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Negative Regulation of the *Saccharomyces cerevisiae ANB1* Gene by Heme, as Mediated by the *ROX1* Gene Product

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In *Saccharomyces cerevisiae* the anaerobic (oxygen-repressed) *ANB1* gene and a group of aerobic (oxygen-induced) genes are coordinately regulated by the *ROX1* gene. We report here that heme, known as an inducer of aerobic genes, also causes inhibition of *ANB1* expression. Thus, in combination with the *ROX1* gene product heme has an opposite effect on the expression of anaerobic and aerobic genes. Accumulation of *ANB1* mRNA was sharply decreased in anaerobic cells grown in the presence of heme. This effect must operate at the level of transcription since heme also inhibited accumulation of *CYC1* mRNA from an *ANB1-CYC1* fusion. Heme precursors did not appear to function either as inhibitors or as activators. Oxygen itself also had no effect on transcription of *ANB1*. Repression by heme cannot be attributed to the respiratory competence conferred by heme since both *ANB1* and the aerobic genes *tr-1* and *CYC1* were regulated normally in [*rho*^{0}] mutants. The results are consistent with a classical allosteric coeffector function for heme, although more indirect explanations are tenable. A role for the *ROX1* gene product in transcriptional regulation can be inferred from the observation that there was no inhibition of *ANB1* expression by heme in *rox1* mutants. Judging from this epistasis the *rox1* phenotype is not due to a defect in heme production; this would indicate that the *ROX1* factor functions by mediating the effect of heme on transcription.

A number of families of coordinately expressed genes have been identified in eucaryotes. It is hypothesized (1) that gene family members dispersed throughout the genome respond to common *trans*-acting factors, which recognize family-specific DNA-binding sites in the upstream region of each gene. An implicit extension of this hypothesis is that genes responding to common regulatory proteins are activated or repressed by the same signalling coeffectors. We sought to test this in the family of oxygen-regulated genes. This group displays a paradoxical pattern of coordinate regulation: while most of its known members are aerobic genes, oxygen-induced genes coding for mitochondrial components, at least one gene, *ANB1*, is an anaerobic gene negatively regulated by oxygen (6, 7). The reason for considering these oppositely regulated genes to be members of a family is that they are controlled by a common *trans*-acting factor, the product of the *ROX1* gene. This was shown by the pleiotropic effect of the *rox1-a1* allele, which causes constitutive expression of both the aerobic gene and *ANB1* (7).

Since *ANB1* and the aerobic genes are regulated in opposite ways in response to oxygen levels, the regulatory system must distinguish between aerobic and anaerobic promoters. The *ROX1* factor could be imagined to operate at the two types of promoter in one of two general ways: (i) aerobic and anaerobic cells might synthesize different metabolites which function as coeffectors to induce *ROX1* activation of aerobic or anaerobic genes, in a mutually exclusive fashion; (ii) alternatively, the *ROX1* factor might activate one set of genes and repress the other in response to a single coeffector produced under aerobic but not anaerobic conditions. Other variations of these basic schemes are possible. In line with the second possibility, we demonstrate here that *ANB1* expression is repressed by heme, which is produced only in the presence of oxygen (9) and which has been shown to be an inducer of aerobic genes (5, 10, 11).

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The identification of heme as both a positive and negative effector supports the conclusion that anaerobic and aerobic genes are coordinately regulated by an interconnected system which includes the *ROX1* gene product. We also conclude here that the constitutive *rox1* phenotype is not due to a disruption of heme metabolism, as shown by the lack of effect of heme on *ANB1* gene expression in *rox1* mutants; this indicates that the *ROX1* gene product is part of the regulatory ensemble rather than a participant in heme metabolism.

