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Evidence for an interaction between the CYP1(HAP1) activator and a cellular factor during heme-dependent transcriptional regulation in the yeast *Saccharomyces cerevisiae*

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Previously, it was shown that the *CYP1(HAP1)* gene product mediates the transcription of several oxygen-regulated genes through a metabolic co-effector, heme, in the yeast *Saccharomyces cerevisiae*. This study investigates the overproduction of the CYP1 protein when the *CYP1(HAP1)* gene is placed under the control of the *GAL10–CYC1* hybrid promoter (either at the locus of the *CYP1(HAP1)* gene or cloned in a high-copy-number plasmid). In these conditions, the CYP1 protein is detected by Western blot analysis and has a molecular mass in agreement with the open reading frame sequence. Band-shift experiments show that the CYP1(HAP1) protein is able to interact specifically with its target sequences *in vitro* without addition of hemin, and forms a large complex with one or several unidentified factors denoted as X. Addition of hemin allows the formation of a new complex which has a lower molecular mass. The internal deletion of the seven repeated amino acid sequences containing the KCPVDH motif in the CYP1(HAP1) protein modifies the heme responsiveness phenomenon observed *in vitro* in the band-shift experiments and *in vivo* in the transcription of the *CYB2, CYC1, CYP3(CYC7)* and *ERG11* genes. On the basis of these data, we propose a new model for heme-induced activation of the CYP1 protein.

Key words: CYP1(HAP1) activator/heme-dependent transcriptional regulation/*S.cerevisiae*/transcription factor

Introduction

In the yeast *Saccharomyces cerevisiae*, a facultative aerobe, the expression of many nuclear genes encoding cytochromes and related proteins that carry out electron transport is regulated according to the oxygen tension in the growth medium. At least two main *trans*-acting regulatory systems have been found to mediate the transcription of these oxygen-regulated genes, either together or separately: the transcriptional activator CYP1 (also called HAP1) (Verdière *et al.*, 1985; Creusot *et al.*, 1988; Pfeifer *et al.*, 1989) and the HAP2/3/4 complex (Hahn and Guarente, 1988; Forsburg and Guarente, 1989). Both exert their effects through a metabolic co-effector, heme, whose biosynthesis is abundant during aerobic growth conditions and low in the absence of oxygen (Labbe-Bois and Labbe, 1990). The transcriptional activator CYP1(HAP1) was the first to be identified and shown to modulate the expression of *CYC1* (Guarente *et al.*, 1984), *CYP3(CYC7)* (Prezant *et al.*, 1987), *CYB2* (Lodi and Guiard, 1991), *CTT1* (Winkler *et al.*, 1988) and *CYT1* (Schneider and Guarente, 1991) by an interaction with specific sequences (UASs) characterized by DNase I footprinting upstream of these genes. *In vivo CYP1*-dependent UASs activity was found to be induced by heme (Guarente *et al.*, 1984; Lodi and Guiard, 1991). Using cellular extracts, *in vitro CYP1(HAP1)* binding to specific target sequences has also been shown to be induced by hemin (Pfeifer *et al.*, 1987a; Winkler *et al.*, 1988; Lodi and Guiard, 1991). *CYP1(HAP1)* has also been reported to be required in the transcriptional activation of *HMG1* (Thorness *et al.*, 1989), *ERG11* (Turi and Loper, 1992) and *ROX1* (Zitomer and Lowry, 1992). The *ROX1* gene product plays a particular role in the regulatory circuitry of genes regulated by heme. Indeed it is involved in the control of the repression of transcription of 'hypoxic' genes such as *ANB1, COX5b, HEME13* and *CYP3(CYC7)* (Lowry *et al.*, 1990) which are expressed more efficiently under conditions of reduced oxygen tension. Consequently, under aerobic conditions, the product of the *CYP1(HAP1)* gene can also modulate the repression of several genes by means of the ROX1 DNA-binding protein.

The sequence of the *CYP1(HAP1)* gene was determined and revealed an uninterrupted open reading frame of 4449 nucleotides able to encode a protein of 1483 amino acid residues (Creusot *et al.*, 1988; Pfeifer *et al.*, 1989). The predicted protein can be divided into at least three functional domains (Creusot *et al.*, 1988; Pfeifer *et al.*, 1989). The N-terminal part (residues 1–148) has the specific DNA-binding sequence. It contains a stretch of 33 amino acids (residues 63–95) which displays a high level of structural identity with several yeast regulatory proteins, including GAL4. For GAL4, a three-dimensional model of the DNA-binding domain has been proposed following NMR and X-ray crystallographic analysis. Two zinc ions are coordinated by six cysteines to form a 'zinc cluster' structure (Baleja *et al.*, 1992; Kraulis *et al.*, 1992; Marmorstein *et al.*, 1992). A second CYP1(HAP1) domain, necessary for its transcriptional activity (Pfeifer *et al.*, 1989), has been located at the carboxyl end of the protein (residues 1308–1483) and contains an acidic region characteristic of transcriptional activation domains. The third region, in the middle of the protein (residues 280–438), contains seven adjacent repeat units including a conserved KCPVDH amino acid motif. This motif, which resembles a metal or heme-binding site (Creusot *et al.*, 1988), has been proposed to be a functional domain which mediates heme control of CYP1(HAP1) activity (Pfeifer *et al.*, 1989; Kim *et al.*, 1990). The identification of multiple functional domains suggests that the CYP1 (HAP1) transcription factor has a modular organization.

The mechanism of induction of UASs activity by CYP1(HAP1) is not known. One hypothesis proposed by Pfeifer *et al.* (1989) is that a cellular factor could mediate heme regulation of CYP1(HAP1) activity by masking its DNA-binding domain, and that heme counteracts this masking. In order to gain an understanding of the mechanism of transcriptional activation mediated by the CYP1(HAP1) protein, we investigated in this work how heme affects the *in vitro* binding of this protein to several target sequences. We have been able to show that in fact CYP1(HAP1) can bind specifically to its upstream activation sites (UASs) in the absence of hemin. The addition of hemin modifies the interactions between CYP1(HAP1) and a cellular factor modifying the DNA complex observed in band-shift experiments. We have also analysed how the *CYP1(HAP1)* product controls the *CYB2, CYC1, CYP3(CYC7)* and *ERG11* transcript levels *in vivo* when its expression is carried out in *HEM1* and *hem1* strains or when *CYP1(HAP1)* itself is modified by an internal deletion, removing the seven adjacent repeat units including the conserved KCPVDH amino acid motif. The implications of our findings are discussed and a synthetic model is presented which aims to explain the mechanism of action of the CYP1(HAP1) protein on genes regulated by heme in yeast.

Results

Overproduction of the CYP1 protein when the CYP1 gene is under the control of the GAL10–CYC1 promoter

Under aerobic growth conditions, the transcription level of the *CYP1* regulatory gene in *S.cerevisiae* is very low and it is not surprising that the detection of the CYP1 protein

