The Transcriptional Regulator Hap1p (Cyp1p) Is Essential for Anaerobic or Heme-Deficient Growth of Saccharomyces cerevisiae: Genetic and Molecular Characterization of an Extragenic Suppressor that Encodes a WD Repeat Protein

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ABSTRACT

We report here that Hap1p (originally named Cyp1p) has an essential function in anaerobic or heme-deficient growth. Analysis of intragenic revertants shows that this function depends on the amino acid preceding the first cysteine residue of the DNA-binding domain of Hap1p. Selection of recessive extragenic suppressors of a hap1Δhem1Δ strain allowed the identification, cloning, and molecular analysis of ASC1 (Cyp1 Absence of growth Suppressor). The sequence of ASC1 reveals that its ORF is interrupted by an intron that shelters the U24 snoRNA. Deletion of the intron, inactivation of the ORF, and molecular localization of the mutations show unambiguously that it is the protein and not the snoRNA that is involved in the suppressor phenotype. ASC1, which is constitutively transcribed, encodes an abundant, cytoplasmically localized 35-kD protein that belongs to the WD repeat family, which is found in a large variety of eucaryotic organisms. Polyome profile analysis supports the involvement of this protein in translation. We propose that the absence of functional Asc1p allows the growth of hap1Δhem1Δ cells by reducing the efficiency of translation. Based on sequence comparisons, we discuss the possibility that the protein intervenes in a kinase-dependent signal transduction pathway involved in this last function.

Saccharomyces cerevisiae is a facultative aerobic organism that possesses two sets of oxygen- and/or heme-regulated genes. One set of genes is transcriptionally activated in the presence of oxygen and/or heme. The second set is transcriptionally repressed by oxygen and/or heme and is efficiently expressed in cells grown at low oxygen tension or in cells synthesizing a limited amount of heme (Zitomer and Lowry 1992). Heme is believed to mediate oxygen-dependent regulation for two reasons: its absence mimics a deficiency in oxygen and its synthesis requires molecular oxygen (Labbe-Bois and Labbe 1990).

Several genes encoding trans-acting factors are known to be involved in this regulation. Among these, HAP1, first identified as CYP1 (Creuot et al. 1988), which belongs to the Zn cluster regulatory family occupies a central position. In heme-sufficient conditions, its product binds to dissimilar DNA sequences and activates a number of genes involved in electron transfer reactions, for example CYC1 and CYC7 (CYP3), which encode iso1- and iso2-cytochrome c, respectively (Creuot et al. 1988; Pfeifer et al. 1989). The function of Hap1p is particularly complex; it has also been shown to be involved in weak, heme-dependent activation of ROX1, which encodes a heme-dependent repressor of the hypoxic genes (Keng 1992). By regulating the ROX1 regulator, Hap1p is responsible, at least in part, for a network of oxygen- and/or heme-dependent regulation. In addition, in the absence of heme, Hap1p participates in the repression of ERG11, which is involved in the metabolism of sterols (Verdiène et al. 1991; Defranoux et al. 1994). In the absence of heme, Hap1p forms a large complex with unidentified factor(s) that interacts specifically with its target sequences and is dissociated by the addition of hemin (Fytlovitch et al. 1993; Zhang and Garen et e 1994). These results suggest that the slow-migrating, Hap1p-containing complex might represent a repression complex that dissociates in the presence of heme, allowing Hap1p to play the role of transcriptional activator.

We report here that Hap1p is essential for anaerobic or heme-deficient growth. This observation is all the more interesting, because cells in which HAP1 is inactive do not present an obvious phenotype in aerobiosis and heme-sufficient conditions. In an attempt to identify the genes involved in this function of Hap1p, we carried out a suppressor analysis of a hap1 mutant strain. Characterization of HAP1 intragenic revertants suggests that it is its binding to CYC1-UAS1-like targets that is necessary for the growth of heme-depleted cells.
We also identified two genes, ASC1 and ASC2, whose inactivation allows the growth of heme-depleted hapl mutant cells. Characterization of ASC1 structure and expression, along with polysome profile analysis, indicate that Asc1p might be involved at the level of translation initiation.