MATERIALS AND METHODS

Strains and plasmids. Strains GM-3C-2, GM-3C-2 (*rox1-a1*), and GM-3C-2 (*rox1-b3*) and plasmids YCp*CYC1*(2.4), YCp*CYC1*(2.4)1/X, and pYe*CYC1*(2.4) are described in reference 7. Plasmid YIp5 (12) was used as a *URA3* probe. Heme mutants, generously provided by E. Gollub (2), were as follows, with the indicated enzyme deficiencies: GL-1 (*hem1*, δ-aminolevulinic acid synthase); GL-4 (*hem2*, δ-aminolevulinic acid dehydratase); GL-6 (*hem4*, coproporphyrinogenase); and RG-10 (*hem10*, ferrochelatase). [*rho*^{0}] mutants were obtained by adaptation of a standard method (overnight growth in YPD medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose) containing 10 μg of ethidium bromide per ml, plating on YPDG plates [0.1% glucose and 2% glycerol], and selecting petite colonies). The mutants, designated GM-3C-2-[*rho*^{0}]-1, -2, etc., were unable to grow on either glycerol or lactate medium (7). They were shown to lack mitochondrial DNA by a Southern blot of *Eco*RI-digested total cellular DNA probed with purified mitochondrial DNA (a gift of T. Pillar). All of several mitochondrial bands recognizable in wild-type DNA were missing in each of five isolates.

Growth conditions and RNA analysis. GM-3C-2 cells were grown to 10^7 cells per ml by shaking at 30°C in YPD medium and either shifted to anaerobic conditions (7) for 90 min or continued in aerobic growth for 60 min. In some experiments YPD medium was supplemented, as indicated, with ergos-

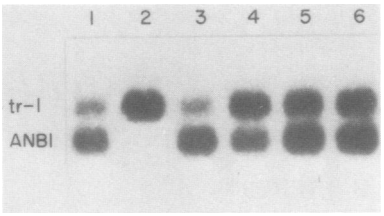

FIG. 1. Effect of heme addition on *ANB1* and *tr-1* expression in anaerobic cells. Cells were grown aerobically in YPD medium to a density of 10^7/ml. Heme was then added as indicated below, and growth was continued under anaerobic conditions for 90 min. All three strains carried plasmid YCp*CYC1*(2.4). The RNA blot was probed with an *Xho*I-*Bam*HI fragment carrying the *ANB1* gene, which has sufficient homology with the *tr-1* gene to hybridize with both the *ANB1* and *tr-1* transcripts. Lanes: 1, GM-3C-2 in YPD; 2, GM-3C-2 in YPD plus heme; 3, GM-3C-2 (*rox1-b3*) in YPD; 4, GM-3C-2 (*rox1-b3*) in YPD plus heme; 5, GM-3C-2 (*rox1-1a*) in YPD; 6, GM-3C-2 (*rox1-α1*) in YPD plus heme.

terol (10 μg/ml), Tween 80 (0.5%), δ-aminolevulinic acid (10 μg/ml), protoporphyrin IX (50 μg/ml), or heme (50 μg/ml). Heme was prepared as described by Maniatis et al. (8). In control experiments the ethylene glycol-heme vehicle had no effect on growth or gene expression. RNA extraction and RNA gel blot analysis were as described previously (7). The gel blot analyses give a semiquantitative relative estimate of steady-state mRNA levels.

RESULTS

Inhibition of *ANB1* expression by heme. Since heme acts as a regulatory coeffector in the induction of aerobic genes it seemed likely to be responsible for the simultaneous repression of the anaerobic gene *ANB1*. To test this, cells were grown anaerobically in the presence or absence of heme. The *ANB1* transcript was detected in untreated anaerobic cells, but not when heme was added (Fig. 1, lanes 1 and 2), suggesting that heme is a negative coeffector of *ANB1* and that oxygen is not directly involved in regulating expression. As a control, RNA from cells grown under these conditions was analyzed for expression of the *URA3* gene. Levels of *URA3* mRNA were unaffected by heme addition (data not shown), indicating that heme does not cause a general depression of mRNA synthesis but is specific in effect. A more convincing demonstration is that heme caused the expected induction of the oxygen-induced *tr-1* gene (Fig. 1, and see below) and of the *CYC1* gene (data not shown).