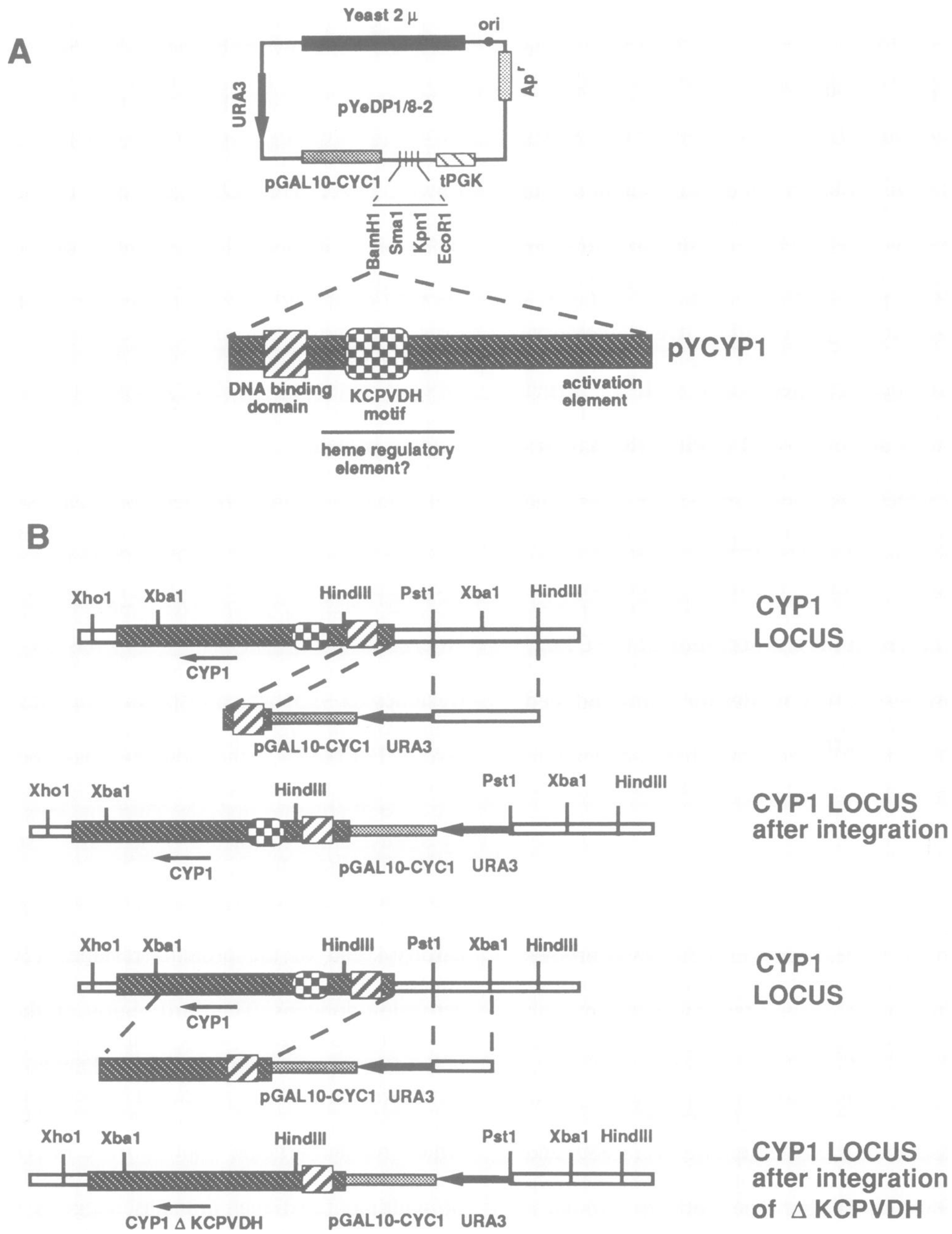

Fig. 1. (**A**) Schematic description of plasmid pYCYP1. The *CYP1* gene was put under the control of the *GAL10–CYC1* promoter by insertion into the expression vector pYpDP1/8-2. (**B**) The *CYP1* gene and the *CYP1ΔK* gene carrying an internal deletion (960–1560) were put under the control of *GAL10–CYC1* promoter by homologous integration.

by Western blotting analysis has not been possible. These observations are consistent with the role of the CYP1 protein as a regulatory factor which must act in the cell at a low concentration. Overproduction of the CYP1 protein in yeast has been investigated and shown to be toxic to the cells (data not shown), as has already been observed in the overproduction of several yeast regulatory proteins. To overcome this problem, we put the transcription of the *CYP1* gene under the control of a *GAL10–CYC1* hybrid yeast promoter (Figure 1) which allowed us to block CYP1 protein synthesis in glucose growth conditions. Subsequently, CYP1 protein synthesis can be specifically induced by a switch to galactose growth conditions and detected by SDS–PAGE and immunoblot analysis (Figure 2). It was possible to express CYP1 protein at two different levels in yeast (Figure 2A). Indeed when the *CYP1* gene, under the control of the *GAL10–CYC1* hybrid promoter, is cloned in the multicopy expression vector pYeDP1/8-2, a very large amount of CYP1 protein is synthesized as compared to the protein production which can be obtained when the *CYP1* gene is put under the control of the *GAL10–CYC1* promoter by homologous integration (Figures 1 and 2A). The estimated value of the relative mass of the synthesized polypeptide is in agreement with a value of 160 kDa calculated on the basis

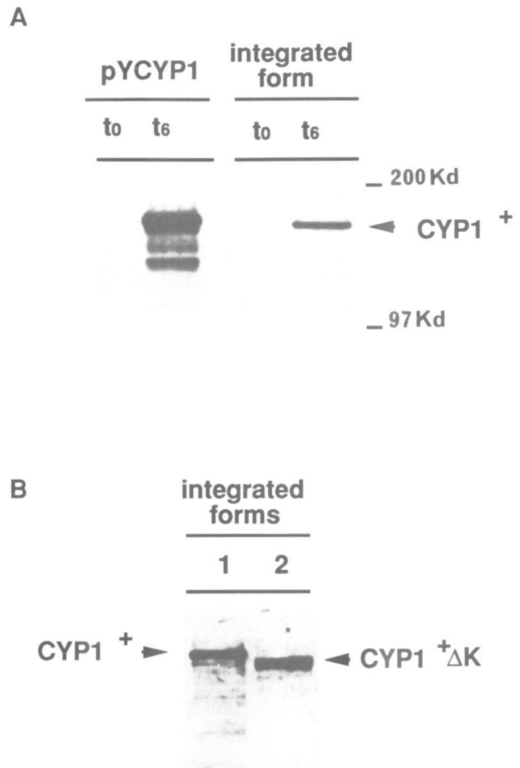

Fig. 2. Overproduction of CYP1 protein: Western blot analysis. Cells were grown in minimum media supplemented with 2% glucose to 1×10^7 cells/ml; 2% galactose was added at $t = 0$ (t0). At $t = 6$ h (t6), yeast protein extracts were prepared, resolved on 6% acrylamide gels containing sodium dodecyl sulphate (SDS), electroblotted to a nitrocellulose sheet and probed with CYP1 antiserum. (**A**) CYP1 protein production was compared between the *S.cerevisiae* strain 334 transformed with the multicopy plasmid pYCYP1 and the *S.cerevisiae* strain 334, where the *CYP1* gene is under the control of the *GAL10–CYC1* promoter. (**B**) The translational level of the *CYP1* and the *CYP1ΔK* genes, both integrated and under the control of the *GAL10–CYC1* promoter, were compared at $t = 6$ h (t6). The proteins were quantified by the method of Bradford (1976).

of the amino acid composition deduced from the open reading frame of 4449 nucleotides composing the *CYP1* gene (Creusot *et al.*, 1988; Pfeifer *et al.*, 1989). Nuclear localization of the overproduced CYP1 protein has been confirmed by immunofluorescence analysis (data not shown).

Gel electrophoresis DNA-binding assay

The CYP1 protein binds to the UAS region of the promoter of different genes and activates their transcription in a heme-dependent manner (Guarente *et al.*, 1984; Lodi and Guiard, 1991). Responsiveness to hemin has been demonstrated *in vitro* using gel retardation DNA-binding assays (Pfeifer *et al.*, 1987a; Winkler *et al.*, 1988; Lodi and Guiard, 1991; Schneider and Guarente, 1991). Protein extracts from yeast cells bearing the *CYP1* gene under the control of a *GAL10–CYC1* promoter, either cloned in a high-copy-number plasmid (pYCYP1) or inserted at its own locus by homologous integration (Figure 1), were prepared and gel-shift analyses were carried out (Figure 3A and B, respectively). The promoter element carrying UAS1-B2 of *CYB2*, encompassing bases −250 to −137, was end-labelled and used as probe. Several oligonucleotides (described in Materials and methods) containing the DNA-binding site of CYP1 characterized by footprinting experiments in the *CYB2* (UAS1-B2), *CYC1* (UAS1-A and UAS1-B) and *CYP3* (UAS′) genes (Pfeifer *et al.*, 1987a,b; Lodi and Guiard, 1991) were used in competition experiments in order to analyse the binding specificity (Figure 3A, lanes 3–6 and Figure 3B, lanes 3 and 5–7). For *ERG11* (UAS-14DM) (Figure 3A, lane 7), the oligonucleotide was chosen because its sequence has been described as a presumptive CYP1 binding site in the *ERG11* promoter (Turi and Loper, 1992). This hypothesis has been confirmed by the observation of a specific interaction of CYP1 with this labelled oligonucleotide (data not shown). The oligonucleotide used as a control in lane 8 of Figure 3A and B is described in Materials and methods. No interaction of CYP1 with this labelled oligonucleotide has been observed (data not shown).

