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Identification of proteins involved in the regulation of yeast Iso-1-cytochrome C expression by oxygen

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On the basis of a gel electrophoresis retardation assay, protein(s) which interact specifically with the upstream activating site (UASc) of the yeast iso-1-cytochrome C (*CYC1*) gene were identified and separated by heparin ultrogel chromatography. DNase I protection experiments indicate that these factors protect a 23-bp sequence overlapping the UASc site previously defined. The specific binding activity is strongly reduced in extracts prepared from a wild-type strain grown anaerobically. It is absent in a mutant strain blocked in the biosynthesis of heme but it is restored upon the addition of the missing precursor, delta amino levulinic acid (dALA) to the growth medium. In contrast, the binding activity does not differ significantly in extracts from a wild-type strain grown in either glucose or glycerol as carbon source. These data strongly argue that the *CYC1* UAS binding protein(s) that we have identified mediate the oxygen and heme control of cytochrome C biosynthesis.

Key words: DNA-protein interaction/upstream activating site/oxygen control/heme/yeast

Introduction

The transcription of the yeast iso-1-cytochrome C gene (*CYC1*) is regulated by a set of physiological signals including oxygen and catabolite repression. The implication of heme as a key regulatory molecule has been suggested by transcription studies employing a mutant blocked in heme biosynthesis (Guarente and Mason, 1983). Oxygen regulation may be acting through heme, the biosynthesis of this molecule is greatly reduced in the absence of oxygen, or be mediated by another controlling factor. The target for heme regulation was mapped ~275 bp upstream of the transcription initiation region and is composed of two upstream activation sequences (UAS1 and UAS2). When linked to β-galactosidase, UAS2 is ~10-fold more active than UAS2 (Guarente *et al.*, 1984). These elements share some properties with enhancers (Yaniv, 1984) in that they can be placed upstream of the transcription initiation site or the region of an heterologous promoter and confer on this promoter the regulation pattern of *CYC1* (Guarente and Mason, 1983). However, it should be noted that contrary to enhancers these sequences are not functional when placed 3′ to a transcription unit (Guarente and Hoar, 1984).

Purified yeast RNA polymerase II initiates transcription *in vitro* on *CYC1* promoter in the same polarity as the initiation site observed *in vivo*, however, a few dozen base pairs upstream (Lescure and Arcangioli, 1984; and Figure 1). This specific initiation required supercoiling of the DNA template. It is also dependent on nucleotides upstream of the TATA box consensus sequence, but not on the far upstream UAS element. These observations have led us to postulate that the UASc could be a target for enzymatic activity that can change the topology of the promoter region. This activity modulated by the metabolic state of the cells could create a promoter configuration similar to that obtained with supercoiled DNA *in vitro*. To test this hypothesis, we were interested in identifying and purifying proteins that interact with the UASc sequences. The presence of such factors interacting specifically with upstream control sequences of several yeast genes can be inferred from the genetic analysis and molecular cloning of several control genes in *Saccharomyces cerevisiae*. Recently the characterization of a DNA binding activity specific for the upstream activating sequence of the GAL1-GAL10 promoter of *S. cerevisiae* (Johnston and Davis, 1984; West *et al.*, 1984; Yocum *et al.*, 1984) has been reported (Bran and Kornberg, 1985; Giniger *et al.*, 1985).

Using a gel electrophoresis retardation assay (Garner and Revzin, 1981; Fried and Crothers, 1981), we characterized DNA binding activities that interact specifically with a DNA fragment containing the upstream activating sequences of the *CYC1* promoter of *S. cerevisiae*. This binding activity is unaffected by growing the cells in the presence of glucose or glycerol as carbon source, but is strongly reduced in a wild-type strain grown anaerobically or in a mutant blocked in heme biosynthesis.

Results

Biochemical evidence for DNA binding activities specific for the UASc1 regulatory region

In an attempt to detect proteins that interact with the *CYC1* control sequences we decided to use the gel retardation technique of Garner and Revzin (1981). It is based on the slower migration of DNA-protein complexes with respect to free DNA on polyacrylamide gels. This approach was recently used by Strauss and Varshavsky (1984) to isolate a nuclear protein that binds specifically to the α-satellite DNA of the monkey. To do so, we cloned the *Sma*I-*Eco*RI fragment which carries the promoter and UASc of *CYC1* between the *Eco*RI and the *Cla*I sites of pBR322 as described in Materials and methods. The resulting plasmid (pAB1) was used to prepare end-labelled or non-radioactive DNA fragments containing the UASc (*Hind*III-*Xho*I) or the promoter (*Xho*I-*Eco*RI) sequences (Figure 1).