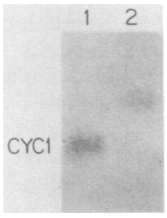

FIG. 2. Effect of heme addition on expression of *ANB1-CYC1* fusion. GM-3C-2 cells carrying plasmid YCp*CYC1*(2.4)1/X (7), which contains a fusion of the upstream control region of *ANB1* to the *CYC1* gene, were grown anaerobically as described in the legend to Fig. 1. The RNA blot was probed with the plasmid pYe*CYC1*(0.6) which carries the coding region of the *CYC1* gene inserted in pBR322. The *CYC1* transcript is at the position indicated. Reprobing with pBR322 demonstrated that the other faint bands shown are transcripts of pBR322 sequences on the plasmid (data not shown). Lanes: 1, GM-3C-2 in YPD; 2, GM-3C-2 in YPD plus heme.

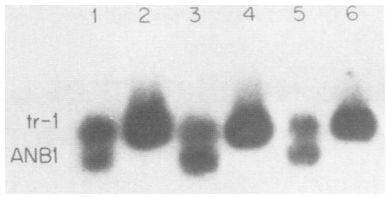

FIG. 3. Expression of *ANB1* and *tr-1* in [*rho*^{0}] cells. Respiratory-deficient (petite) mutants lacking mitochondrial DNA were derived from strain GM-3C-2(YCp*CYC1*(2.4)) as described in Materials and Methods. Cells of two of these mutant strains, still carrying plasmid YCp*CYC1*(2.4), and the parent strain were grown aerobically or anaerobically as described in the legend to Fig. 1. The RNA blot was probed as described in the legend to Fig. 1. Lanes: 1, GM-3C-2-[*rho*^{0}]-1, anaerobic; 2, GM-3C-2-[*rho*^{0}]-1, aerobic; 3, GM-3C-2-[*rho*^{0}]-2, anaerobic; 4, GM-3C-2-[*rho*^{0}]-2, aerobic; 5, GM-3C-2, anaerobic; 6, GM-3C-2, aerobic.

***ROX1* gene product is epistatic to heme.** Mutations in the *ROX1* gene were originally selected for constitutive expression of *ANB1* (7). While it has been assumed that these *trans*-acting mutations affect the regulatory apparatus, it was possible that the phenotype resulted from decreased production of heme, leading to derepressed expression during aerobic growth. However, when mutant cells were grown anaerobically as above, heme no longer inhibited expression of *ANB1* in either the *rox1-b3* (Fig. 1, lanes 3 and 4) or *rox1-a1* (lanes 5 and 6) strains, in contrast to wild type (lanes 1 and 2). Thus, both mutations cause a lack of sensitivity to added heme. To be sure that heme penetrates the mutant strains, we also determined the pattern of expression of the *tr-1* gene. Figure 1 shows the transcript of this gene, which is induced in the presence of oxygen. Although *tr-1* is unlinked to *ANB1* and regulated in an opposite fashion, the two genes share extensive homology (6; C. V. Lowry, R. H. Lieber, and R. S. Zitomer, unpublished data) and their transcripts both hybridized to the *ANB1* probe in this experiment. The RNA blot shows that *tr-1* expression was stimulated by added heme in *rox1-b3* cells just as in wild type, indicating substantial heme uptake. Hence, in *rox1* mutants *ANB1* is expressed in the presence of intracellular heme. The epistasis of the constitutive mutation to heme argues against a role for the *ROX1* factor in heme synthesis, since heme uptake would be expected to circumvent a deficit in production. The constitutive phenotype is most easily explained by heme insensitivity within the regulatory apparatus rather than by an effect on heme metabolism, indicating that the *ROX1* gene product is part of the regulatory system.