Surprisingly, the retardation complexes obtained with protein extracts from yeast cells transformed with the high-copy-number plasmid bearing the *CYP1* gene under the control of the *GAL10–CYC1* promoter (pYCYP1) do not require the presence of hemin in the binding reaction (Figure 3A, lanes 2 and 8). Under our experimental conditions, all the probe can be titrated to yield a CYP1-dependent complex.

When similar experiments are carried out with cell extracts from a strain where the CYP1 protein is encoded by one copy of the *CYP1* gene under the control of the *GAL10–CYC1* promoter (Figure 1B), giving a moderated CYP1 protein expression (Figure 2A), two main observations are made (Figure 3B). First, in the absence of heme, the CYP1 protein is able to interact specifically with the target sequence, as is shown by the titration of the probe used in this experiment (Figure 3B, lane 2). A diffuse band shift with a weak migration in the gel can be observed and an important part of the complex remains in the well after 2 h of electrophoresis. Secondly, in the presence of 25 μM hemin, the same specific titration of the probe is also observed, but a well-defined band shift is generated (Figure 3B, lanes 4 and 8). In both cases (± hemin), competition experiments using excess amounts of unlabelled oligonucleotides (described in Materials and methods) containing

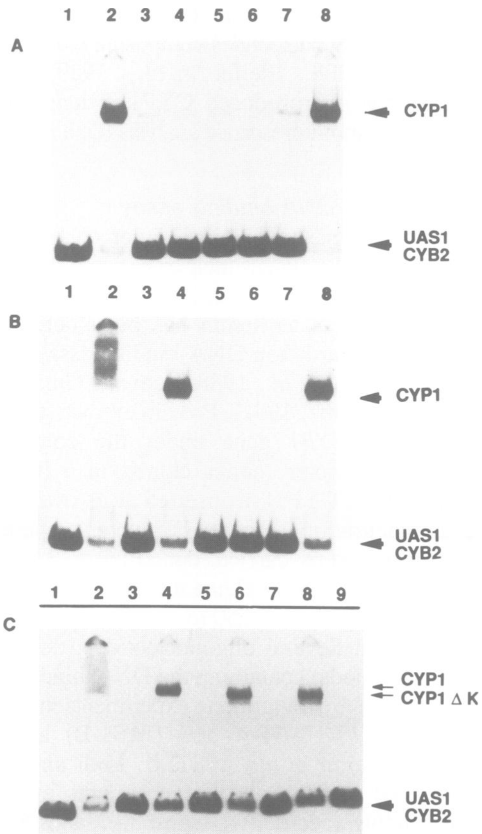

Fig. 3. Band-shift analysis with the *in vivo* overexpressed CYP1 protein. Gel retardation assays were carried out with the linear upstream DNA fragment (−250 to −137) containing the UAS1-B2 of the *CYB2* promoter end labelled with $\gamma^{32}P$. Sequences of competitor oligonucleotides are given in Materials and methods. Electrophoresis was carried out at 5°C. (**A**) Band-shift analysis with the *in vivo* overexpressed CYP1 protein extracted from *S.cerevisiae* strain 334 transformed with pYCYP1. Extracts (3 μg protein) were incubated for 15 min at 0°C with end-labelled probe and assayed as described in Materials and methods. Lane 1, probe without extract; lane 2, binding reaction; lanes 3, 4, 5, 6 and 7 binding reaction in the presence of 100 ng of competitor oligonucleotide UAS1-B2 (*CYB2*), UAS1-B (*CYC1*), UAS1-A (*CYC1*), UAS′ (*CYP3*), UAS1 (*ERG11*), respectively; lane 8, control competitor. Competitor and extract were added simultaneously. (**B**) Band-shift analysis with the *in vivo* overexpressed CYP1 protein extracted from the *S.cerevisiae* strain 334 in which the *CYP1* gene was placed under the control of the *GAL10−CYC1* promoter. Extracts (40 μg protein) were incubated for 15 min at 0°C with the end-labelled probe and assayed as described in Materials and methods. Lane 1, probe without extract; lane 2, binding reaction; lane 3, binding reaction in the presence of 100 ng of competitor oligonucleotide UAS1-B2 (*CYB2*); lane 4, binding reaction in the presence of 25 μM hemin; lanes 5, 6, 7 and 8, binding reaction in the presence of 25 μM hemin and 100 ng of the corresponding competitor oligonucleotide UAS1-B2 (*CYB2*), UAS1-B (*CYC1*), UAS′ (*CYP3*), respectively; lane 8, control competitor. (**C**) Effects of the deletion of the domain containing the repeated KCPVDH motif. The band-shift analyses were carried out with the *in vivo* overexpressed CYP1 protein extracted from the *S.cerevisiae* strain 334 where the *CYP1* locus (lanes 2, 3, 4 and 5) and the *CYP1ΔK* locus with an internal deletion (960−1560) (lanes 6, 7, 8 and 9) had been put under the control of the *GAL10−CYC1* promoter by homologous integration (Figure 1). Protein extracts (40 μg) were incubated for 15 min at 0°C with end-labelled probe and assayed as described in Materials and methods. Lane 1, probe without extract; lanes 2, 3, 6 and 7, binding reactions without addition of heme; lanes 4, 5, 8 and 9, binding reactions in the presence of 25 μM hemin; 100 ng of oligonucleotide containing UAS1-B2 (*CYB2*) was used as competitor in lanes 3, 5, 7 and 9.

the DNA-binding sites of CYP1 confirm the specificity of the interactions (Figure 3B, lanes 3 and 5−7). Addition of hemin at the same time, or 10 min later than the cell extracts, was also tested and had the same effect on band-shift formation.

In vitro effects of the internal deletion 247−444 in CYP1 protein on this heme responsiveness

The repeated amino acid sequence containing the KCPVDH motif (located in the 247−444 interval), which has been proposed to be the heme-responsive domain of CYP1 (Creusot *et al.*, 1988; Pfeifer *et al.*, 1989), was removed to give the CYP1ΔK construction. The modified gene was integrated at the CYP1 locus under the transcriptional control of the *GAL10−CYC1* promoter (Figure 1). The synthesis of the truncated CYP1ΔK protein was confirmed by Western blotting. As expected, it presents a lower mass as compared to the CYP1 protein (Figure 2B) and the level of expression of both proteins is not significantly different, which means that the internal deletion removing amino acids 247−444 has no detectable effect *in vivo* on the turnover of the truncated protein as compared to CYP1. The internal deletion of the KCPVDH domain does not affect the *in vitro* formation of a CYP1-dependent complex with a labelled DNA fragment containing UAS1−*CYB2*, but in this case the behaviour of the retardation complex is heme independent (Figure 3C, lanes 6 and 8) with a faster migration as compared to the CYP1-dependent complex observed in the presence of heme (Figure 3C, lane 4). This heme independence is quite similar to that of the retardation complex observed with yeast protein extracts from the strain transformed with pYCYP1 (Figure 3A). Excess amounts of the 30 bp unlabelled oligonucleotides containing UAS-B2 of *CYB2* were used as a competitor to confirm the specificity of the interaction between the truncated CYP1ΔK protein and the DNA-binding site of CYP1 on the *CYB2* promoter (Figure 3C, lanes 7 and 9).

For all experiments illustrated in Figure 3, the amount of yeast protein extract was adjusted to obtain an equivalent titration of the probe (3 μg for 3A, 40 μg for 3B and 3C). The results obtained (presented in Figure 3) were confirmed with two other promoter elements carrying UAS′ of *CYP3* and UAS1 of *CYC1* (data not shown).

