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Regulation of hypoxic gene expression in yeast

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Regulation of hypoxic gene expression in yeast. Baker's yeast, *Saccharomyces cerevisiae*, can adapt to growth under severe oxygen limitation. Two regulatory systems are described here that control this adaptation. The first involves a heme-dependent repression mechanism. Cells sense hypoxia through the inability to maintain oxygen-dependent heme biosynthesis. Under aerobic conditions, heme accumulates and serves as an effector for the transcriptional activator Hap1. The heme-Hap1 complex activates transcription of the *ROX1* gene that encodes a repressor of one set of hypoxic genes. Under hypoxic conditions, heme levels fall, and a heme-deficient Hap1 complex represses *ROX1* expression. As a consequence, the hypoxic genes are derepressed. The second regulatory system activates gene expression in response to a variety of stress conditions, including oxygen limitation. Oxygen sensing in this system is heme-independent. The same DNA sequence mediates transcriptional activation of each stress signal.

Over the past decade, Baker's yeast, *Saccharomyces cerevisiae*, has emerged as a surprisingly accurate model system for many of the basic eukaryotic cellular processes that it shares with human cells. Not only are the components involved in such processes as transcription, RNA splicing, translation, signal transduction, protein compartmentalization, and chromosome structure, to name a few examples, highly conserved at the sequence level between yeast and humans, but in many cases they are functionally interchangeable. Therefore, it is reasonable to ask whether yeast might not serve as a model system for studying the mechanisms by which cells sense and respond to limitation in oxygen levels. Here we will discuss two of the mechanisms through which yeast cells respond to hypoxia; one represents a specific response, and the other is part of a global stress response. Neither system contains a homologue of the recently discovered mammalian HIF-1 transcriptional activator that is responsible for the induction of hypoxic genes [1].

While there are likely to be other oxygen sensing mechanisms in both yeast and humans, it is not surprising that there are some fundamental differences. Baker's yeast is a facultative aerobe, capable of growth in the complete absence of molecular oxygen. Consequently, the specific hypoxic response of yeast described below occurs at vanishingly low oxygen concentrations, conditions under which yeast cells can grow but under which most mammalian cells, let alone the intact organism, could not long survive. It is perhaps important when exploiting yeast as a model for mammalian cells to differentiate between basic cellular processes and those processes that allow the organism to interface with its environment and exploit its ecological niche. These latter processes are subject to very different selective pressures in such diverse organisms, and it would not be surprising if the molecular mechanisms that govern them are different.

Heme-dependent hypoxic response

Oxygen serves as an electron acceptor for a number of metabolic reactions in yeast cells. These include respiration and the formation of conjugated carbon bonds in sterol, fatty acid, and heme biothsyntheses. While cells can generate energy solely through fermentation to bypass the need for oxygen in respiration, strictly anaerobic growth requires a sugar and thus limits the range of energy sources that anaerobically-grown cells can use compared to those grown aerobically. Also, cells cannot bypass the requirement for oxygen in sterol and fatty acid biosyntheses, and these compounds must be present in the media for cells to assemble membranes anaerobically [2, 3]. Hence, it is advantageous to cells grown under hypoxia to maximize the use of the limiting oxygen. One mechanism for achieving this end is to increase the levels of enzymes that require oxygen as a substrate, thus maintaining high reaction rates as substrate concentration falls. Table 1 lists those genes whose expression is known to be induced by hypoxia, and, consistent with the above rationale, the *ERG11*, *HEM13*, and *OLE1* genes each of whose products use oxygen as a substrate are all induced by hypoxia. Also included among the hypoxic genes, are *COX5b*, an alternate cytochrome oxidase V subunit and *CYC7*, an alternate cytochrome c. Cytochrome chains in which these two proteins replaced their corresponding aerobic isoforms have a higher turnover number for oxygen utilization, consistent with the rationale that hypoxic genes allow more efficient use of a limiting substrate [4]. Other hypoxic genes encode functions in sterol uptake (*SUT1*), a requirement for anaerobic growth, sterol biosynthesis (*HMG2* and *CPR1*), and ATP/ADP transport into the mitochondria (*AAC3*).