Action of heme at the level of transcription. The RNA blots shown reflect net mRNA accumulation—the balance of rates of transcription and degradation. To determine which process is regulated by heme, we monitored expression of an *ANB1-CYC1* gene fusion. Cells transformed with plasmid YCp*CYC1*(2.4)1/X, which carries a fusion of the *ANB1* upstream control region to the *CYC1* gene, were tested under anaerobic conditions. *CYC1* mRNA was synthesized only in the absence of added heme (Fig. 2). The structure of the fusion is such that the resulting *CYC1* mRNA is transcribed entirely from the *CYC1* gene and contains no *ANB1* leader or coding sequence (6); hence any degradative mechanism specifically recognizing *ANB1* mRNA would be inoperative on the fusion transcript. Since heme inhibits accumulation of message from the *ANB1-CYC1* fusion as well as from *ANB1* the specific regulatory effect must be exerted through the untranscribed 5′ region of the *ANB1* gene and

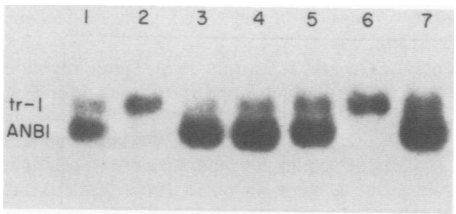

FIG. 4. Expression of *ANB1* and *tr-1* in heme deficient mutants. Mutants lacking various enzymes of the heme synthetic pathway were grown aerobically in YPD medium, with supplements as indicated below. The blot was probed as described in the legend to Fig. 1. Lanes: 1, GL-1 in YPD plus Tween and ergosterol; 2, GL-1 in YPD plus Tween, ergosterol, and δ-aminolevulinate; 3, GL-4 in YPD plus Tween and ergosterol; 4, GL-6 in YPD plus Tween and ergosterol; 5, RG-10 in YPD; 6, RG-10 in YPD plus heme; 7, RG-10 in YPD plus protoporphyrin IX.

operate at the level of transcriptional initiation. This does not rule out an effect on degradation by an independent mechanism.

Respiratory products do not inhibit *ANB1* expression. In accounting for regulation by heme, it was possible that inhibition of expression is actually caused by the presence of competent mitochondria, which are dependent on heme for function. In this view mitochondria containing heme (and heme-induced proteins) could produce a signal or metabolite which inhibits *ANB1* transcription. To help rule out this possibility, petite mutants ([*rho*^{0}]), lacking mitochondrial DNA and consequently not engaged in respiration, were tested for their ability to express *ANB1*. We expected that if respiratory function was required to inhibit *ANB1* expression, then [*rho*^{0}] cells would be constitutive. However, the two mutants tested were regulated normally by oxygen in the expression of *ANB1* and *tr-1* (Fig. 3, lanes 1 through 4) compared with the wild type (lanes 5 and 6). We assume that heme is produced in aerobically grown [*rho*^{0}] cells, since cell growth requires heme catalysis of the synthesis of sterols and unsaturated fatty acids (2). Since heme is presumed present and the rest of the respiratory pathway is nonfunctional, the simplest explanation of the normal *ANB1* regulation observed in these cells is that heme affects transcription independently of the respiratory apparatus. However, it is still possible that either a heme derivative or a nonrespiratory metabolite whose production is dependent on heme is the actual coeffector in the *ROX1* system.

Heme precursors are not coeffectors. Although heme is a negative coeffector of *ANB1* expression, it was possible that a positive coeffector was also involved. Heme precursors were likely candidates for such a role since heme synthesis requires oxygen only at the penultimate step in the pathway (8), and several porphyrin precursors could occur in anaerobic cells and function as activators. Both a possible positive and negative role of precursors was tested in mutants deficient in heme synthesis.