Antibody effects on the CYP1-dependent band-shift formation

Experiments using antibodies to supershift the protein−DNA complexes observed when the *CYP1(HAP1)* gene is placed under the control of the *GAL10−CYC1* hybrid promoter were carried out to confirm that the observed complexes contain the CYP1 protein. Band-shift analysis was performed with or without hemin, in the presence of antibodies against the N-terminal part of the CYP1 protein (residues 1−247 (Figure 4A, lanes 5−7 and 11−13). The antibody−antigen interactions do not interfere with the complex formation between CYP1 and the UAS1−*CYB2* target sequence, as is shown by the titration of the labelled probe. In the presence of antibodies, the formation of high mol. wt complexes (labelled probe + CYP1 protein + antibodies) is observed. Such immunocomplexes remain in the well at the highest amount of added CYP1 antibodies. The control serum has no effect on the band-shift experiment (Figure 4A, lanes 3−4 and 9−10). The same results were obtained with protein

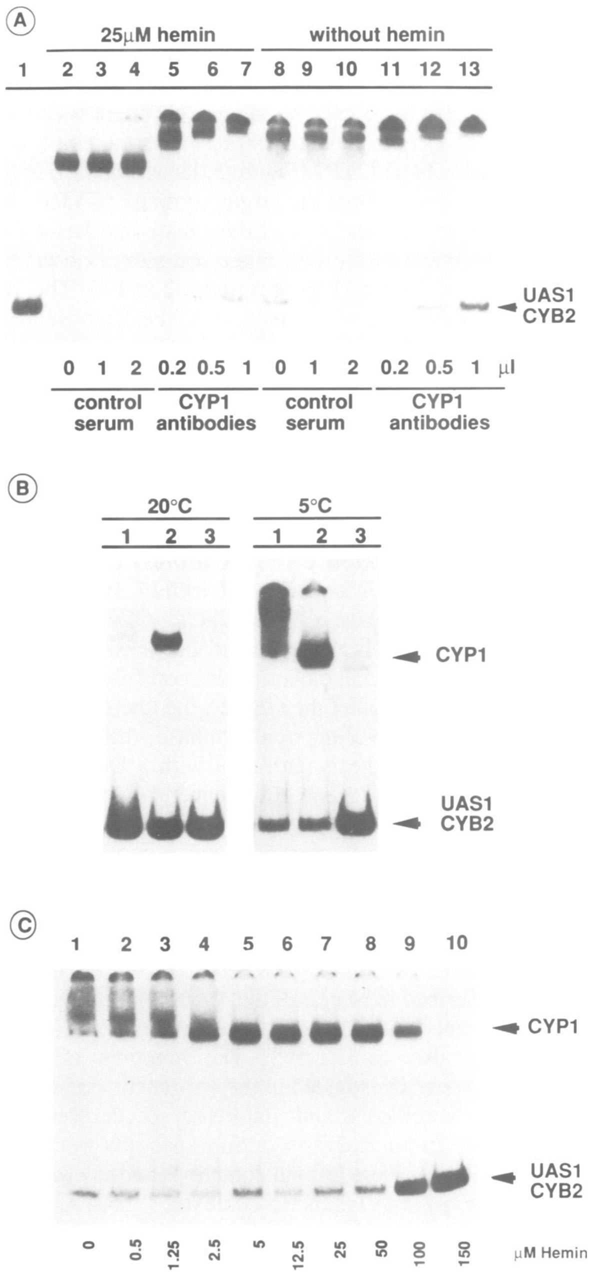

Fig. 4. Analysis of CYP1−DNA complexes: effects of antibodies, temperature and hemin concentration. Gel retardation assays were carried out with labelled linear upstream DNA fragment (−250 to −137) containing the UAS1-B2 of the *CYB2* promoter as a probe. Protein extracts (40 μg) from the *S.cerevisiae* strain 334 where the gene *CYP1* had been placed under the control of *GAL10−CYC1* promoter (Figure 1) were incubated with end-labelled probe and assayed as described in Materials and methods. (**A**) Antibody effects on the CYP1-dependent band-shift formation. Protein extracts were incubated for 15 min at 0°C with end-labelled probe. Electrophoresis was carried out at 5°C. Lane 1, probe without extract; lanes 2−7, band-shift analysis developed with hemin, in the absence (lanes 2−4) or in the presence of CYP1 antibodies (lanes 5−7) and in the presence of control serum (lanes 3−4); lanes 8−13, band-shift analysis developed without hemin, in the absence (lanes 8−10) or in the presence of CYP1 antibodies (lanes 11−13) and in the presence of control serum (lanes 9−10). (**B**) Temperature effect on the CYP1-dependent band-shift formation. Protein extracts (40 μg) were either incubated for 15 min at 20°C with end-labelled probe and the band-shift gel was developed at room temperature, or incubated at 0°C with end-labelled probe and electrophoresis was carried out at 5°C. Lane 1, binding reaction without addition of heme; lane 2, binding reaction in the presence of 25 μM hemin; lane 3, binding reaction in the presence of 25 μM hemin and 100 ng of competitor oligonucleotide UAS1-B2 (*CYB2*). (**C**) Heme effect on the CYP1-dependent band-shift formation. Protein extracts were incubated for 15 min at 0°C with end-labelled probe and electrophoretic separation was carried out at 5°C. The effect of hemin concentration was analysed in the 0−150 μM range.

extracts from cells transformed with pYCYP1. These experiments show that the DNA−protein complexes observed in our previous experiments and described in Figure 3 contained the CYP1 protein. This result is also corroborated by several other observations; the different complexes cannot be observed in a *cyp1* strain or when using extracts obtained from cells cultured under glucose growth condition when CYP1 is under the control of the hybrid promoter *GAL10−CYC1* (integrated or plasmid-borne form). Furthermore, as previously observed, the retardation complex is affected when the *CYP1* gene is modified by the internal deletion of the KCPVDH domain; it becomes heme independent with a faster migration as compared to the CYP1-dependent complex observed in the presence of heme (Figure 3C).

Temperature effects on the CYP1-dependent band-shift formation

Most of the band-shift experiments carried out previously by us and several groups to analyse the interactions between CYP1 protein and its target sequences have been carried out at room temperature (Pfeifer *et al.*, 1987a; Winkler *et al.*, 1988; Lodi and Guiard, 1991). In the present study, we have modified the experimental conditions by an incubation on ice for 15 min and an electrophoretic fractionation in a cold room at 5°C. The better stability of the complex in the presence or absence of heme in these new conditions is illustrated in Figure 4B. These observations explain why nobody has been able to observe a CYP1 complex in the absence of heme until now (especially with crude yeast protein extracts obtained from wild-type cells where the amount of CYP1 protein is low). In these new experimental conditions, we do not observe any significant difference in the titration of the probe in the presence and absence of hemin (Figure 3B, lanes 2 and 4; Figure 4B, lanes 1−2 at 5°C).

Heme concentration effects on the CYP1-dependent band-shift formation

The effects of hemin concentration on the CYP1-dependent complex formation were investigated (Figure 4C). In this experiment, hemin was added before the protein extracts. In this case, the concentration of heme giving maximal stimulation is in the 5−50 μM range. Hemin at concentrations >100 μM inhibits complex formation in extracts from cells where the *CYP1* locus is under the control of the *GAL10−CYC1* promoter and from cells transformed with the high-copy-number plasmid bearing the *CYP1* gene under control of the *GAL10−CYC1* promoter. There is one obvious explanation for the interpretation of these data: low concentrations of hemin (<100 μM) could play a specific role in the formation of the well-defined CYP1-dependent complex observed in the band-shift experiment (Figure 4C, lanes 5−8). At concentrations >100 μM, hemin, due to its

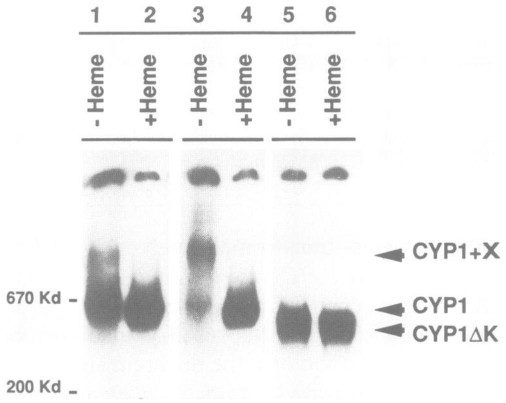

Fig. 5. Molecular size of the CYP1 complexes characterized in band-shift experiments. Band-shift analysis with the *in vivo* overexpressed CYP1 proteins extracted from *S.cerevisiae* strain 334 (i) transformed with pYCYP1 (**lanes 1** and **2**), (ii) where the *CYP1* gene has been placed under the control of the *GAL10–CYC1* promoter (**lanes 3** and **4**), (iii) in which the *CYP1ΔK* gene with an internal deletion (960–1560) has been placed under the control of the *GAL10–CYC1* promoter (**lanes 5** and **6**). Extracts (lanes 1 and 2, 8 μg; lanes 3–6, 40 μg) were incubated for 15 min at 0°C with end-labelled oligonucleotide containing UAS1–CYB2 without addition of hemin (lanes 1, 3 and 5) or in the presence of 25 μM hemin (lanes 2, 4 and 6). Electrophoresis in a non-denaturing polyacrylamide gel of graded porosity (5–20% acrylamide) was performed for 20 h at 5°C in buffer containing 45 mM Tris–HCl, 40 mM H_3BO_3 and 1.25 mM EDTA. On the left side, the apparent molecular masses of bovine thyroglobulin (670 kDa) and sweet potato β-amylase (200 kDa) are indicated.

hydrophobic properties, might be absorbed aspecifically by the surface of the CYP1 protein, inhibiting the formation of the CYP1–DNA complex (Figure 4C, lanes 9 and 10).