**MATERIALS AND METHODS**

**Strains and media:** The bacterial strains DH5α and XL1-Blue were used in this study. Growth conditions, transformation, and DNA preparation were as described in Sambrook et al. (1989). Yeast strains are presented in Table 1. All the strains listed are isogenic to W303-1B, except YJ7, YJ-14B, FJ1-17A, FJ1.11B/H/C, VP/H, and its derivatives, VP/18 and VP/18/H. Yeast cells were grown at 28°C in either rich, YPD, or synthetic media, SC, supplemented with specific growth requirements, as described by Sherman et al. (1986). Growth of anaerobic or heme-deficient cells requires sterol and fatty acid supplements (Andrews and Stier 1953, 1954). For growth in these conditions, YPD medium was supplemented with 30 mg/liter of ergosterol and 0.2% (v/v) of Tween 80 and was named TE. When necessary, to obtain heme-sufficient growth, Δhem1 strains were grown on YPD (YPALA) or SC (SCALA) media supplemented with 40 mg/liter of δ-aminolevulinate (ALA). Anaerobic cultures were described in Verdière et al. (1991). Yeast transformations were carried out as described in Chen et al. (1992). Spectroscopy on whole cells was as described in Claisse et al. (1970). For growth tests, cultures in the stationary phase were centrifuged for 5 min at 3000 g, cells were washed three times with Ringer’s solution, resuspended in YPD, and 5 μl of appropriate dilutions were dropped on plates. For YPD and YPALA plates, photographs were taken after 1 day of growth, and for TE plates, photographs were taken after 2 or 3 days of growth.

**Genetic techniques:** Mutagenesis was carried out in the strain VP/18/H using ethyl methyl sulfonate (Eastman, Rochester, NY), as described by Hawthorne (1969), to a survival of 50%. Gap repair was performed as described in Orr-Weaver et al. (1983). Two truncated derivatives of HAP1 were used: YCpCYP-ΔPH is derived from YcpCYP1 and contains a deletion in part of the promoter and DNA-binding domain (DBD: residues 1-118; Defranoux et al. 1994). YCpCYP1-18K is derived from YcpCYP1-18; it contains a deletion from residues 247–444 and was a generous gift from S. Fyllovich (unpublished results). These plasmids were digested with PstI, HindIII and BstEII-SalI, respectively, and introduced into VP/18. The information used to repair the gap and regenerate a replicating plasmid is usually from chromosomal DNA. Total DNA from a pool of Ura+ transformants was extracted and used to transform Escherichia coli. In each experiment, the restriction pattern of plasmids obtained from four independent bacterial transformants was analyzed. The plasmids containing the repaired genes were used to transform VP/18/H, and the repaired domain of those that allowed growth of the recipient strain on TE was sequenced. To clone ASC1, a genomic partial BamH1 library constructed in YCBL1 was used to transform YJ1-4B. About 30,000 transformants were plated on selective medium supplemented with ALA. To starve the cells of the accumulated heme, two intermediate replicas were performed on media devoid of ALA, followed by a third replica on TE. One hundred transformants that had lost the ability to grow in heme-deficient conditions were further analyzed. Plasmid loss was induced, and for 20 transformants, the loss of growth on TE cosegregated with loss of the plasmid marker. Restriction analysis showed a common insert of 7.5 kb that was subcloned into pRS316. The ability of the sub-

<table>
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<th>Strain</th>
<th>Genotype</th>
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<tr>
<td>FJ1.11B/H/C</td>
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<td>N. Defranoux et al.</td>
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<td>J. Verdière et al.</td>
</tr>
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<td>B. J. Thomas and Rotstein</td>
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**TABLE 1**

Strains used in this study
cloned inserts to abolish the suppressor phenotype of REM24 was then examined.

**Nucleic acid analysis:** Gel electrophoresis and Southern blotting methods were essentially as described in Sambróok et al. (1989).