Crude extracts were prepared by mechanical disruption of cells desalted on Sephadex G-50 columns and used to test the binding activity with the *Hind*III-*Xho*I DNA fragment containing the regulatory region of the *CYC1* gene. When several micrograms of the protein fraction were mixed with a radioactively labelled fragment, all the radioactivity remained at the origin of the gel after electrophoresis. Addition of a large excess of non-labelled carrier DNA (100- to 1000-fold weight excess of salmon sperm DNA) reduced the level of non-specific DNA binding and permitted the detection of specific DNA-protein bands migrating slower than the free DNA. Each DNA binding activity was designated according to the position on the gel of the specific complex (band) it formed with the UAS-containing DNA fragment. The incubation time was <10 min to prevent proteolysis but >1 min to allow specific binding. The pattern of slowly

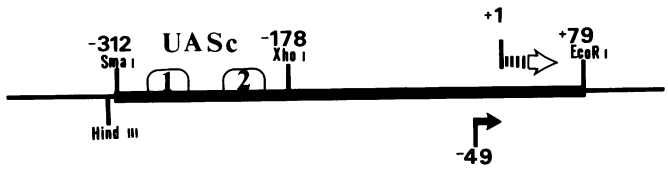

Fig. 1. Diagram of the *Sma*I-*Eco*RI restriction fragment carrying the promoter and the UAS sites of the *CYC1* gene. The position +1 corresponds to the first A of the most upstream of the heterogenous initiation site of *CYC1* RNA *in vivo* (Faye *et al.*, 1981; Guarente and Mason, 1983). The location of the two distinct UASc sites (Guarente *et al.*, 1984) is indicated. The arrow at position −49 corresponds to the strong initiation site for *in vitro* transcription by purified yeast RNA polymerase II (Lescure and Arcangioli, 1984).

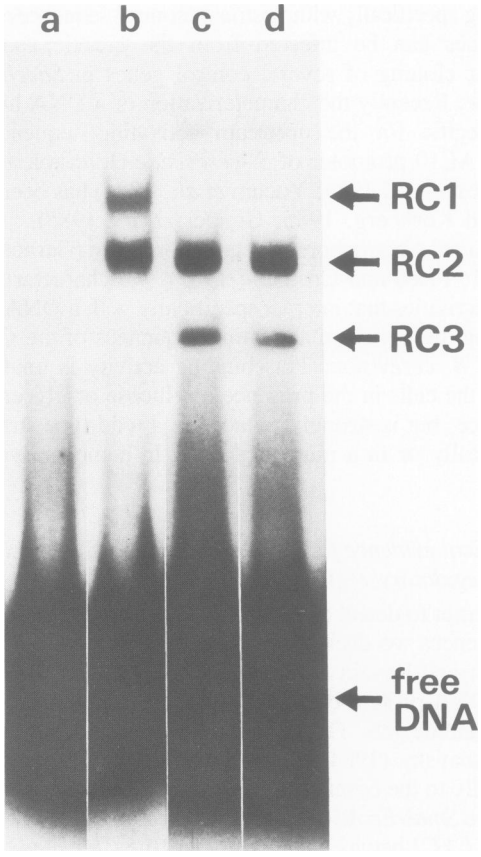

Fig. 2. UASc binding activities in crude extracts. Crude extracts were prepared as described in Materials and methods. The comparative studies of the UASc binding activity were achieved using 10 μg of proteins of crude extract in the electrophoresis binding assay. **(a)** No protein added; **(b)** Eaton press disruption; **(c)** Manton Gaulin disruption; **(d)** glass beads disruption.

migrating DNA-protein complexes observed in the electrophoretic assay was dependent on the method used to break the cells (Figure 2). We compared three different methods used to prepare extracts from yeast cells. Using the Eaton-press to disrupt pep4 cells, two complexes (regulatory complex 1-RC1 and regulatory complex 2-RC2) were observed with crude extracts (lane b). A partially different pattern was observed after breakage of these same cells with a Manton-Gaulin homogenizer or with glass beads (Figure 2, lanes c and d). In the last two cases, the activity corresponding to the RC1 band previously described was strongly reduced whereas an additional complex (RC3) which migrates faster than RC1 and RC2 complexes was observed. However, the major RC2 activity appeared unchanged whatever method was used to disrupt the cells.

For further studies, cells grown to mid-log phase were broken using the Eaton-press to yield a crude extract as described in Materials and methods. The extract was adjusted to an ionic strength equal to 50 mM ammonium sulfate and loaded onto a heparin ultrogel column that usually retains DNA-binding proteins. Approximately 90% of the proteins were recovered in the flow-through fraction. The remaining proteins were eluted with a linear gradient between 50 and 700 mM ammonium sulfate. To test the specific binding activity, aliquots were mixed with the radioactively labelled *Hind*III-*Xho*I fragment containing the UASc sequences in the presence of a 100-fold excess of non-radioactive salmon sperm DNA and the samples were applied to a polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed. As shown in Figure 3, two overlapping peaks of binding activity can be detected in the gradient fractions. The first was eluted around 200 mM $(NH_4)_2SO_4$ (RC1) and a second centered around 300 mM $(NH_4)_2SO_4$ (RC2). No activity was detected in the flow-through. The addition of carrier DNA was crucial for the detection of the binding activity even in this step. In its absence, all the radioactive DNA was retained at the origin of the gel, certainly due to the presence of many non-specific DNA-binding proteins in the column eluate. Fractions containing the separated RC1 or RC2 activities were pooled and used for the subsequent experiments.

To demonstrate that the DNA binding activities we detected were in fact sequence specific we undertook competition experiments. As labelled probe we used again the UASc-containing *Hind*III-*Xho*I DNA fragment and as competitor the non-labelled homologous DNA fragment or the non-labelled *Xho*I-*Eco*RI DNA fragment which contains the promoter region of the *CYC1* gene (Figure 1). When increasing amounts of the homologous fragment were added to the binding test, the visible radioactive complex progressively decreased for both RC1 and RC2 and completely disappeared after addition of 100-fold excess of competitor DNA (Figure 4, lanes a−h). In contrast the complex formation was only slightly affected after addition of 100 ng of the competitor heterologous DNA fragment (Figure 4, lanes k and m). Under similar experimental conditions, using crude extracts, we observed that the formation of RC1, RC2 and RC3 complexes (Figure 2) was competed specifically by the non-labelled homologous DNA fragments (not shown). These results clearly demonstrate that RC1, RC2 and RC3 activities bind specifically to the *Hind*III- *Xho*I DNA fragment which contains the UASc sequences.