The hypoxic genes fall into at least two classes in terms of the strategies employed for the regulation of protein levels. One class is comprised of single copy genes and includes *OLE1*, *ERG11*, *CPR1*, *HEM13*, and *SUT1*. These genes encode enzymes expressed at low levels under aerobic conditions, but as oxygen becomes limiting, expression levels increase. The second class represents gene-pairs, where one is expressed aerobically and the other under hypoxic conditions. These include *COX5a/COX5b*, *CYC1/CYC7*, *HMG1/HMG2*, and *AAC1/AAC2/AAC3*, and *TIF51a/ANB1*, whose function is yet to be clearly defined.

Oxygen availability is sensed through cellular heme levels

For the regulation of these hypoxic genes, the cell senses oxygen availability through cellular heme levels. Molecular oxygen is

Table 1. Heme-repressed hypoxic genes

Gene	Function	Aerobic homologue	References
Heme synthesis			
HEM13	Coproporphyrinogen III oxidase	None	[38, 39]
Sterol synthesis			
ERG11	Cytochrome P450, Lanosterol 14α-demethylase	None	[40]
HMG2	3-Hydroxy-3-methylglutaryl CoA reductase	*HMG1*	[41]
CPR1	NADPH-cytochrome P450 reductase	None	[40]
Sterol uptake			
SUT1		None	[42]
Fatty acid synthesis			
OLE1	Δ9 fatty acid desaturase	None	[43]
Electron transport			
COX5b	Subunit Vb of cytochrome c oxidase	*COX5a*	[44, 45]
CYC7	Iso-2-cytochrome c	*CYC1*	[46]
AAC3	ADP/ATP translocator	*AAC1*, *AAC2*	[47]
Function unknown			
ANB1	eIF5A	*TIF51a*	[19]

required as a substrate in two consecutive steps of heme biosynthesis catalyzed by the enzymes coproporphyrinogen oxidase and protoporphyrinogen IX oxidase [5]. Consequently, under hypoxic conditions, heme levels are reduced. However, it should be noted that these two enzymes have K_m values for oxygen in the range of 0.1 μM; thus, for yeast hypoxia represents very low oxygen levels indeed [5].

A diagram of how changes in heme levels are sensed and translated into changes in gene expression is presented in Figure 1. In the presence of oxygen (aerobic growth), heme accumulates and binds to the transcriptional activator Hap1. Hap1 is a protein composed of a zinc finger DNA binding domain at the N-terminus, a heme binding domain within the central region, and a transcriptional activation domain at the C-terminus [6–8]. When heme is bound, Hap1 acts as a transcriptional activator of genes containing its recognition site (5'CGGN$_6$CGG) [9–11], for the most part, genes encoding a variety of respiratory and oxidative stress functions [reviewed in 12]. In addition, the *ROX1* gene encoding the repressor of hypoxic genes is also activated by the Hap1-heme complex [13, 14]. The Rox1 repressor binds to its cognate site upstream of the hypoxic genes to repress their transcription [15, 16]. Under hypoxic or anaerobic growth conditions, heme levels are reduced. Hap1 still binds to its cognate site, but in the absence of heme, additional proteins bind to Hap1 creating a larger complex that represses transcription [14, 17, 18]. Consequently, under hypoxic conditions, *ROX1* expression is repressed resulting in the derepression of the hypoxic genes.

The important features of this scheme are supported by a number lines of evidence. First, when heme is added to anaerobically growing cells, the hypoxic genes are repressed, and when heme-deficient mutants are grown aerobically in the absence of heme, the hypoxic genes are induced, demonstrating that heme is the effector molecule [19]. Second, in a *hap1* deletion mutant, *ROX1* expression is decreased in the presence of heme and increased in its absence, indicating that Hap1 serves to both activate and repress *ROX1* expression [13, 14]. Third, when *ROX1* was expressed from a heterologous promoter in the absence of heme, the hypoxic genes were repressed, indicating that heme is required only for expression and not for the Rox1 function [13]. Fourth, the hypoxic genes are expressed constitutively in a *rox1* deletion mutant, indicating that Rox1 serves as a repressor [13, 14].