**Plasmid construction:** YCBL1 is a centromeric vector containing the TRP1 gene; the yeast genomic partial BamHI library was constructed in this plasmid by Eric Petrochilo (personal communication). The rol1::HIS3 construction is described in Amill et al. (1995). The hem1::ADE2 construction was a generous gift from Rosine Labbé-Bois. The constructions used to inactivate ASC1 were created in two steps. First, the 1.7-kb BamHI-KpnI fragment of ASC1 was subcloned into the same sites in pUC19 to create pBK. Second, the 517-bp BglII fragment was removed and substituted by the URA3 containing a 1096-bp BglII fragment from pFL38 or by the TRP1 containing a 840-bp BglII fragment from pFL39 (Bonneaud et al. 1991), giving the p1ΔM and p1ΔT plasmids, respectively. These plasmids were digested with BamHI and KpnI before yeast transformation. PCR with p316K* was generated by digestion of pRS316 (Sikorski and Hieter 1989) with KpnI, and the linearized plasmid was treated with Mung Bean Nuclease and then ligated with T4 DNA ligase. The A and D oligonucleotides were used in PCR to amplify the ASC1 gene from VP/18 DNA extract, and the 2.1-kb PCR fragment was digested with XbaI and cloned into the same site of p316K* to create the pASC1°+ plasmid. Construction of the pΔINT plasmid is described in Figure 5. For the second step of PCR, exon-containing fragments were used in equimolar amounts, and to facilitate initiation of the reaction, pASC1°+ was added in a 1/1000 ratio. The resulting 1.8-kb PCR fragment was digested with XbaI and cloned into the same site of p316K° to create the plasmid pΔINT. pΔORF and pasc1° were generated by digesting pASC1°+ and pΔINT, respectively, with Acc65 I, filling in with Klenow fragment, and ligating. The frameshift was verified by sequencing. pASC1°+′ was constructed as follows: A 1.1-kb fragment containing the TRP1 gene and a sequence coding for the HA epitope in frame with the 3′ end of the ASC1 ORF was formatted by PCR using the oligonucleotides Tag1 and Tag2 and the plasmid pYiFcT as a template (constructed by J. M. Rouillard, unpublished results). This fragment was used in cotransformation with pASC1° to transform the W303 strain. Three Trp+ colonies were tested, and in all cases, cosegregation experiments indicated an integration of the PCR fragment in the pASC1°+ plasmid. The in-frame junction was verified by sequencing.

**DNA sequencing:** DNA sequencing was done using the dideoxy chain termination method (Sanger et al. 1977) on double-stranded DNA templates. Concerning the ASC1 gene, the sequence of the entire 2.6-kb BamHI-SpeI fragment was determined on both strands using different restriction fragments subcloned into pUC19 and digested with HindIII to create nested deletions. The universal and reverse primers were used. The mutated ASC1 alleles were amplified by PCR using the A and D oligonucleotides (see plasmid construction). Two independent PCR amplifications per allele were sequenced using the Amplifycicle Sequencing Kit (Perkin Elmer, Norwalk, CT).

**PCR and oligonucleotides:** PCR reactions were carried out in a total volume of 80 μl with 1 unit of Taq DNA polymerase (Appligene) according to the manufacturer’s recommendations. After 5 min at 95°C, the tubes were subjected to 30 cycles as follows: 60 sec at 95°C, 60 sec at 55°C, and 90 sec at 72°C, followed by a final cycle of 60 sec at 95°C, 60 sec at 55°C, and 5 min at 72°C. The oligonucleotides A, B, C, and D were used for the ASC1 constructions. A (5′-CAGCTCAAATTTTATCCCATGAC ACC-3′) hybridized at position −429 and contained a substitution (T460C) that created an Xbal site, D (5′-CTGCTAGAATGGGGGTTTAGCTCATGAAAGG-3′) hybridized at position +1623 and contained an XbaI site at its extremity B (5′-TTAAGGCTACGCAATCTTTTTGTGTTACGG-3′) and C (5′-ACA AATGGTTAAAGCCTTGAACTAAACCAAT GCC-3′) were hybrid oligonucleotides used for the construction of pΔINT. Tag1 and Tag2 were hybrid oligonucleotides used to construct pASC1°+H. Tag1 (5′-GGCTCATTTAGATTGGCAGATGTA CGTCCTATAACCCATACGACCTCC-3′) fused with the extremity of the ASC1 ORF and the region encoding the HA epitope. Tag2 (5′-ATATTTACACTAAATAATAGAAATATTTTTCTTGG ATCTGGCAAGTGAC-3′) targeted integration in ASC1 at position +1337.

**Northern blot analysis:** Cells were grown overnight in YPAL to log phase (OD600 = 1), centrifuged for 5 min at 3000 g, washed three times with Ringer’s solution, and resuspended. These cells were then used to inoculate either YPAL or TE medium to OD600 = 0.12, and the medium was incubated at 28°C for the time necessary to reach OD600 = 1 (about three generations). Total yeast RNA was extracted and processed for Northern blot analysis as described by Nasmuth et al. (1980). Gel lanes were loaded with 30 μg of total RNA, as determined by A260. Hybridizations were carried out overnight at 42°C. 32P-labeled probes were generated using random-priming DNA labeling (Boehringer Mannheim, Indianapolis, IN). Probes used in this study were a 1.4-kb BamHI-HindIII fragment for ACT1, a 1.6-kb BamHI-KpnI fragment for ASC1, a 4.6-kb BamHI-EcoRI fragment for HAP1, and a 1.9-kb ClaI-Xbal fragment for R0X1.