To map precisely the region of DNA which interacts with RC1 and RC2, DNase I protection experiments were undertaken. The *Hind*III-*Xho*I fragment, 3′-end-labelled at the *Hind*III or *Xho*I sites, was incubated with RC1 or RC2, briefly treated with DNase I and then fractionated by electrophoresis on polyacrylamide gel as described in Materials and methods. The labelled DNA engaged in a complex was eluted and analyzed by electrophoresis on an 8% polyacrylamide sequencing gel under denaturing conditions. Non-complexed labelled DNA was also eluted from the bottom of the gel to serve as control for the DNase I treatment. For non-purified DNA-binding activities, this approach allows a correlation between the DNase I protection results and a given complex, characterized by the gel electrophoretic assay, to be established. The results of DNase I protection are shown in Figure

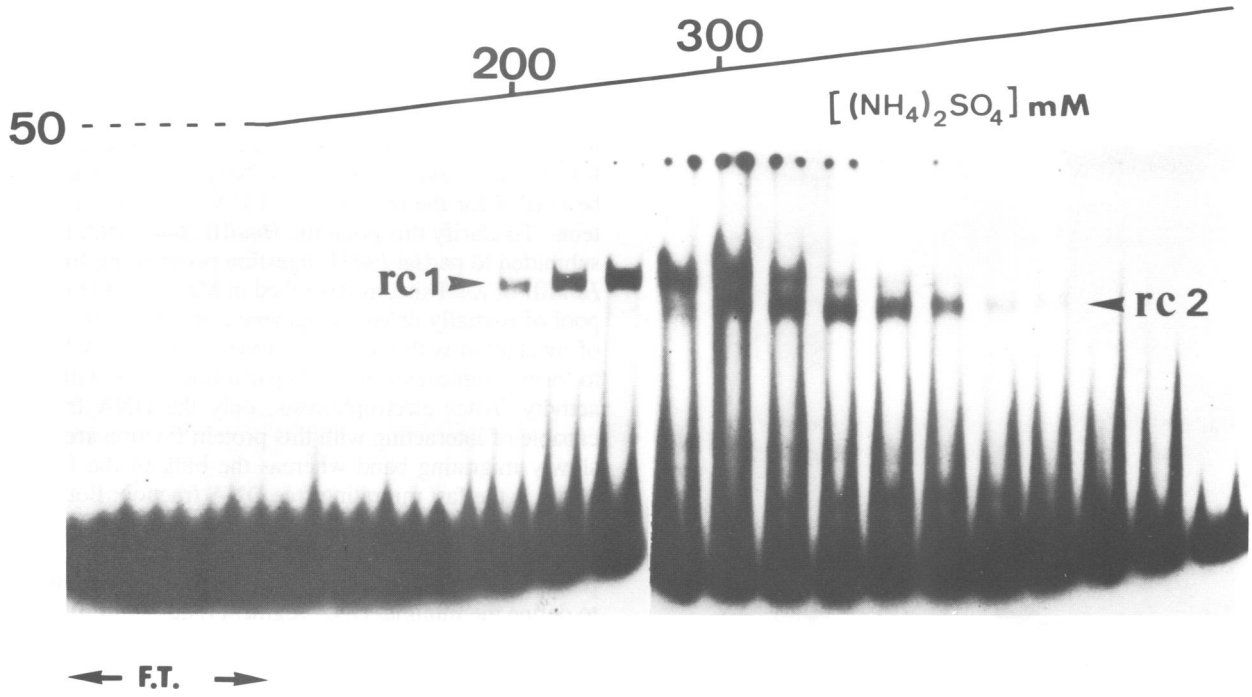

Fig. 3. Heparin ultrogel filtration. A crude extract from pep4 cells broken by the Eaton press was chromatographed on heparin ultrogel as described in Materials and methods. The binding activity was tested by the electrophoretic assay using 2 μg of proteins of each fraction in the standard assay. The ionic strength corresponding to the elution of RC1 and RC2 is indicated. F.T. (flow-through fraction).