Physiology of Rox1-mediated repression

An additional feature of *ROX1* repression that is not represented in the scheme in Figure 1 is that it is autorepressed [14]. In the absence of a functional Rox1, *ROX1* mRNA levels are increased 15-fold, and the mutant, non-functional protein accumulates at much higher than wild-type levels. This autorepression results from Rox1 binding to its own promoter as demonstrated by gel-retardation experiments. We believe that this autorepression is important for adjusting Rox1 concentrations in the cell. As indicated above, a number of the hypoxic genes are part of a gene-pair where the other homologue is expressed aerobically. These hypoxic genes can be completely repressed during aerobic growth. However, other hypoxic genes like *CPR1*, *ERG11*, *HEM13*, and *OLE1* do not have aerobically expressed homologues, but encode functions that are required aerobically. Were these genes to be completely repressed, aerobic growth would cease. While one strategy for such differential repression involves placing weak Rox1 binding sites upstream of the genes whose expression is required aerobically and strong sites upstream of those that are completely repressed, were Rox1 levels to rise too high, these single copy genes could still be over-repressed. However, this problem is circumvented when the repressor is autorepressed by a weak binding site within its own upstream region.

To yeast, hypoxia represents a stress, the starvation for a central metabolite. Hence, the response is rapid; *ANB1* mRNA levels become detectable within 30 minutes after purging cultures of oxygen and reach fully induced levels within 90 minutes, less than one generation [15]. The speed of the hypoxic response is achieved through the combination of a quick drop in the transcription of the *ROX1* gene [15], and the short half-life of the Rox1 protein [20]. Immunoblot measurements of Rox1 levels during the transition to hypoxia indicate that within 30 minutes, there is no Rox1 protein detectable in the cell. This rapid turnover of Rox1 can account for the rapid induction of hypoxic gene expression, and is consistent with the findings that the presence/absence of Rox1 is the determining factor in whether the hypoxic genes are expressed. Interestingly, Rox1 is not only highly labile in yeast, but also in *E.coli* and *Drosophila* cells, presenting problems for its purification (unpublished data).

Rox1 DNA binding properties

Rox1 consists of 368 amino acids that can be divided into three domains (Fig. 2) [16, 21]. The first third of the protein consists of an HMG DNA binding motif. This motif is found in a number of DNA binding and bending proteins including both sequence-specific regulatory proteins and general DNA binding chromatin proteins [reviewed in 22]. The ability of these proteins to bend DNA has led to their designation as architectural proteins, proteins that induce conformations in DNA that may be important for their function. The HMG domain of Rox1 was expressed