Molecular size of the complexes characterized with band-shift experiments

CYP1 complexes with the end-labelled 30 bp oligonucleotide containing UAS1–CYB2 (Figure 5) were obtained in the absence or presence of hemin and separated by electrophoresis on a non-denaturing 4–20% acrylamide gradient gel at 5°C for 20 h at 15 V/cm. The CYP1 complexes from cell extracts from yeast strains transformed with pYCYP1, or strains in which the *CYP1* and *CYP1ΔK* genes have been placed under the control of the *GAL10–CYC1* promoter, are shown in Figure 5. This method allows stable and well-defined complexes to be observed even after an electrophoretic run of 20 h. Differences in migration distance of the complexes can be detected, but their molecular mass has not been precisely determined as we have some doubts about the exact relationship between the mol. wt of the standards used and the relative mobility of the detected complexes. The specificity of the DNA–protein complexes was confirmed by competition experiments using excess amounts of unlabelled oligonucleotides containing the *CYB2* promoter DNA-binding site of CYP1 (data not shown). The heme responsiveness observed is in agreement with our previous results presented in Figure 3. In the absence of hemin and with 40 μg of protein extract from a yeast strain in which the *CYP1* gene had been placed under the control of the *GAL10–CYC1* promoter (Figure 5, lane 3), a major complex (CYP1+X) with a high mol. wt is observed. In the same conditions, but in the presence of hemin (Figure 5, lane 4), this complex disappears and a new one (CYP1) with a lower mol. wt appears. The same experiment was carried out with 8 μg of protein extract from a yeast strain transformed with pYCYP1 (Figure 5, lanes 1 and 2). In these experimental conditions, the larger complex (CYP1+X) is present (Figure 5, lane 1) and the major one has a lower mol. wt identical to the two other complexes observed in the presence of hemin (Figure 5, lanes 2 and 4). The lower intensity of the larger complex (CYP1+X) observed in lane 1 of Figure 5, as compared to the same complex present in lane 3 of Figure 5, is only due to the fact that a lower amount of protein extract was used in this experiment. When the KCPVDH domain is removed, the complexes have an identical and lower mol. wt with or without the addition of hemin.

Effect of the truncated CYP1ΔK protein on CYB2, CYC1, CYP3(CYC7) and ERG11 mRNA synthesis

Analysis of the *in vivo CYB2, CYC1, CYP3(CYC7)* and *ERG11* mRNA levels were carried out in several genetic contexts. The *hem1* mutant strain was constructed by partial deletion and disruption of the *HEM1* gene (the structural gene encoding δ-aminolevulinic acid synthase, the first enzyme in the heme biosynthesis pathway) with a DNA fragment containing the *LEU2* gene. Consequently, the cells were deficient in heme biosynthesis and unable to respire. The *cyp1* mutant strain was obtained after a partial deletion of the *CYP1* gene and disruption with a DNA fragment containing the *URA3* gene (Figure 6B). Integration of the sequence deleted for the KCPVDH domain in the protein was carried out by homologous recombination at the *CYP1* locus as described in Materials and methods (see Figure 6B). Double mutants having the genotype *hem1 CYP1ΔK* or *hem1 cyp1* were also constructed. The amounts of various transcripts were investigated in the six genetic contexts with respect to aerobiosis and galactose as carbon source (Figure 6A). In this analysis, actin transcripts were probed as control. Under these growth conditions and in *cyp1, CYP1 hem1* and *cyp1 hem1* genetic contexts, *CYB2, CYC1* and *CYP3(CYC7)* transcripts cannot be detected. The *ERG11* mRNA level is low in *HEM1* strains under galactose growth conditions. The CYP1-dependent activation is not clear under these conditions as compared to the results obtained by Turi and Loper (1992), who showed that *ERG11* transcription is reduced by half in the *hap1* strain in glucose growth conditions. In fact, optimum transcription was obtained during the semi-anaerobic growth conditions with glucose as carbon source. In the *hem1* strain, *ERG11* is abundantly transcribed in the absence of CYP1, as has already been observed by Verdière *et al.* (1991) in glucose growth conditions. The absence of the KCPVDH domain in the CYP1 protein has two main consequences: (i) the amounts of *CYB2, CYC1* and *CYP3(CYC7)* transcripts are increased in *CYP1ΔK HEM1* as compared to the *CYP1 HEM1* strain and (ii) *CYB2, CYC1* and *CYP3(CYC7)* transcription is not affected in the absence of heme biosynthesis in the *CYP1ΔK* genetic context and an overproduction, already described in the *CYP1ΔK HEM1* strain, is observed. Moreover, *ERG11* is also strongly activated in the *CYP1ΔK hem1* strain. We have determined the extent of *CYP1* and *CYP1ΔK* transcription under these conditions. Northern blotting analysis

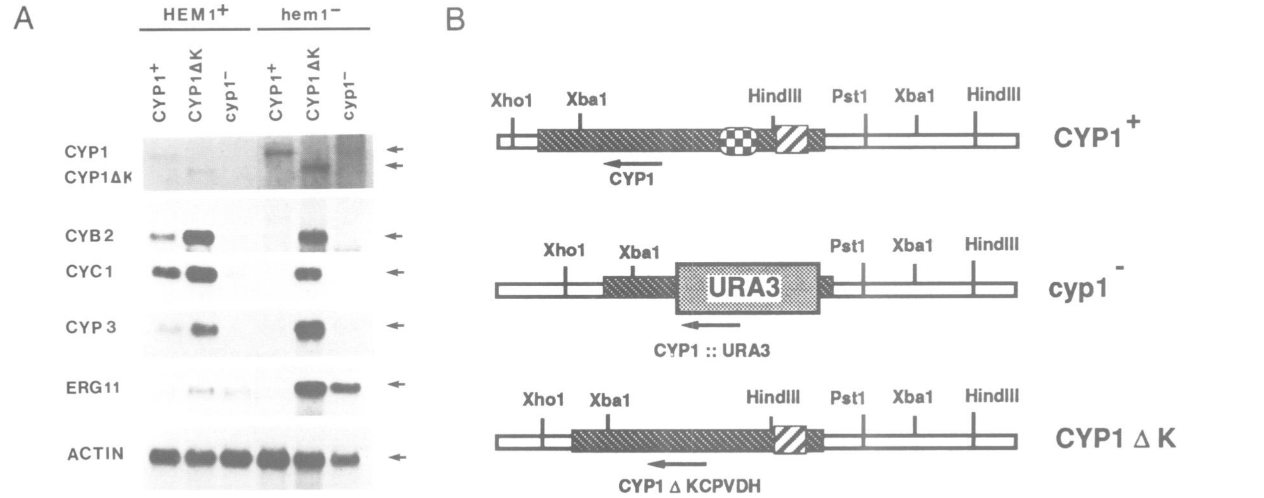

Fig. 6. (**A**) Northern blot analysis of *CYP1, CYP1ΔK, CYB2, CYC1, CYP3* and *ERG111* transcripts from strains W303-1B, W303-CYP1ΔK, W303-ΔCYP1, W303-ΔHEM1, W303-CYP1ΔK-ΔHEM1 and W303-ΔCYP1-ΔHEM1. Cells were grown in rich medium [1% (w/v) yeast extract, 1% (w/v) Bacto-Peptone] plus 2% galactose to two generations before stationary phase. Poly(A)$^+$ RNAs were extracted, purified and fractionated on 1.1% agarose–formaldehyde gels, transferred to nitrocellulose membranes and sequentially hybridized with appropriate nick-translated probes. The lower panel represents the same blot rehybridized with an actin (*ACT1*) probe. (**B**) Schematic description of *CYP1* loci.