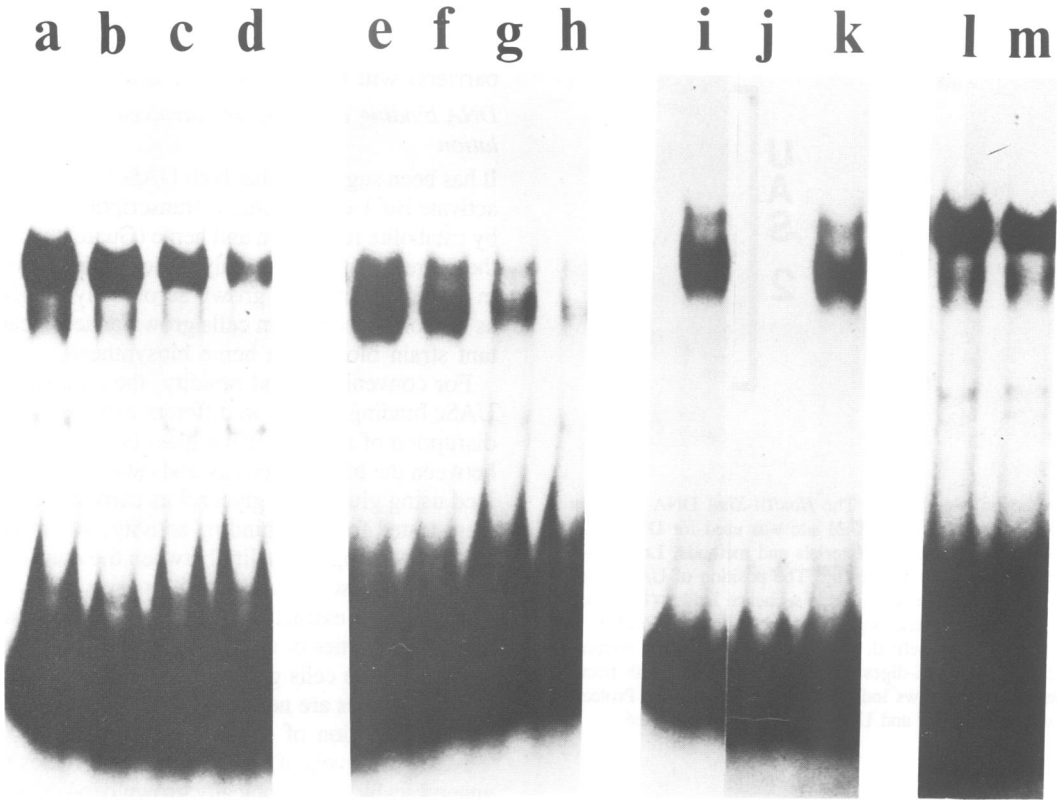

Fig. 4. Binding competition experiments. The binding activity was tested under standard conditions using 2 μg of pooled RC1 and RC2 fractions and 1 ng of 3'-end-labelled *Hind*III-*Xho*I DNA fragment. Various amounts of competitor DNA were added in addition to 1 μg of salmon sperm DNA and the binding reaction was started by the addition of proteins. **(a)** and **(l)** RC1 control; **(b)** RC1, 2 ng of UAS fragment (*Hind*III-*Xho*I DNA fragment, see Figure 1); **(c)** RC1, 10 ng of UAS; **(d)** RC1, 40 ng of UAS; **(e)** and **(i)** RC2 control; **(f)** RC2, 2 ng of UAS; **(g)** RC2, 10 ng of UAS; **(h)** RC2, 40 ng of UAS; **(j)** RC2, 100 ng of UAS; **(k)** RC2, 100 ng of *Xho*I-*Eco*RI DNA fragment (see Figure 1); **(m)** RC1, 100 ng of *Xho*I-*Eco*RI DNA fragment.