**Western blot analysis:** Cells were grown overnight in YPD to log phase (OD600 = 1), centrifuged for 10 min at 3000 g, and washed twice with 1 ml of ice-cold, double-distilled water. Cell pellets were suspended in 200 ml of lysis buffer (50 mm Tris, pH 8.0, 0.3 M NaCl, 0.5% SDS, 10 mm DTT, PMSF) with 1 volume of HC1-washed glass beads. Extractions were made by vortexing three times for 1 min followed by incubation on ice for 5 min. After centrifugation for 30 min at 10,000 g at 4°C, the supernatants were collected and frozen at −70°C. Proteins were separated by SDS polyacrylamide gel electrophoresis on 10% gels and electrobotted onto nitrocellulose membrane (Amersham, Arlington Heights, IL) using a Semi-Dry Transfer Unit (Hoefer Scientific Instruments, San Francisco, CA) as recommended by the manufacturer. Membranes were blocked for 1 hr in TBST (to mm Tris, pH 7.5, 150 mm NaCl, 0.1% Tween 20) containing 5% (w/v) nonfat dry milk and then incubated at 4°C overnight with anti-HA antibody (Boehringer Mannheim) diluted 1:1000 in the same buffer. After three washes for 10 min in TBST, membranes were incubated for 1 hr in TBST/5% nonfat milk containing the secondary antibody, an anti-mouse IgG HRP conjugate (Sigma, St. Louis, MO) used at 1:2000. Finally, membranes were washed three times for 10 min in TBST before being developed with the ECL Western blotting detection kit (Amersham).

**Indirect immunofluorescence:** Cells were grown overnight in YPD to log phase (OD600 = 1), and preparation of cells for immunofluorescence was as described in Berkower et al. (1994). Incubation with the primary antibody was performed overnight at 4°C with anti-HA antibody diluted 1:1000 in PBS (PBS supplemented with bovin serum albumin at 400 μg/ml). Incubation with the secondary antibody was performed for 1 h at 4°C with anti-mouse IgG FITC conjugate antibody (Sigma) diluted 1:200 in PBS.

**Gradient analysis of yeast polysomes:** We used the method described by Petitetjean et al. (1995) to prepare the polysome extracts for gradient analysis. For immunoblot analysis, gradients were fractionated from the top, and 0.6-ml fractions were collected, concentrated by trichloroacetic acid precipitation, and resuspended by Laemmli loading buffer.
RESULTS

The DBD of Hap1p is involved in the efficiency of growth of heme-depleted cells: In the course of a previous study, we observed that anaerobic growth of hap1-mutated strains occurs after a lag time of several days (J. Verdier and M. Gaïne, unpublished results). To gain some insight into this phenomenon, we repeated the experiment with a CYP1-18 strain. The allele CYP1-18, renamed HAP1-18 (Pfeifer et al. 1987), has already been described (Verdière et al. 1988). It encodes a protein that presents unusual features: substitution of the wild-type serine for arginine at the amino acid immediately preceding the first cysteine of the zinc cluster does not affect Hap1p binding to CYC7-UAS, but it abolishes binding to CYC1-UAS1 (Pfeifer et al. 1987). Moreover, binding to the CYC7-UAS results in considerable activation of CYC7 and consequent overproduction of iso2-cytochrome c by a mechanism that is not understood, making it quite easy to distinguish in the absence of iso1-cytochrome c (cyc1-1), the wild type of the mutated allele. A cyc1-1 HAP1-18 (VP/18) anaerobic culture, which began to divide after a lag time of 5 days and had reached stationary phase, was aerated for 30 min to induce the synthesis of cytochromes before the cells were harvested. Low-temperature spectroscopy on whole cells showed a wild-type amount of iso2-cytochrome c that was characteristic of Hap1+-dependent regulation. We concluded that a reversion of HAP1-18 to pseudo-wild type had occurred, allowing anaerobic growth (Figure 1A). To verify that the role of Hap1p is also essential in heme-depleted cells, we inactivated the gene in a Δhem1 strain: the double disruptant was unable to grow unless supplemented with ALA, confirming that Hap1p is essential in both anaerobiosis and in the absence of heme. We previously showed that the amino acid preceding the first cysteine residue (position 63) of the Hap1p DBD mutated in HAP1-18 is critical for the efficiency of regulation both in the presence and absence of heme (Defranoux et al. 1994). To determine if this position is also essential for the growth of heme-depleted cells, several constructions substituting position 63 were introduced in the hap1 hem1 strain FJ1-11B/H/C. The positive and negative controls were the pFL38 vector with or without an insertion of the wild-type allele, respectively. As shown in Figure 1B, the nature of the residue present at position 63 is critical for the efficiency of growth on TE medium: serine, alanine, isoleucine, and glycine allow complete or partial growth, while arginine, lysine, and aspartate do not permit cell growth.