(Figure 6) clearly indicates that *CYP1* and *CYP1ΔK* transcripts are more abundant in the *hem1* genetic context, and that the deletion of the region encoding the KCPVDH domain does not affect the stability of the *CYP1ΔK* transcript as compared to the *CYP1* mRNA.

Discussion

In spite of the toxicity of overproduction of the yeast CYP1 regulatory protein, we have succeeded in overexpressing this protein in *S.cerevisiae* by placing the *CYP1* gene under the control of a stringently regulated *GAL10–CYC1* hybrid promoter. The extent of CYP1 protein expression was measured by employing an immunological approach, using antibodies elicited against the N-terminal part of the molecule. To effect our studies, an acrylamide gel electrophoresis procedure used in band-shift experiments was used together with an improved method which allows the DNA–protein complexes to be analysed according to their size.

The analysis of CYP1 behaviour when the chromosomal *CYP1* gene expression is under the control of the *GAL10–CYC1* hybrid promoter allowed us to make several observations. First, the CYP1 protein is able to interact specifically *in vitro* with its target sequences without addition of hemin. Under these experimental conditions, a very large complex can be observed which could be the result of an interaction between the CYP1 protein and an unidentified X factor. Secondly, addition of hemin allows the formation of a new complex which has a lower molecular mass and which could be a multimer of the CYP1 protein. These results suggest that an additional cellular factor interacts with the CYP1 protein in the absence of hemin. This factor, composed of one or several proteins, would be present in a limited amount in the cell and would be titrated when the *CYP1* gene, cloned on a multicopy plasmid and under the control of the *GAL10–CYC1* hybrid promoter, is expressed. Under these experimental conditions, the CYP1 protein is produced in large quantities, as was observed by Western blotting. Our observations also support the notion that the interaction between the CYP1–X complex and its target DNA sequences (UAS elements) occurs *in vitro*, even in the absence of hemin. This result is not in agreement with the hypothesis proposed by Pfeifer *et al.* (1989), according to which an internal region mediates the heme control by masking the DNA-binding domain of CYP1 in the absence of heme.

When the seven repeated amino acid sequences containing the KCPVDH motif (located in the 247–444 interval) are removed, the interaction between CYP1 protein and the putative X factor does not occur *in vitro*. This result suggests that the X factor could interact directly with the CYP1 protein by means of this motif. Obviously, we cannot exclude the possibility that this internal deletion of 198 amino acid residues indirectly affects the interaction between CYP1 protein and the X factor.

We have shown that the deletion of the repeated amino acid sequence containing the KCPVDH motif affects the *in vivo* transcription of *CYB2, CYC1, CYP3(CYC7)* and *ERG11*, to a large extent. When the intracellular concentration of heme is strongly reduced by the *hem1* mutation, the transcription of most of the genes under CYP1 control is not detected. In these conditions, a high transcript level of the *CYB2, CYC1, CYP3(CYC7)* and *ERG11* genes can be observed when the KCPVDH repeated motif (247–444) is absent from the CYP1 protein. These *in vivo* results support the idea proposed by Pfeifer *et al.* (1989) that the polypeptidic region including the KCPVDH repeated motif could be the heme-responsive domain of the CYP1 protein. An overtranscription of the *CYB2, CYC1* and *CYP3(CYC7)* genes is also observed when the repeated amino acid sequence containing the KCPVDH motif of CYP1 is removed in a *HEM1* genetic context. This result suggests that this structural modification induces the loss of some negative control on CYP1 activity which could be mediated via protein–protein interaction or improves the contact between CYP1 and the transcriptional machinery. Finally, in a *hem1* strain, the *ERG11* transcripts cannot be detected in the presence of CYP1 and are accumulated in the absence of this protein. This finding is consistent with the previous

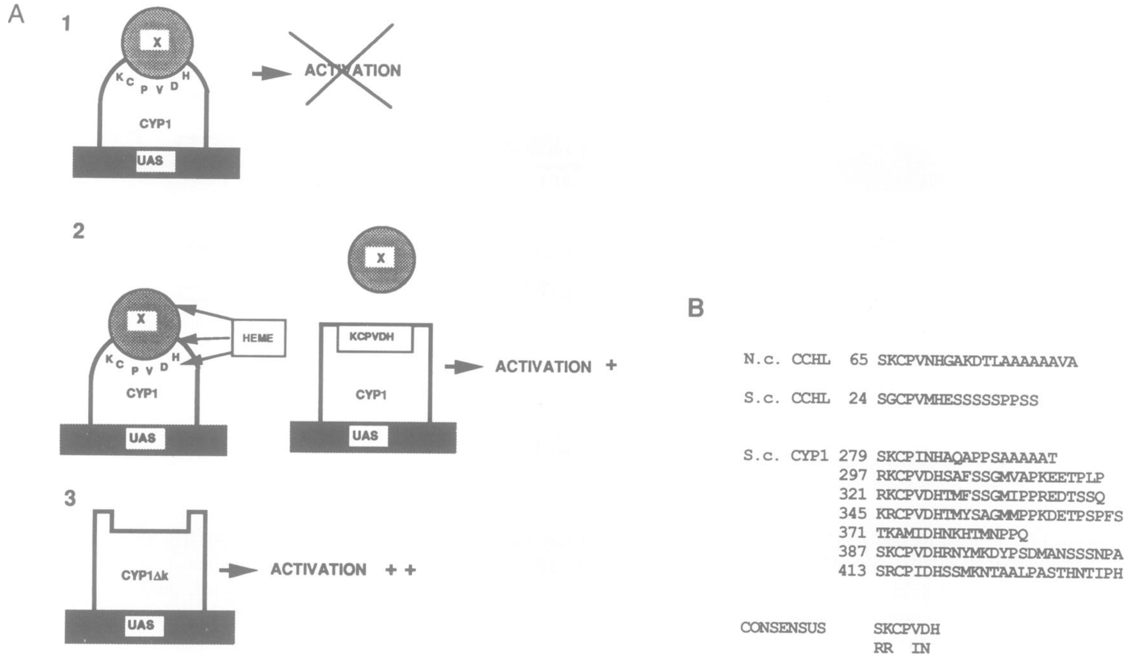

Fig. 7. Importance of the KCPVDH region in the control of the transcriptional activity of the CYP1 activator. (**A**) A model explaining the interaction between the transcriptional activator CYP1 protein and the putative inhibitory X element. CYP1 is shown bound at the UAS by its DNA-binding domain and making protein−protein contacts with the X-element via the repeated motif KCPVDH. (**B**) Amino acid sequence (279−439) of the heme regulating element of the CYP1 protein is shown (S. c. CYP1) (Creusot *et al.*, 1988; Pfeifer *et al.*, 1989). We have aligned the seven repeats of the motif S/R-K/R-C-P-V/I-D/N-H and the similar unit sequence found in the cytochrome c heme lyase from *N.crassa* (N. c. CCHL) (Drygas *et al.*, 1989) and in the cytochrome c heme lyase from *S.cerevisiae* (S. c. CCHL) (Dumont *et al.*, 1987).

observation reported by Verdière *et al.* (1991). Considering the results of the *in vitro* binding experiments reported here, we propose that CYP1 is able to interact *in vivo* with its target sequence in heme-depleted cells and acts negatively on the *ERG11* transcription mechanism in these conditions.

Taken together, the *in vitro* and *in vivo* observations allow us to propose a working model presented in Figure 7A. We think, as is the case of GAL80 for GAL4 (Lue *et al.*, 1987; Chasman and Kornberg, 1990), that the X factor binds to the CYP1 protein. This interaction occurs when the intracellular heme concentration is low, but does not affect the binding of the CYP1 protein to its DNA target sequences. In this interaction, the part of the CYP1 molecule containing the KCPVDH motif could play a major role, such as acting as an interface between CYP1 and the X factor. According to our model, the transcriptional activity of the CYP1 protein is antagonized by the X factor by protein−protein interaction similar to that when GAL80 interferes with the GAL4 activator function in the absence of galactose (Nogi *et al.*, 1984). In this hypothesis, the negative control of CYP1 could result from an intra- or intermolecular masking of activating domain(s). When hemin is added *in vitro*, or in a *HEM1* genetic context *in vivo*, there is dissociation of the X factor from the CYP1 protein. Several hypotheses can be proposed to explain such a mechanism. The heme co-factor could act through an interaction with either the X factor or with CYP1, inducing dissociation of the two or more proteins. The possibility that the KCPVDH motifs and their adjacent amino acid sequences could bind heme directly has already been suggested (Creusot *et al.*, 1988; Pfeifer *et al.*, 1989). The heme could also act at the interface of the interacting proteins. After the dissociation of the X factor, the CYP1 protein would be able to play its role of transcriptional activator.