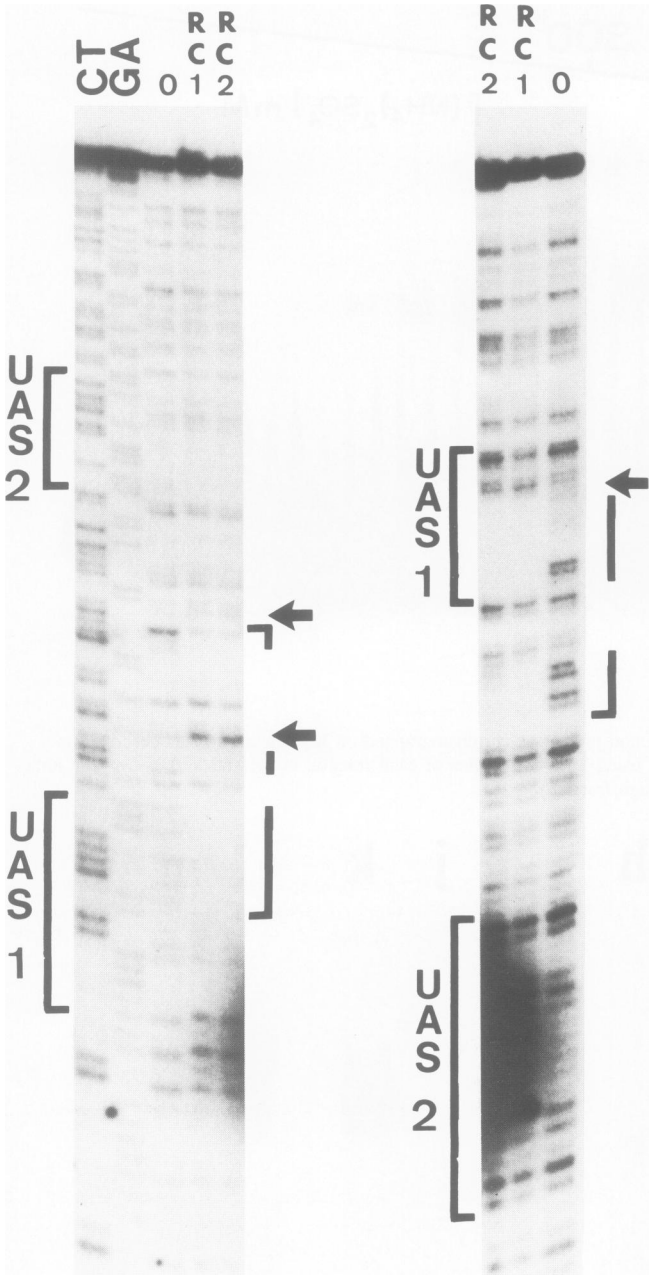

Fig. 5. DNase I protection experiments. The *Hind*III-*Xho*I DNA fragment (Figure 1) 3′ labelled at the *Hind*III or *Xho*I site was used for DNase I protection experiments as described in Materials and methods. **Left panel:** *Hind*III labelling; **right panel:** *Xho*I labelling. The position of UAS1 and UAS2 on a corresponding Maxam and Gilbert sequence ladder (1980) are indicated. C+T and G+A: chemical degradation procedure of *Hind*III 3′-end-labelled fragment **(left panel)**; 0: DNase I digestion of the bottom gel band; RC1 and RC2: DNase I-digested material associated with fraction RC1 and RC2, respectively. Arrows indicate hypersensitive sites. Protected nucleotides are boxed and UASc1 and UASc2 regions are indicated alongside.

5 for both RC1 and RC2. Modification of the DNase I digestion pattern was observed in an identical region partially overlapping the UASc1 sequence (Figure 7). The protected region encompasses 22–23 bp on both strands with several accessible residues in its middle. The cleavage at some neighbouring sites is increased relative to the control. No protection was seen along the UASc2 element.

Characterization of sequences required for the formation of the RC2 complex

DNase I footprinting experiments allowed us to define a protected DNA sequence in the RC1 and RC2 complexes. However, this protected region is not necessarily the same as that required for the formation of complexes. Neighbouring sequences could be needed for the recognition of UASc1 by the regulatory proteins. To clarify this point the *Hind*III-*Xho*I DNA fragment was submitted to partial *Bal*31 digestion progressing from either the *Hind*III or *Xho*I site, as described in Materials and methods. The pool of partially deleted fragments, obtained after a given time of incubation with the exonuclease, was end-labelled and used to form complexes with the heparin fraction containing the RC2 activity. After electrophoresis, only the DNA fragments still capable of interacting with this protein fraction are found in the slowly migrating band whereas the bulk of the fragments are found in the fast migrating free DNA fraction. Both bands were eluted from the gel and analyzed for their size on a polyacrylamide gel under denaturing conditions (Figure 6). This comparison allowed us to confirm the DNase I footprinting data and to define the minimal DNA segment required for the recognition of UAS1 by the RC2 activity. Figure 7 summarizes the data obtained from DNase I protection and *Bal*31 deletion experiments. A remarkable correlation was observed between the protected region and the minimal sequence required for the formation of complexes. One and five additional base pairs that are not protected against DNase I cleavage are required on 5′ and 3′ sides for the formation of the complex. However, since each series of deletions was made unidirectionally, it is not known whether a fragment containing only 31 bp (from the left to the right *Bal*31 barriers) will bind to the RC1 activity.

DNA binding activities are involved in heme and oxygen regulation

It has been suggested that both UASc1 and UASc2 independently activate iso-1-cytochrome C transcription and mediate regulation by catabolite repression and heme (Guarente *et al.*, 1984). It was, therefore, of interest to analyze the binding activities we observed in extracts from cells grown aerobically on glycerol or glucose as carbon source, from cells grown anaerobically or from a mutant strain blocked in heme biosynthesis.

For convenience and rapidity, the comparative studies of the UASc binding activity in different extracts were carried out after disruption of the cells using glass beads. A potential correlation between the binding activity and catabolite repression was examined using glucose or glycerol as carbon source. Crude extracts were tested for their binding activity, at a protein concentration allowing a proportionality between the quantity of crude extract used in the test and the activity observed. Similar activity was found in both extracts, however a slight perturbation in the migration properties of the RC2 complex was observed with extract prepared from cells grown in gycerol (Figure 8, lanes a and b). Further studies are necessary to test if this observation is related to the regulation of *CYC1* transcription.

Next, the levels of UASc binding activities were compared in anaerobically and aerobically grown pep4 cells. As shown in Figure 8 (lanes c and d), this activity was reduced at least 20-fold in crude extracts prepared from anaerobically grown cells.

Heme has been implicated in the biosynthesis of cytochromes in *S. cerevisiae* (Gollub *et al.*, 1977) and recent studies have suggested that this molecule regulates transcription of the *CYC1* gene via the UASc sequences (Guarente and Mason, 1983). We have therefore investigated the UASc binding activities in crude