Inactivation of ROX1 does not restore growth of a Δhap1Δhem1 strain: ROX1 encodes a heme-dependent repressor of hypoxic genes that is induced by Hap1p among others (Keng 1992). In the absence of heme or oxygen, ROX1 transcripts are apparently repressed by Hap1p, leading to an absence of transcription of the hypoxic genes in hap1-mutated strains (Ushinsky and Keng 1994; Deckert et al. 1995; Amill et al. 1995). This might explain the essential function of Hap1p in this physiological context. To test this hypothesis, we first confirmed by Northern blot the above observations in our strains (Figure 2B). We then inactivated ROX1 in the hem1− hap1− strain W4/HC. The absence of Rox1p did not restore growth on TE (Figure 2A). There are two alternative explanations for this result: either Rox1p is not involved in the requirement for a functional Hap1p in the absence of heme, or it is only one of several Hap1p-dependent genes implicated in this phenotype. If the latter hypothesis is correct, the only way to suppress hap1 mutations would be to modulate metabolism at a more general level.

Isolation and genetic characterization of revertants of a HAP1-18 Δhem1 strain: In an attempt to gain insight into the function of Hap1p in heme-depleted cells, we performed a suppressor analysis.

Characterization of intragenic revertants confirms the importance of the DBD for growth in heme-depleted cells: The HAP1-18 hem1 strain VP/18/H was mutagenized with ethyl methyl sulfonate, and 24 mutants that were able to grow on TE were selected. Eight were dominant for this phenotype. Spectroscopy on whole cells showed that...
these mutants have lost the HAP1-18 iso2-cytochrome c-overproducing phenotype, suggesting that a mutation might have occurred in HAP1. Disruption of HAP1 led to the loss of the suppressor phenotype, which was not restored by transformation with a centromeric plasmid carrying HAP1-18 (YCpCYP1-18). Gap repair experiments (see materials and methods) located the suppressor mutation to the DBD, which was entirely sequenced. All the reversions affect codon 63: they substitute the arginine present in Hap1-18p either by a serine, restoring a wild-type sequence, or by one of the amino acids already identified by us and others as compatible with a wild-type or a pseudo-wild-type function of the protein (Kim et al. 1989; Defranoux et al. 1994). Our results, therefore, support the idea that binding to CYC1-UAS1-like target(s) is necessary for this function of Hap1p, and that the binding depends only on the structure of the Zn cluster. It must be stressed that, unlike the remaining recessive mutations that only partially restored the ability to grow on TE, all the dominant mutations restored wild-type growth.

Characterization of extragenic recessive suppressors: In addition to their initial HAP1-18 Δhem1 suppressor phenotype, a lag time corresponding to about two generations was observed for five of the 16 remaining recessive mutants. This lag was followed by a wild-type rate of division, and stationary phase was reached one generation before the wild type. One of them was further analyzed. After crossing to F1-17A, random spore analysis of the Δhem1 progeny indicated a 2:2 segregation for the TE growth phenotype, suggesting that only one gene was involved in the suppression. This was confirmed by tetrad analysis in which the HEM1 gene was disrupted in each of the two heme-sufficient spores from four tetrads. Growth on TE could not be dissociated from delayed growth on YPALA, suggesting that the active product of the suppressor gene facilitates aerobic metabolism (Figure 3). These data define the suppressor as a single nuclear gene, which we have designated ASC1. The mutated allele present in the mutant was named asc1-24.