Furthermore, a sequence presenting some similarities with the CYP1 KCPVDH repeat motif has been observed at the N-terminus of two heme lyases from *S.cerevisiae* and *Neurospora crassa*, respectively (Dumont *et al.*, 1987; Drygas *et al.*, 1989). The comparison between the two heme lyases and the seven adjacent repeat units of CYP1 is shown in Figure 7B. The amino acid sequence adjacent to the KCPVDH motif also displays a few similarities and is particularly abundant in alanine and serine residues. The two heme lyases have two main functions: they interact with apocytochrome c with high affinity (Nicholson *et al.*, 1988) and catalyse the covalent fixation of the heme co-factor to apocytochrome c. This mechanism suggests a direct interaction between the heme lyase and the heme co-factor, and requires that the two proteins interact specifically and reversibly (Henning and Neupert, 1983; Dumont *et al.*, 1991). Formation of the holo-cytochrome c could induce the dissociation of the complex between the two proteins. There is an interesting analogy between this system and the heme effect we have observed in the interaction between the CYP1 protein and the X factor. This observation suggests that the KCPVDH motif could play a role in the heme fixation on these proteins.

Our investigations provide new *in vitro* and *in vivo* data which allow a better understanding of the role of the CYP1 activator, and demonstrate some interesting relations between the structure and the function of this protein. The identification of the X factor by biochemical and genetic approaches will be attempted.

Materials and methods

Bacterial and yeast strains

Escherichia coli DH5αF′/*endA1 hsdR17*($r_K - m_{K+}$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* (φ*80dlac*Δ(*lacZ*)*M15*) was used for plasmid propagation and maintenance. The *S.cerevisiae* strains used in this study were 334 (*MATa pep4-3 prb1-1122 ura3-52 leu2-3,112 reg1-501 gal1*) from B.Sclafani, and three strains generated from 334, 334-Δcyp1 (*MATa pep4-3 prb1-1122 ura3-52 reg1-501 gal1 cyp1::LEU2*), 334-GAL10−CYC1::CYP1 (*MATa pep4-3 prb1-1122 leu2-3,112 reg1-501 gal1 GAL10−CYC1::CYP1*) and 334-GAL10−CYC1::CYP1ΔK (*MATa pep4-3 prb1-1122 leu2-3,112 reg1-501 gal1 GAL10−CYC1:CYP1ΔK*) which were constructed for this work, W303-1B (*MATα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*) from Thomas and Rostein, and strains generated from W303B, W303-CYP1ΔK (*MATα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 cyp1ΔK*), W303-ΔCYP1 (*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 can1-100 cyp1::URA3*), W303-ΔHEM1 (*MATα ade2-1 ura3-1 his3-11,15 trp1-1 can1-100 hem1::LEU2*), W303-CYP1ΔK-ΔHEM1 (*MATα ade2-1 ura3-1 his3-11,15 trp1-1 can1-100 cyp1ΔK hem1::LEU2*) and W303-ΔCYP1-ΔHEM1 (*MATα* ade2-1 his3-11,15 tp1-1 can1-100 cyp1::URA3 hem1::LEU2) which were constructed for this work.

Preparation and analysis of RNA

Total RNA was prepared according to the method of Maccecheni *et al.* (1979). Poly(A)$^+$ RNA was purified as described by Fraser (1975). The steady-state level of transcripts was analysed by Northern blots as described by Maniatis *et al.* (1982). The RNA was hybridized with the ^{32}P-labelled nick-translated DNA probes: the fragments used as probes are: *CYP1*: *Hin*dIII−*Xba*I fragment (3.7 kb) (Creusot *et al.*, 1988); *CYB2*: *Bam*HI−*Bgl*II fragment (1.2 kb) (Guiard, 1985); *CYC1*: *Eco*RI−*Hin*dIII fragment (0.6 kb); *CYP3(CYC7)*: *Eco*RI fragment (2.0 kb) (Verdière *et al.*, 1988); *ERG11*: *Eco*RI−*Bgl*II fragment (1.2 kb) (Turi and Loper, 1992). In order to normalize for the amounts of mRNA, the blot was hybridized with an actin (ACT1) probe (a *Bam*HI−*Hin*dIII fragment of 1.2 kb), which provides a reliable standard.

Oligonucleotide synthesis

Oligonucleotides were synthesized with a model 7500 DNA synthesizer (Milligen) and purified by gel electrophoresis. All the described double-stranded synthetic oligonucleotides were used as competitors in protein−DNA binding. The 30 bp oligonucleotide containing UAS1-B2 of *CYB2*, labelled with [γ-^{32}P]ATP and polynucleotide kinase was used as a radioactive probe in the gel shift experiment. Only the top strand of the oligonucleotides is shown below. The 30 bp oligonucleotide containing the *CYB2* UAS1-B2: 5′-CAAAAAGCCTGCCGATATCTCCTTGCCCCC-3′ (Lodi and Guiard, 1991). The 24 bp oligonucleotide containing the *CYC1* UAS1-A: 5′-GATGTTTCACCGATCTTTCCGGTC-3′ (Pfeifer *et al.*, 1987a). The 32 bp oligonucleotide containing the *CYC1* UAS1-B: 5′-GGTCTCTTTGGCCGGGGTTTACGGACGATGAC-3′ (Pfeifer *et al.*, 1987a). The 29 bp oligonucleotide containing the *CYP3* UAS′: 5′-CAAAGCTAATAGCGATAATAGCGAGGGCATTT-3′ (Pfeifer *et al.*, 1987b). The 31 bp oligonucleotide containing the *ERG11* UAS1: 5′-GTGCCGCGCCCGGGAATTACCGGGGGCACAG-3′ (Turi and Loper, 1992). The 32 bp oligonucleotide: 5′-TTACTAATTGCTATTA-TCATTGTTGGCGCGAC-3′ used as control. Underlined sequences represent DNA sequences protected from DNase I by the CYP1 protein.

Plasmid constructions

pBC1. The plasmid pBC1 was constructed using two complementary *CYP1* DNA fragments: *Pst*I−*Hin*dIII (570 bp) and *Hin*dIII−*Xho*I (4665 bp) derived from pVGC10 (Verdière *et al.*, 1988) and YepUX5.4 (Creusot *et al.*, 1988), respectively. The fragment *Pst*I−*Hin*dIII which contains the ATG start codon at position +225 was redigested with *Taq*I at position +228. The 343 bp fragment encoding the amino terminal part of CYP1 was ligated to a synthetic oligonucleotide adaptor (5′-GATCCAATGT-3′/3′-GTTACAGC-5′) in order to restore the *Taq*I site, the original codon ATG and to create a new *Bam*HI site. The resulting *Bam*HI−*Hin*dIII DNA fragment was then inserted into the *Bam*HI and *Hin*dIII sites of pUC18 (Yanisch-Perron *et al.*, 1985), to give the pBI construct. The *Hin*dIII−*Xho*I DNA fragment (4665 bp) containing the carboxyl portion of CYP1 was subcloned into the *Hin*dIII and *Sal*I sites of pUC19 (Yanisch-Perron *et al.*, 1985), to give the pB2 construct. Taking advantage of the *Sca*I site in the Amp region of the pUC vectors, the pB1 and pB2 constructs were digested with *Sca*I and *Hin*dIII. The *Sca*I−*Hin*dIII DNA fragment encoding the 5′ end of *CYP1* in pB1 was purified, and ligated to the *Sca*I−*Hin*dIII DNA fragment encoding the 3′ end of *CYP1* in pB2 to give the pBC1 plasmid. In this construct, the full open reading frame of the *CYP1* gene was restored and was flanked by two *Bam*HI sites. The structure of the plasmid pBC1 was confirmed by restriction enzyme mapping and sequence analysis.

pYCYP1. The *CYP1* gene was put under the control of the *GAL10−CYC1* hybrid promoter by the insertion of the *Bam*HI DNA fragment from the pBC1 construct into the 2μ shuttle vector pYeDP1/8-2 (Cullin and Pompon, 1988) to give the recombinant plasmid pYCYP1 (Figure 1). Consequently, *CYP1* transcription could be induced by addition of galactose to the growth medium.