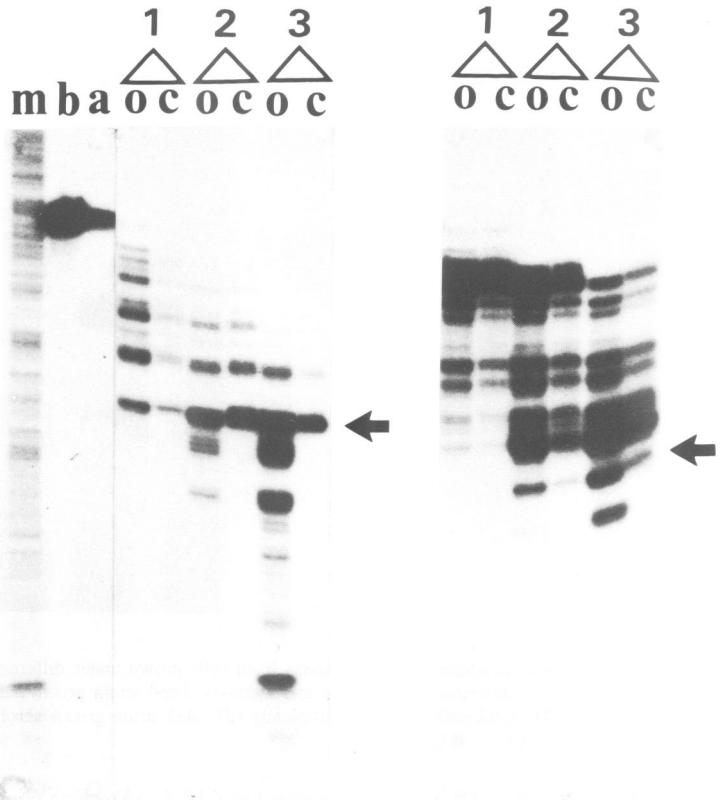

Fig. 6. Sequences required for the formation of specific complex. *Bal*31 digestions, complex formation and analysis of functional sequences are described in the text and in Materials and methods. **Left panel:** *Bal*31 digestions from the *Hind*III site; **right panel:** from the *Xho*I site (see Figure 1); 1,2,3: correspond to increasing times of digestion with the exonuclease; 0: bulk of deleted fragments corresponding to a given time of *Bal*31 digestion; c: comparative pattern of deleted fragments engaged in DNA protein complex; m: sequence channel used as size marker; B and A are respectively undigested DNA without and with protein. The arrows indicate the 5′ and 3′ limit of the functional sequence.

extracts from the ole3 strain (Woods *et al.*, 1975) deficient in the synthesis of the heme precursor dALA. As shown in Figure 8 (lane e) such a mutation exerts the same drastic reduction in the DNA binding activities specific for UASc as does anaerobic growth. The activity was restored when the growth medium was supplemented with dALA (lane f). The pattern of complexes observed using the electrophoresis retardation assay was identical to that obtained with the pep4 strain. Similar to the results obtained with the pep4 strain, when the ole3 strain supplemented with dALA was grown anaerobically, a strong reduction in the DNA binding activity was observed (lane g). To test if heme is a ligand that is required for the formation of a complex between the target sequences and the protein(s) present in the extract, we added heme to the extract obtained from the ole3 cells grown in the absence of dALA. This addition did not restore the specific binding activity (data not shown).

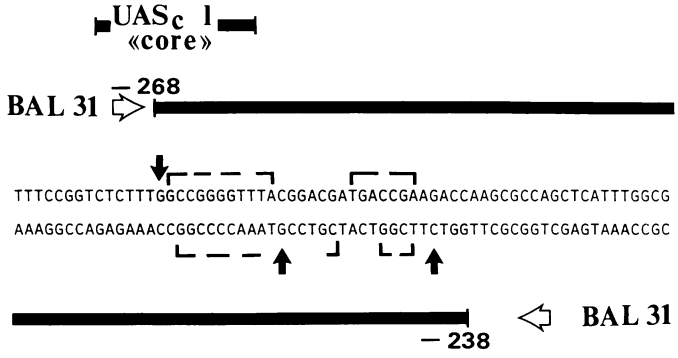

Fig. 7. Diagram showing the limit of functional sequences and the DNase I-protected region. The position +1 refers to the *CYC1* mRNA initiating at the most upstream location (Guarente and Mason, 1983). The dotted lines and the arrows indicate the DNase I-protected and hypersensitive sites, respectively. The full line corresponds to the minimal sequences required for the formation of the RC1 complex.

Discussion

The transcription of the *CYC1* gene in yeast cells occurs only under aerobic conditions. It is blocked in mutants defective in heme biosynthesis and is partially repressed by growth in glucose. The studies by the group of Guarente have also established that an upstream activation site (UASc) composed of two separate elements, UASc1 and UASc2, is the target for the physiological transcription regulation of this gene.

Using a gel electrophoresis migration retardation assay, we searched for the proteins involved in the regulation of *CYC1* gene expression. Our results reveal DNA binding activities which specifically interact with one of the distinct tandem upstream activation sites (UASc1) of the yeast *CYC1* gene. These activies were characterized by conferring distinct migration properties in the gel assay to a DNA fragment containing the upstream regulatory sequences. The major activity, RC2, was invariant, whereas the two other activities (RC1 and RC3) were found in different proportions according to the method used to break the cells.

The possibility of isolating a specific complex by gel electrophoresis also permitted the identification of the nucleotide se-