To determine which of the mutants obtained affects ASC1, a HAP1-18 Δhem1 asc1-24 spore (YJ1-4B) was mated to all the recessive suppressors, and the diploids were scored for growth on TE. The four revertants, which also presented a delayed growth phenotype on YPALA, did not complement asc1-24, strongly suggesting that they are alleles of the ASC1 gene.

Cloning, sequence, and transcription analysis of the ASC1 gene: The ASC1 gene was isolated from a yeast genomic library (see materials and methods). ASC1 was localized to a BamHI-SpeI fragment of 2.6 kb that was entirely sequenced, revealing a gene of 957 bp encoding a protein of 319 amino acids. The gene corresponds to the ORF YMR116C (EMBL accession number Z49702). A putative TATA box is located at position -99 upstream the ATG. An intron of 273 bp, which has recently been characterized as containing the U24 small nucleolar RNA (snoRNA) coding region (Qu et al. 1995), interrupts the sequence at position 538 after the first ATG (Figure 4). snoRNAs are metabolically stable, small nucleolar RNAs associated with a set of nucleolar proteins in snoRNPs (Maxwell and Fournier 1995). U24 presents two complementarity regions with the 25S rRNA, and it has recently been shown that this snoRNA is required for sites-specific 2′-o-methylation of rRNA (Kiss-Laszlo et al. 1996). Northern blot experiments using an ASC1-specific probe show that its tran-
Inactivation of the gene and identification of the functional element: To determine if the ASC1 ORF or snoRNA is involved in the heme-dependent phenotypes, ASC1 was first inactivated by deletion of both the snoRNA and part of the ORF. Tetrad analysis and Southern blot experiments confirmed that the delayed growth phenotype is associated with the \textit{D\textasciitilde}asc1 allele (Figure 5). Introduction of pRS-ASC1, which carries the entire gene into a \textit{D\textasciitilde}asc1-disrupted strain, restored normal growth on YPALA (data not shown). Thus, ASC1 is not essential for yeast, but it is required for normal growth in aerobiosis. As expected, the inactivation of ASC1 in the hap1\textasciitilde}hap1\textasciitilde} strain W3/H/C resulted in growth on TE and in delayed growth in YPALA.

To assign functions to the different elements that constitute ASC1, we used four PCR-generated constructions cloned into the pRS316 vector: (1) the entire gene (pASC1), (2) the gene carrying a frameshift mutation in the first exon (p\textasciitilde}ORF), (3) the gene deleted for the intronic sequence (p\textasciitilde}INT; Figure 6A), and (4) the gene carrying both the frameshift mutation and the intron deletion (pasc1\textasciitilde}; see materials and methods). These four constructions were introduced in the hem1\textasciitilde}hap1\textasciitilde}asc1\textasciitilde} strain W3/H/C/A. The transformants were tested for their ability to grow in the presence or absence of heme. Only two of them, pASC1\textasciitilde} and p\textasciitilde}INT, restore the wild-type phenotype under all growth conditions (Figure 6B). These results clearly show that only the ORF is involved in normal growth in aerobiosis and in the inability to grow on TE in the absence of Hap1p and heme. Our results confirm the observation of Kiss-Laszlo et al. (1996), that the U24 snoRNA is not essential.

Involvement of the ASC1 ORF is confirmed by identification of the mutations: The alleles present in four asc1 mutants (REM6, 12, 14, and 24) were entirely sequenced (see materials and methods). The positions of the four corresponding point mutations are indicated in Figure 4. REM12 and 14 carry missense mutations that alter two strictly conserved amino acids in the seventh WD motif (see next section and Figure 7).
of the elements GH and WD of the repeats, and 127 residues are strictly invariable among these four proteins. Two of them, Ser291 and Gly304, are replaced in the asc1-12 and asc1-14 alleles, respectively. Since most of these protein sequences are derived from cDNA sequences, there is almost no information concerning their function. Nevertheless, it has been shown that in Mus musculus, the P205 gene is strongly expressed in the embryonic and early postnatal brain (Imai et al. 1994), and that in tobacco, the expression of the ARCA gene is induced by auxin (Ishida et al. 1993). The LACK gene if leishmania is expressed constitutively (Mougeot et al. 1995). The Cpc2p and Rack1p homologues have been functionally studied: Rack1p is a protein that binds activated protein kinase C (PKC), suggesting a role in PKC-mediated signal transduction (Ron et al. 1994), and Cpc2p is involved in general amino acid control and in the formation of protoperithecia (Krüger et al. 1990).