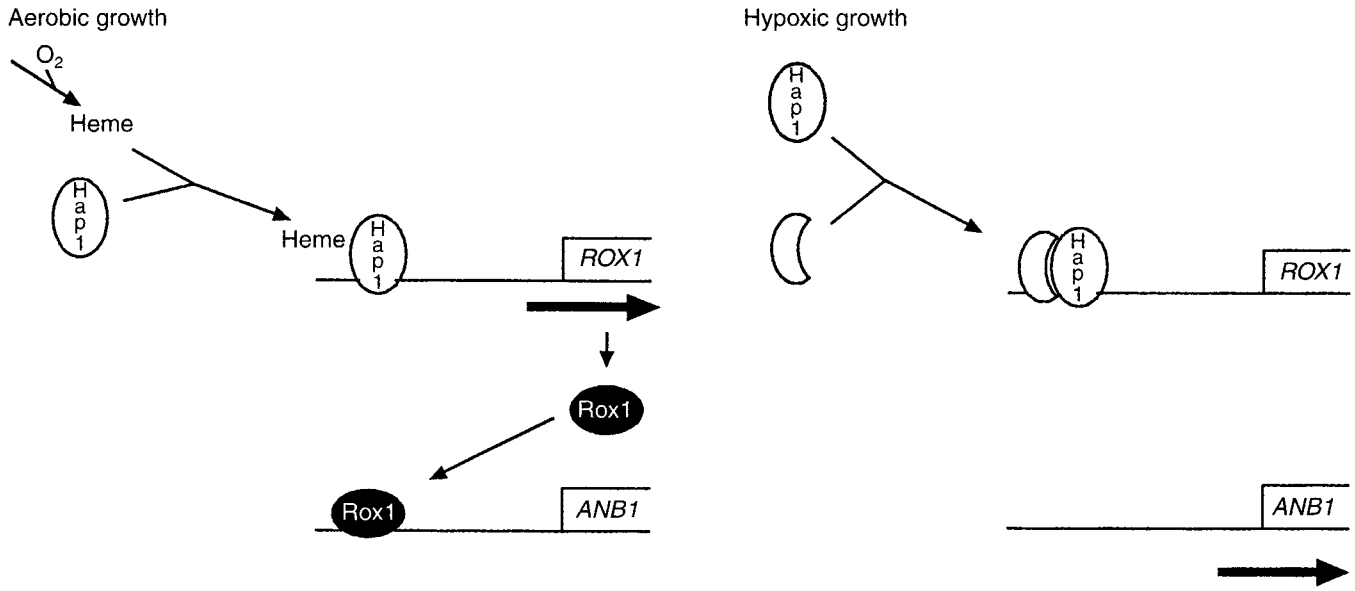

Fig. 1. *Scheme for heme-dependent repression of the hypoxic genes.* Cells grown under aerobic conditions synthesize heme which begins the regulatory cascade that ultimately results in repression of the hypoxic genes as illustrated. Cells grown under hypoxic conditions do not accumulate heme, resulting in the derepression of the hypoxic genes.

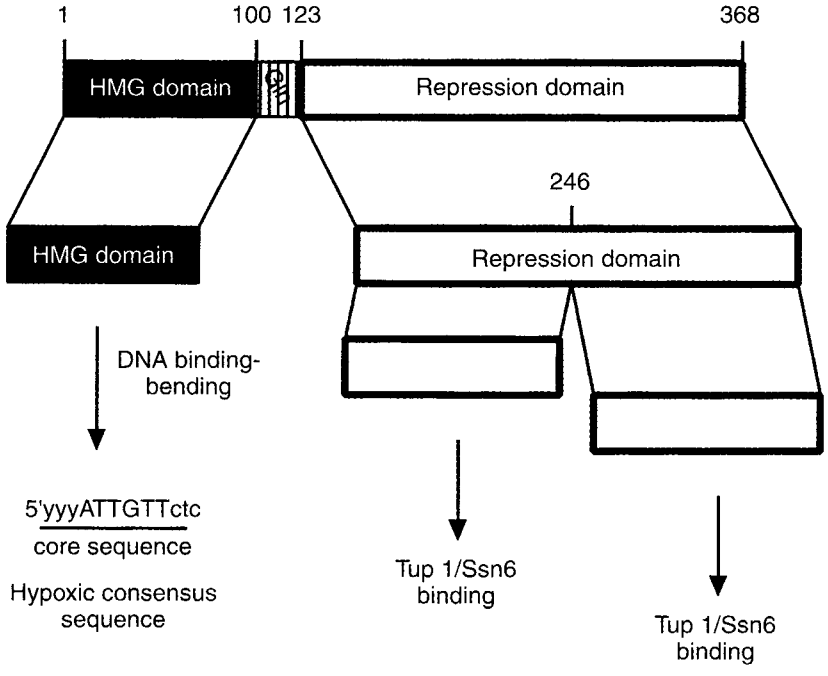

Fig. 2. *Organization of the Rox1 protein.* Evidence for the functions of the various domains of the Rox1 protein are presented in the text. Gln represents the glutamine-rich sequence. Numbers refer to amino acid residues. Y in the consensus sequence represents pyrimidine.

in and purified from *E. coli* cells. Using gel retardation and DNase protection studies, this domain was found to bind as a monomer to the previously mapped operator sites in the *ANB1* gene with a dissociation constant of about 20 nM [16, 20, unpublished data]. These results, combined with a comparison of the operator sequences in other hypoxic genes presented in Table 2, led to the formulation of the hypoxic consensus sequence indicated in Figure 2. A synthetic double stranded DNA containing the sequence 5'CCCATTGTTCTC bound purified Rox1 [16], and mutations in any of the base pairs within the core sequence (Fig. 2) resulted in sharp reduction of DNA binding (unpublished data). These results confirm that the consensus sequence does bind Rox1. Interestingly, this recognition sequence is remarkably conserved among the HMG proteins in organisms as divergent as yeast and humans [22].