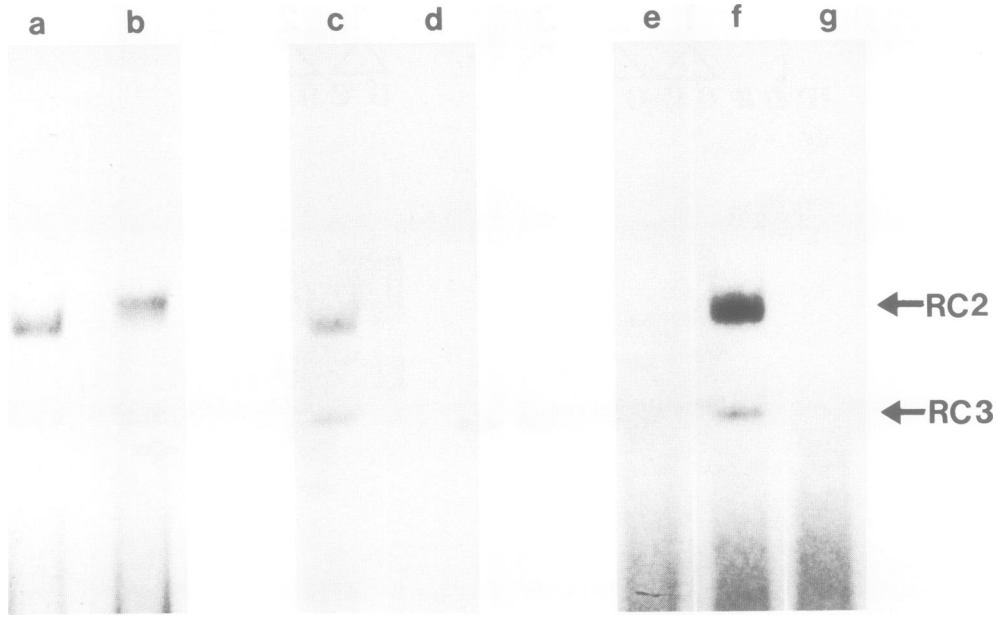

Fig. 8. UASc DNA binding activity under various metabolic conditions. Crude extracts from cells grown under different metabolic conditions or of different strains were tested for their UASc1 DNA binding activity as described in Materials and methods. Pep4 strain grown aerobically in minimum medium 2% glucose **(a)** or 2% glycerol **(b)**; pep4 strain grown in YPD, aerobically **(c)** or anaerobically **(d)**; ole3 strain grown aerobically in YPD, in the absence **(e)** or presence of dALA **(f)**; ole3 strain grown anaerobically in YPD + dALA **(g)**.

quence involved in protein-DNA contacts by DNase I footprinting. Both RC1 and RC2 fractions, separated on the heparin ultrogel column, gave identical footprints on UASc1 protecting the 3′-proximal part of this element. No protection was found along the other upstream activating site UASc2. Another approach, employing partial *Bal*31 digestion of the specific DNA fragment before binding, confirmed the assignment of the DNA sequences recognized by the specific DNA-binding protein(s). Only one to five extra nucleotides outside the domain protected against DNase I digestion are required for the formation of a stable complex between RC2 and UASc1.

The differences between RC1, RC2 and RC3 complexes are unknown at present. They could correspond to modified forms of the same protein or to different species of proteins. This last possibility is difficult to reconcile with the similarity in the DNase I protection pattern observed with RC1 and RC2. Alternatively, they could differ from each other by interaction of a unique protein with other cellular components which are differentially released from the cells depending on the breakage method used. In this context, we have observed that, at high salt concentration, RC1 and RC2 activities sedimented on a glycerol gradient at a similar position, corresponding to an apparent mol. wt. of ~70 000 – 100 000 daltons. When the centrifugation was made at a lower salt concentration, RC1 sedimented faster than yeast RNA polymerase II (mol. wt. 600 000 daltons) whereas the sedimentation constant of RC2 was only slightly affected (unpublished results).

A strong decrease in the activity that binds with high affinity to the cytochrome C UASc1 element was detected in a wild-type strain grown anaerobically or in a mutant strain blocked in heme biosynthesis. This observation, together with the demonstration that a specific sequence included in the UASc1 element is protected against DNase I and required to form the complexes, strongly supports the notion that the binding actitives we observed are related to the positive regulation of *CYC1* gene expression. Guarente *et al.* (1984) have considered the possibility that the control of *CYC1* expression could be mediated by a single protein-heme complex analogous to CAP-cyclic AMP in *Escherichia coli*. It does not seem that the DNA binding activities we observed act in such a way, since their association with UASc1 was independent of heme addition to the incubation mixture. Moreover, addition of heme to extracts from the ole3 mutant failed to restore a binding activity on the UASc1 site (data not shown). However, these observations do not exclude the possibility that *CYC1* regulation *via* UASc1 could be achieved by a hemoprotein that can constitute the DNA binding activities we observe. The stability or the synthesis of the corresponding apoprotein could be affected by the intracellular level of heme. Several *trans*-acting mutations affecting *CYC1* gene expression have been reported (Clavilier *et al.*, 1976; Guarente *et al.*, 1984; Lowry and Zitomer, 1984). It will be interesting to investigate whether any of these mutations affect the DNA binding activity we observe. This DNA binding activity is not cytochrome C1, the product of the *CYC1* gene, the RC protein(s) are distinct from this small hemoprotein (results not shown). The DNase I footprint and the *Bal*31 digestion results show that only the 3′ part of the UASc1 control element is interacting with RC1 or RC2. It is possible that another protein that we failed to detect (or eventually RC3) interacts with the 5′ part of this element and that at least two proteins are required for the function of UASc1 *in vivo*.

At first glance, the specific DNA binding activities we detect are not implied in the catabolic repression of the *CYC1* gene that decreases 10-fold the activity of the UASc1 element since the activity appears to be similar in cells grown in glucose or glycerol. Further studies are, however, necessary to exclude this possibility. A small molecule, affecting the affinity for the UAS1 site and present at different levels in cells grown in glucose or glycerol, could be lost during the preparation of extracts.

Finally, the availability of tests allowing detection of a specific DNA binding activity in a crude extract or partially purified fraction and which precisely map the nucleotides involved will be useful for studies of *trans*-acting factors interacting with *cis*-acti-

vating sequences. The purification and biochemical studies of RC1 and RC2 are under way.