**Asc1p is localized in the cytoplasm:** Because all the intronic snoRNA coding sequences that have been characterized are located in parent genes that specify proteins involved in nucleolar function, ribosome structure, or protein synthesis (Maxwell and Fourrier 1995), it was of interest to identify the cellular localization of Asc1p. We tagged Asc1 at the C terminus with the HA epitope (see materials and methods). The residual growth on TE of the cells transformed with pASC1-HA and pΔINT is caused by the high level of plasmid loss. The photographs were taken after 1 day of growth on YP-ALA medium and after 3 days on TE medium.

REM12, serine 291 is replaced by phenylalanine, and in REM14, glycine 304 is substituted by aspartate. In REM24, a tryptophan codon is converted to a UGA stop codon, and REM6 contains a frameshift mutation. All four mutations affect the ORF and confirm that it is indeed the protein and not the snoRAN that is involved in the phenotypes observed.

**Asc1p belongs to a highly conserved subgroup of the WD repeat family of proteins:** Asc1p belongs to the WD repeat family. Proteins containing these repeats are involved in a wide variety of regulatory functions, including gene transcriptional regulation, RNA splicing, signal transduction, and cell division. A role in the formation of multiprotein complexes has been shown for some of them (for review see Neer et al. 1994). Asc1p is entirely composed of seven repeats of the WD motif and shows a strong similarity to >20 proteins present in organisms from yeast to humans (>53% identical residues over the entire sequence). In the interest of clarity, we have adopted for all organisms the genetic nomenclature used in yeast. The alignment presented in Figure 7 shows Asc1p and three of these proteins: Cpc2p from Neurospora crassa (Müller et al. 1995), Rack1p from Rattus norvegicus (Ron et al. 1994), and arcAp from Nicotiana tabacum (Ishida et al. 1993). Sequence conservation is more pronounced in the region of the elements GH and WD of the repeats, and 127 residues are strictly invariable among these four proteins. Two of them, Ser291 and Gly304, are replaced in the asc1-12 and asc1-14 alleles, respectively. Since most of these protein sequences are derived from cDNA sequences, there is almost no information concerning their function. Nevertheless, it has been shown that in Mus musculus, the P205 gene is strongly expressed in the embryonic and early postnatal brain (Imai et al. 1994), and that in tobacco, the expression of the ARCA gene is induced by auxin (Ishida et al. 1993). The LACK gene if leishmania is expressed constitutively (Mougeot et al. 1995). The Cpc2p and Rack1p homologues have been functionally studied: Rack1p is a protein that binds activated protein kinase C (PKC), suggesting a role in PKC-mediated signal transduction (Ron et al. 1994), and Cpc2p is involved in general amino acid control and in the formation of protoperithecia (Krüger et al. 1990).

**Polysome profiles are indicative of a translational function for Asc1:** We carried out polysome profile analysis on two diploid strains: YJ60 (Δasc1::TRP1/ASC1) and YJ70 (Δasc1:: TRP1/asc1-24); asc1-24 is a nonsense mutation. The choice of these strains allowed us to work in a HAP1+ HEM1+ context and to avoid any possible additional effects of the mutagen in REM24. Compared with that of wild-type cells, the polysome profile of asc1 mutant cells contained discrete additional peaks (Figure 9B, vertical arrows) sedimenting in the gradient at positions intermediate to the polyribosomal peaks containing an integral number of ribosomes (Heller et al. 1981). Western blot analyses show that HA-tagged Asc1p cofractionates with the 40S ribosomal subunit but is not detected in the 60S subunit. This observation was confirmed using antibodies against Ssm1p, a component of the 60S subunit (Figure 10). In conclusion, Asc1p harbors a snoRNA, is a ho-
mologue of Cpc1, and is associated to translating ribosomes. Taken together, these data strongly indicate that Asc1p is involved in the efficiency of translation.

**DISCUSSION**

The observation that Hap1p is essential for growth on TE in anaerobiosis and/or heme-deficient conditions led us to look for the possible involvement of Rox1p in this function of Hap1p. The disruption of Rox1p in a Δhap1Δhem1 strain did not restore growth on TE, allowing us to rule out this hypothesis. A possible interpretation is that Rox1p is not the unique Hap1p target involved in anaerobic metabolism. The existence of another repressor regulating the hypoxic gene ERG11 has been proposed by Turi and Loper (1992). It is not excluded that this postulated factor is also regulated by Hap1p. If several target genes are involved, the only way to overcome the absence of Hap1p would be by a more general change in metabolism. Thus, in agreement with our results, one would expect to isolate suppressors involved in the efficiency of transcription or translation.