pBC2. Plasmid pYCYP1 was digested with *Hin*dIII. The resulting DNA fragment, which carries the *URA3* gene and the *GAL10−CYC1* hybrid promoter fused to the amino terminal part of *CYP1*, was subcloned into the pUC19 vector to give the pB3 construct. The *Hin*dIII site upstream of the *URA3* gene was eliminated following partial digestion with *Hin*dIII, filled in with Klenow fragment and religated in the presence of T4 DNA ligase, to give the subclone pB3-1. The *Hin*dIII−*Pst*I DNA fragment (3000 bp) encoding the upstream part of the *CYP1* promoter isolated from the plasmid YepCX15.2 (Creusot *et al.*, 1988) was purified and cloned into pUC18 to give the pB4 construct. The pB3-1 and pB4 plasmids were digested with *Sca*I and *Sal*I. The *Sca*I−*Sal*I DNA fragment from pB4 carrying the upstream part of the *CYP1* promoter was purified and ligated to the *Sca*I−*Sal*I DNA fragment from pB3-1 encoding the *URA3* gene and the *GAL10−CYC1* hybrid promoter fused to the first 220 bp of the *CYP1* coding sequence, to give the integrative pBC2 construct. The fused elements were flanked by two *Hin*dIII sites. In order to put the transcription of the chromosomal *CYP1* gene under the control of the *GAL10−CYC1* hybrid promoter, pBC2 was digested with *Hin*dIII, the corresponding *ura*$^-$ yeast strain was transformed with the DNA fragment (Figure 1) and plated onto a selective medium. Positive colonies were further analysed by Western blotting to confirm the expression of the protein (Figure 2). Integration was confirmed by Southern analysis.

YCpCYP1ΔK. The 384 bp *Bam*HI−*Sal*I DNA fragment encoding an internal part of *CYP1* was purified and ligated with a synthetic oligonucleotide adaptor (5′-GTGACCAGTCCG-3′/3′-GATCCGGACTG-5′) to restore the *Bam*HI site and to create a new *Bst*EII site. This DNA fragment was then substituted for the fragment *Bst*EII−*Sal*I of YCpCYP1 (N.Defranoux, unpublished results) which contained the complete *CYP1* gene (8300 bp) cloned in pFL38 (Bonneaud *et al.*, 1991), to give the YCpCYP1ΔK construct. The internal deletion created in YCpCYP1ΔK consequently removed amino acid residues 247−445 of the CYP1 protein containing the seven repeated units which include the KCPVDH motif. To introduce this deletion into the wild-type *CYP1* gene, the YCpCYP1ΔK construct was digested with *Pst*I and *Xba*I, and the strain *cyp1::URA3* (Figure 6) was transformed with the resulting *Pst*I−*Xba*I DNA fragment (4100 bp). Cells were selected for the loss of the *URA3* marker (Figure 6) and the integration was confirmed by Southern analysis.

pBC2ΔK. The YCpCYP1ΔK construct was cleaved with *Hin*dIII and *Sal*I. The *Hin*dIII−*Sal*I DNA fragment (770 bp) encoding the internal part of CYP1 protein, including the internal deletion of residues 247−445, was cloned into pUC18 to give the pB5 construct. The 3 kb *Hin*dIII DNA fragment isolated from pBC2, which carried the upstream part of the *CYP1* promoter, the *URA3* gene and the *GAL10−CYC1* hybrid promoter fused to the first 220 bp of the *CYP1* coding sequence, was inserted into the *Hin*dIII site of pB5 to place the two N-terminal parts of *CYP1* gene in phase, and gave the pBC2ΔK construct. In order to put the transcription of the chromosomal *CYP1* locus under the control of the *GAL10−CYC1* hybrid promoter, and to introduce the internal deletion into the *CYP1* gene, the plasmid pBC2ΔK was digested with *Xba*I, and the corresponding *ura*$^-$ yeast strain was transformed with the resulting *Xba*I DNA fragment (Figure 1) and plated onto a selective medium. Positive colonies were further analysed by Western blotting to confirm expression of the protein (Figure 2). Integration was confirmed by Southern analysis.

Probe for gel electrophoresis DNA-binding assays

The linear *Bgl*II−*Bgl*II DNA fragment containing UAS1-B2(*CYB2*) was inserted into the *Bam*HI site of the pUC19 polylinker to give the plasmid pGB1303; the DNA probe containing UAS1-B2(*CYB2*) was prepared by digesting the plasmid pGB1303 with *Nhe*I and *Hin*dIII. Probes were end labelled with polynucleotide kinase and [γ-^{32}P]ATP and purified by gel electrophoresis according to standard methods (Maniatis *et al.*, 1982).

Preparation of yeast extracts for DNA-binding assays

Saccharomyces cerevisiae strain 334 transformed with pYCYP1 was grown in liquid minimum medium containing 2% glucose as carbon source to a cell density of A_{600} = 1.5. CYP1 expression was induced for 4 h by the addition of galactose (final concentration 2%). Cultures of *S.cerevisiae* strain 334 containing the chromosomal *CYP1* gene under the control of the *GAL10−CYC1* promoter were grown in liquid complete medium containing 2% glucose as carbon source and grown to a cell density of A_{600} = 1.

CYP1 expression was then induced for 5 h by the addition of galactose (final concentration 2.5%). Cells were harvested by centrifugation and washed in extraction buffer [200 mM Tris–HCl, pH 7.5, 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PMSF)]. The cells were disrupted in the extraction buffer by vortexing at 4°C in the presence of an equal volume of glass beads (0.45 mm diameter): the extracts were centrifuged for 3 min at 1500 *g* and the supernatants were re-centrifuged for 1 h at 100 000 *g*. The supernatant was collected and proteins were precipitated by the addition of 100% saturated $(NH_4)_2SO_4$ (pH 7.5) to a final concentration of 50%. The extracts were incubated for 30 min at 0°C and then centrifuged for 20 min at 27 000 *g*. The protein pellet was resuspended in protein buffer (20 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 20% glycerol and 1 mM PMSF). Protein concentrations were determined using a Coomassie Blue assay supplied by the Bio-Rad company.

Gel electrophoresis DNA-binding assays

The gel retardation assays were carried out according to Pfeifer *et al.* (1987b) with several modifications. $[\gamma\text{-}^{32}P]$ATP-labelled fragments were incubated with the cell extract in 20 ml of the incubation buffer (20 mM Hepes, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 6% glycerol), containing 0.5–5 μg of sonicated salmon sperm DNA as a non-specific competitor. Binding reactions were carried out at 4°C for 15 min. Protein–DNA complexes were resolved on polyacrylamide gels as follows: the 20 μl binding reaction was loaded onto a 4% polyacrylamide gel in TBE buffer (45 mM Tris, 45 mM H_3BO_3, 1.2 mM EDTA, 2% glycerol), the gels were run at 12 mA at 4°C, then dried and autoradiographed.

Preparation of CYP1 antibodies

The open reading frame encoding CYP1 N-terminus residues 1–256 was cloned into the expression vector pUHE21-2 (H.Bujard, unpublished results). The overproduced polypeptide was separated electrophoretically on an SDS–polyacrylamide gel, electroeluted and finally used to immunize rabbits.

Western analysis

For immunoblot analysis, proteins were separated electrophoretically on SDS–polyacrylamide gel and electroblotted to nitrocellulose. The CYP1 protein and its derivatives were detected using polyclonal rabbit anti-CYP1 antiserum. The primary antibodies were detected using anti-rabbit horseradish peroxidase-labelled IgG using the enhanced chemiluminescence (ECL) Western blotting detection system from Amersham.

Molecular analysis

Techniques used in general DNA preparation and manipulation were as described in Maniatis *et al.* (1982). Restriction endonucleases, T4 DNA ligase, alkaline phosphatase from calf intestine and T4 polynucleotide kinase were obtained from Biolabs and Boehringer, and used in accordance with the suppliers' recommendations.

Other methods

Escherichia coli was transformed according to the technique of Mandel and Higa (1970). Yeast transformation was carried out by the LiCl procedure of Ito *et al.* (1983) and yeast media were prepared according to Sherman *et al.* (1986).

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