10% acetic acid, 10% methanol, transferred to Whatman 3MM paper, dried and autoradiographed.

DNase I footprinting

For DNase I protection experiments, which were carried out as described by Arcangioli and Lescure (1985), binding reactions were scaled up 10-fold except that 300 fmol of labelled DNA fragment was used. Furthermore, 40 μM hemin was added in all cases. After a 15 min incubation at 20°C, 10 μl DNase I solution (50 ng/μl DNase I, 100 mM $CaCl_2$) was added. After 30 s the reaction was stopped by adding 10 μl 500 mM EDTA. Electrophoresis was carried out as above and bound and free DNA was visualized by autoradiography of the wet gel at 4°C overnight. The bands corresponding to free and HAP1-protein-bound DNA were cut out and the DNA was eluted with 3 ml of 0.3 M NaCl, 20 mM Tris−HCl, pH 7.5, 1 mM EDTA, 0.5 μg/ml sonicated herring sperm DNA, by shaking at 37°C for 6 h. The supernatant solution was then replaced by fresh buffer and elution was continued overnight. Eluted DNA was purified and concentrated by passage over Elutip-d columns (Schleicher and Schüll), extraction with phenol and chloroform and ethanol precipitation. The DNA was dissolved in water, denaturing buffer was added, the samples were heated for 5 min in a boiling water bath, chilled on ice and loaded onto 7% polyacrylamide gels containing 7.3 M urea and TBE. Gels were calibrated by sequencing aliquots of end-labelled DNA fragments according to Maxam and Gilbert (1980). The gels were treated with 10% acetic acid, 10% methanol, transferred to Whatman 3MM paper, dried and autoradiographed.

Methylation interference

Methylation interference of protein binding (Siebenlist and Gilbert, 1980; Hendrickson and Schleif, 1985) was tested with DNA (~300 fmol) methylated in 200 μl 50 mM Na-cacodylate, pH 8.0, 0.1 mM EDTA, 10 mM $MgCl_2$, by addition of 1 μl dimethyl sulfate (DMS) and incubation for 1 min at room temperature. Methylation was stopped by addition of 50 μl of G-stop buffer (1 M Tris−HCl, pH 7.5, 1 M β-mercaptoethanol, 1.5 M sodium acetate, 50 mM magnesium acetate, 1 mM EDTA, 40 μg tRNA/ml). The methylated DNA was precipitated twice with ethanol and used in a scaled up gel retardation assay (see DNase I footprinting). The DNA was purified as described above. To cleave the DNA after methylating purines it was dissolved in 43 μl 20 mM sodium acetate, 7.5 μl 1 M NaOH was added and the sample was incubated at 95°C for 30 min. The mixture was neutralized with 1 M HCl, 20 μl 20 mM Tris−HCl, pH 7.5, and 4 μg tRNA was added, and the samples were precipitated with ethanol and electrophoresed on 7% sequencing gels as described above.

Yeast transformation

Strains BWG1-7A and BWG1-7A-hap1-1 were transformed with plasmid pF14/1 and two deletion derivatives of this plasmid essentially as described by Beggs (1978).

β-Galactosidase assays

β-Galactosidase activity of extracts prepared by breakage of yeast transformants with glass beads (Rose and Botstein, 1983) was assayed using *o*-nitro-phenyl-β-D-galactoside as substrate (Miller, 1972). Protein concentrations of extracts were assayed according to Bradford (1976).

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References

Arcangioli,B. and Lescure,B. (1985) *EMBO J.*, **4**, 2627−2633.
Beggs,J.D. (1978) *Nature*, **275**, 104−109.
Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248−254.
Clavilier,L., Pere Aubert,G., Somlo,M. and Slonimski,P.P. (1976) *Biochimie*, **58**, 155−172.
Galas,D.J. and Schmitz.A. (1978) *Nucleic Acids Res.*, **5**, 3157−3170.
Garner,M.M. and Revzin,A. (1981) *Nucleic Acids Res.*, **2**, 1047−1060.
Guarente,L. and Mason,T. (1983) *Cell*, **32**, 1279−1286.
Guarente,L., Lalonde,B., Clifford,P. and Alani,E. (1984) *Cell*, **36**, 503−511.
Gudenus,R., Spence,A., Hartig,A., Smith,M. and Ruis,H. (1984) *Curr. Genet.*, **8**, 45−48.
Hendrickson,W. and Schleif,R. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3129−3133.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499−560.
Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Pfeifer,K., Arcangioli,B. and Guarente,L. (1987a) *Cell*, **49**, 9−18.
Pfeifer,K., Prezant,T. and Guarente,L. (1987b) *Cell*, **49**, 19−27.
Rose,M. and Botstein,D. (1983) *Methods Enzymol.*, **101**, 167−180.
Sherman,F., Fink,G. and Hicks,J.B. (1986) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Siebenlist,U. and Gilbert,W. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 122−126.
Spevak,W., Hartig,A., Meindl,P. and Ruis,H. (1986) *Mol. Gen. Genet.*, **203**, 73−78.
Verdiere,J., Greusot,F., Guarente,L. and Slonimski,P. (1986) *Curr. Genet.*, **10**, 339−342.
Zimniak,P., Hartter,E., Woloszczuk,W. and Ruis,H. (1976) *Eur. J. Biochem.*, **71**, 393−398.

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