Cells carrying the *hem1* mutation lack porphyrins and porphyrin precursors because the pathway is blocked at the synthesis of δ-aminolevulinic acid. Aerobically grown *hem1* cells accumulated substantial levels of *ANB1* mRNA (Fig. 4, lane 1), indicating that the heme precursors missing in this strain are not required for expression. The fact that *ANB1* is expressed during aerobic growth also shows that oxygen is not itself an inhibitor. These conclusions require that the *hem1* strain, GL-1, is otherwise normally regulated for *ANB1*; this was shown by inhibition of expression after the addition of the missing precursor, δ-aminolevulinic acid (lane 2), or of heme (data not shown).

In an attempt to show that heme is specifically required for inhibition, we also analyzed heme-deficient mutants blocked at three later steps in the synthetic pathway. Two of these mutants, GL-6 and RG-10, accumulate porphyrin precursors (2). It was reasoned that if any precursors were inhibitors, then expression might be reduced in mutants blocked either before the critical oxygenase step (GL-6) or after (RG-10). However, each mutant accumulated *ANB1* mRNA when grown aerobically (Fig. 4, lanes 3 to 5), suggesting that heme itself must be present to turn off expression. Most convincing is the expression of *ANB1* in the *hem5* mutant, RG-10 (lane 5), which is blocked at the last step of heme synthesis, the addition of iron to protophorphyrin IX by ferrochelatase. Again, the addition of heme (lane 6) but not of the precursor protoporphyrin IX (lane 7) restored inhibition of expression.

These observations confirm that a product of the heme pathway is necessary and sufficient to signal inhibition of *ANB1* expression. The fact that the *hem5* mutation permits expression even in cells supplemented with protoporphyrin IX suggests that heme is the only porphyrin capable of carrying out the inhibitory function and that inclusion of the iron atom is necessary for signal recognition.

DISCUSSION

Given that the *ROX1* gene product regulates the transcription of both aerobic and anaerobic genes (7), it was hypothesized that a common coeffector might play a corresponding dual role, inducing expression of one group and inhibiting that of the other. Evidence presented here suggests that heme is both necessary and sufficient for such a function.

Identification of a negative effector of *ANB1* also permitted a test of whether the *ROX1* factor is directly involved in the regulation of transcription. We showed here that the constitutive effects of *rox1* mutations are independent of (i.e., epistatic to) heme levels, indicating that the factor must act at some point in the regulatory pathway other than heme synthesis. It is possible that the factor, in association with heme, forms part of a chromatin complex which regulates the initiation of transcription, although other equivalent roles are still under consideration.

This leaves the question of how the *ROX1* factor and heme act together to cause opposite effects on anaerobic and aerobic genes. For a protein to respond to a single coeffector by repressing at one locus and activating at another seems to demand an unusual versatility, although there is a comparable precedent in *Escherichia coli* (3). An alternative explanation is that other "adaptor" factors recognize the two classes of DNA site, forming complexes which are then activated or inhibited by the *ROX1* factor-heme complex. If such site-specific factors are required for *ANB1* transcription they may be identified by *trans*-acting mutations of noninducible phenotype.

The regulatory signal presented by heme to the transcriptional apparatus may be indirect; it is premature to conclude that heme regulates by interaction with *ROX1*-regulated transcription complexes. However, we strengthened the implication of heme in a direct role by elimination of one alternative possibility, showing that respiration does not itself produce the regulatory signal. Our finding of normal regulation of aerobic and anaerobic genes in [*rho*^{0}] mutants parallels the observation by Guarente et al. (4) of induction of *CYC1* by the inactive heme analog deuteroporphyrin. The parallel is that heme itself is gratuitous in respiratory mu-

tants, showing its regulatory effect despite apparent metabolic inactivity. Both findings are somewhat inconclusive because heme or its analogs could modulate transcription via functions outside the electron transport chain. Uncertainties about the role of heme may soon be resolved by observation of the interaction between heme and regulatory factors in an in vitro system.

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