Complementation analysis of the recessive mutants allowed us to identify at least two genes whose products are involved in the TE phenotype. One of them ASC1, is defined by five mutants that present a delayed growth phenotype in heme-sufficient conditions. The remaining mutants (with the exception of REM9 and REM11) all belong to a single complementation group that we have named ASC2. Their efficiency of suppression is slightly weaker than that of the asc1 mutants, and their growth is not delayed in heme-sufficient conditions. Even though both asc1 and asc2 mutations are recessive, complementation analysis between mutants in these genes did not totally cancel growth on TE in the heterozygous diploids, suggesting that Asc1 and Asc2 interfere functionally (Y. Chantrel, M. Gaisne and J. Verdier, unpublished results). Identification of ASC2 should provide additional insight concerning the function of ASC1.

ASC1 is interrupted by an intron that shelters the U24 snoRNA. Deletion of the intron, inactivation of the ORF, and molecular localization of the mutations show unambiguously that it is the protein that is involved in the identified phenotypes.

Asc1p belongs to the WD repeat family of proteins. Sequence comparison criteria defined by Neer et al. (1994) classified the WD proteins into four groups: the G3 subunits, the neurogenic Groucho-related proteins, the PR55 and CDC55 phosphatase subunit proteins, and a group of proteins that include, among others, Cbp1p, Rack1p, ArcAp, and Cpc2p (for review see Neer et al. 1994). Asc1p belongs to this last group.

Even though this class of proteins is highly conserved and is found in a large variety of eucaryotic organisms, its function is not well defined. Functional data are available for only two members: Cpc2p of Neurospora crassa and rat Rack1p.
CPC2 was isolated as encoding a positive trans-acting factor involved in general amino acid control. Its genomic sequence indicates that the ORF is interrupted by four introns (Müller et al. 1995). Intriguingly, in addition to the high degree of similarity between Cpc2p and Asc1p, the fourth intron found in CPC2 interrupts the ORF at precisely the same position as the intron found in ASC1. We performed an extensive analysis of the sequence of CPC2, which revealed that this intron contains a sequence flanked by box C and box D motifs, which are conserved sequences required for the formation of specific complexes and for the accumulation of stable snRNAs (Cafarelli et al. 1996). Moreover, it contains a 12-nt-long sequence complementarity to 25S rRNA that is strikingly similar to that of yeast U24. Thus, the fourth intron of CPC2 may encode a functional snoRNA. The involvement of Cpc2p in the translational control of CPC1, the Neurospora homolog of GCN4, has been suggested. It has also been shown to be necessary for the formation of protoperithecia (Krüger et al. 1990). The polysome profile and the Western blot analysis we performed show that halfmer
polysomes accumulate in an asc1-24 mutant and that Asc1p is associated with the 40S ribosomal subunit, implying that Asc1p is involved in translation. Yeast halmers are thought to be a 43S complex consisting of the 40S subunit with attached initiating factors awaiting the addition of the 60S ribosomal subunit (Helser et al. 1981), suggesting that Asc1p, although not essential, improves translation efficiency.

Rack1p was isolated from a cDNA expression library during a screen for proteins that bind PKC in the presence of PKC activators. The recombinant Rack1p, which is expressed in bacteria, met all the criteria so far established for intracellular receptors for activated C kinase, suggesting that it has a role in PKC-mediated signal transduction (Ron et al. 1994). Like Asc1p, Rack1p is located in the cytoplasm (Ron et al. 1995); also, when anti-Rack1 antibodies are used to probe total yeast proteins in a Western blot, a single band of 35 kD, consistent with the size of Asc1p, is detected (Kuo et al. 1995).

In summary, Cpc2p is involved in general amino acid control, Rack1p binds activated PKC, and the absence of functional Asc1p modifies the polysome profile and allows heme-deficient growth of hap1- strains. Considering the very high levels of sequence conservation among these proteins (>50% identical and 70% similar amino acids between yeast and humans), it seems likely that they modulate common processes that can nonetheless occur in their absence. We propose that Asc1p, Cpc2p, and Rack1p have similar functions, and that they are all involved in kinase signal transduction pathways that modulate the efficiency of translation. This hypothesis is currently under investigation.

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