Materials and methods

Yeast strains and growth conditions

Pep4 strain (Zubenko and Jones, 1979) was grown at 30°C in YPD medium or in minimal medium of yeast nitrogen base without amino acids supplemented with 2% carbon source. Ole3 strain (Woods *et al.*, 1975) was grown at 30°C in YPD medium supplemented with Tween 80 and ergosterol and when indicated with 50 μg/l of dALA. Anaerobic conditions were established by flushing the glass fermentor vessel with nitrogen. Before harvesting, the anaerobic culture was cooled to 0°C and poisoned with 200 μg/ml cycloheximide.

Preparation of yeast extracts

Cells grown to an A_{600} value of two were harvested by centrifugation and washed with cold water then with the buffer A (200 mM Tris-HCl pH 8, 300 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 7 mM mercaptoethanol, 10% glycerol). The cell pellet was resuspended with an equal volume of buffer A containing 1 mM PMSF, then disrupted at −70°C in a Eaton-press or at 0°C by passing twice through the pressure chamber of a Manton Gaulin homogenizer or at 0°C by agitation for 5 min with a vortex mixer at maximum speed with an equal volume of glass beads (0.45 mm diameter). The extracts were cleared by centrifugation for 1 h at 100 000 *g*. The supernatant was collected and desalted on a Sephadex G-50 column in buffer B containing 20 mM Hepes pH 7.8, 100 mM NaCl, 2 mM EDTA, 7 mM mercaptoethanol, 1 mM PMSF and 10% glycerol. The final extract contained 5 − 15 mg of protein/ml and could be frozen for several months at −70°C without any loss of binding activity.

Heparin ultrogel chromatography

10 g of pep4 cells were broken in an Eaton press in 10 ml of buffer A. The cell lysate was centrifuged in a SW41 rotor at 21 000 r.p.m. for 1 h. The supernatant was collected, the ionic strength adjusted to 50 mM $(NH_4)_2SO_4$ by dilution in buffer B without salt, then loaded on a 25 ml heparin ultrogel A4R (LKB) column. The column was washed with 50 ml of 10 mM Hepes pH 7.8, 50 mM $(NH_4)_2SO_4$, 2 mM EDTA, 1 mM PMSF, 7 mM mercaptoethanol and 10% glycerol, then a 100 ml gradient from 50 to 700 mM $(NH_4)_2SO_4$ in the same buffer was applied. Fractions of 1.5 ml were collected and tested for their DNA binding activities. All operations were done at 4°C.

Electrophoresis binding assay

Binding tests were carried out in 20 μl of buffer B containing 5 mM $MgCl_2$, 1 ng of *Hind*III-*Xho*I DNA fragment (Figure 1) labelled at its 3′ end with ^{32}P (10^7 c.p.m./ μg), 1 − 5 μg of sonicated double-stranded salmon sperm DNA as carrier and 1 − 20 μg of proteins. The reaction mixtures were incubated at 30°C for 10 min and immediately loaded on a 4% vertical polyacrylamide gel under tension (180 V) in TBE buffer (TBE: 90 mM Tris, 90 mM H_3BO_3, 2.5 mM EDTA) at room temperature. 5 min after loading, the voltage was reduced to 120 V. After migration for 2 h, the gels were treated for 10 min in 10% acetic acid/10% methanol mixture, dried and autoradiographed.

DNase I protection experiments

Following 10 min of incubation at 30°C of the RC1 or RC2 fractions (see Figure 1B) with the UASc-containing DNA fragment 3′-end-labelled at the *Hind*III or the *Xho*I sites, the incubation mixtures were treated for 30 s with 5 ng of DNase I (Worthington) in the presence of 0.5 mM $CaCl_2$. DNase I action was stopped wtih 5 μl of 100 mM EDTA and the samples were immediately loaded on a polyacrylamide gel. Following electrophoresis, the gel was autoradiographed for 5 h, the area containing the labelled DNA engaged in a complex or the free DNA was excised and the DNA was eluted from the acrylamide by shaking overnight at 37°C in 10 mM Tris pH 7.8, 1 mM EDTA, 0.2% SDS; 0.3 M NaCl and 1 μg/ml of sonicated double-stranded salmon sperm DNA as carrier. The eluate was filtered through a 0.22 μm nitrocellulose filter (Millipore), resuspended in 5 μl of loading denaturing buffer, heated for 2 min at 90°C and electrophoresed on an 8% polyacrylamide gel containing 7 M urea and TBE buffer. The gel was dried and autoradiographed with lighting plus intensifying screen at −70°C.

Characterization of sequences required for the UASc binding activities

The plasmid pAB1 was linearized with *Hind*III (or *Xho*I), then digested with the *Bal*31 exonuclease (Boehringer) for increasing time periods. The DNA was repaired with the *E. coli* DNA polymerase Klenow fragment, digested with *Xho*I (or *Hind*III) and the single-stranded ends were filled in by using the *E. coli* DNA polymerase Klenow fragment in the presence of [^{32}P]dATP and [^{32}P]dGTP. The bulk of UASc-containing DNA fragments deleted from either the *Hind*III or the *Xho*I sites was purified on a preparative gel and used for the electrophoresis binding assay as described above. Following electrophoresis the gel was autoradiographed, and the labelled DNA engaged in the complex or the free DNA fragment was eluted and analyzed on a 8% polyacrylamide gel under denaturing conditions.

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