As with other HMG proteins, Rox1 bends DNA [21]. Using a gel-retardation assay with DNA fragments containing an hypoxic operator in various positions along the sequence allows an analysis of the topology of the DNA in the bound complex. From such experiments, it is possible to calculate the bending angle to

Table 2. Hypoxic operator sequences

Gene	Operator[a]			Evidence[b]	References
HEM13	−476	TCAATTGTTTAG	−465	Deletion analysis	[48]
	−238	TGCTTTGTTCAA	−249		
	−185	CCCATTGTTCTC	−174		
ERG11	−358	CCTATTGTGCAT	−347	Consensus	[40]
CPR1	−95	TCATTTGTTCCT	−84	Consensus	[40]
HMG2	−282	CGCATTGTTTTG	−271	Consensus	Yeast Genome Database
	−224	CTTATTGTTCTC	−235	Consensus	
SUT1	−243	GTTTTTGTTCCT	−232	Consensus	[42]
	−342	AGCTTTGTTCTT	−331		
OLE1	−272	CCTATTGTTACG	−261	Consensus	[43]
COX5b	−228	TGTATTGTTCGA	−217	Consensus	[49]
CYC7	−333	CCTATTGTATTA	−322	Consensus	[46]
AAC3	−197	TTCATTGTTTGG	−186	Deletion analysis	[47]
	−145	TCCATTGTTCTT	−134		
ANB1	−316	TCCATTGTTCGT	−305	Deletion analysis, gel retardation, DNase protection	[16, 20, 50]
	−285	CCTATTGTTCTC	−274		
	−218	TCCATTGTTCTC	−207		
	−197	CTCATTGTTGCT	−186		
ROX1	−397	CCTATTGTTGCT	−386	Deletion analysis, gel retardation	[14]
	−364	CGTATTGTCTTG	−353		
Consensus sequence		YYYATTGTTCTC[c]			

[a] Sequences are numbered with the first base in the coding strand immediately 5′ to the ATG initiation codon as −1

[b] Consensus indicates the operators were identified solely by sequence homology

[c] Y represents pyrimidine

be 90°. Experiments with chimeric proteins suggests that this bend is not essential but enhances repression activity.

The tertiary structure of the protein-DNA complex for the HMG domain of SRY, a transcriptional activator protein encoded on the human Y chromosome and essential for male determination, has been resolved by NMR [23]. Computer modeling using the SRY coordinates indicates that the HMG domain of Rox1 fits this structure (unpublished data). The conclusion from the modeling studies was reinforced by a mutational analysis of *ROX1* involving over 25 missense mutations in the HMG domain that caused loss or weakened DNA binding activity [21, unpublished data]. The most severe lesions fell in residues that were conserved between Rox1 and SRY, and almost all of the mutations altered residues that either contacted DNA directly or were important for the structural integrity of SRY. Thus, it is highly probable that Rox1 shares a common structure with SRY.

Rox1 repression domain

The Rox1 protein consists of at least two additional domains. There is a glutamine rich region following the HMG domain, extending from residues 100 to 123. Deletion of this region does not affect either the function or the lability of Rox1, and its purpose is not known [21].

The remainder of the protein represents the repression domain (Fig. 2). Deletion of this domain results in a DNA binding protein with no repression activity, and fusion of this domain to a heterologous DNA binding protein (the amino terminus of the yeast protein Gal4) results in a chimeric protein with repressor activity [21]. This repression domain is comprised of redundant information. Deletion of either half (Δ100–245 or Δ247–368) does not dramatically affect repression activity of the remaining protein, and fusion of either half (124–247 or 246–368) to the Gal4 DNA binding domain results in chimeric repressors, each of which is equal in activity to the fusion containing the entire region (124–368) [21]. Despite this functional redundancy, there is no obvious sequence repetition. This region is presumed to interact with the general repressor Tup1/Ssn6 complex as described below.

Rox1 repression requires the general repressor Tup1/Ssn6 complex

Eukaryotic promoters occupy large stretches of DNA sequences. In yeast, sites for the binding of transcriptional activators often lie hundreds of base pairs from the TATA box, where the basal transcriptional machinery and RNA polymerase II bind. In addition, many genes have multiple, differentially regulated activators that can act individually and additively to set transcription levels. Such promoters preclude repression mechanisms that simply act through competitive binding of the repressor with an activator or RNA polymerase, and suggest that repression must be an active process. The organization of the Rox1 protein, with its separate DNA binding and repression domains reinforces this view. In fact, Rox1 binding to DNA alone is not sufficient to repress hypoxic gene transcription. A general repression complex consisting of two proteins, Tup1 and Ssn6, is also required [16, 24]. This general repression complex has no specific DNA binding properties, but is anchored to the DNA through the interaction with gene-specific repressors, such as Rox1 [25]. Once tethered to a promoter, the complex appears to be capable of both repressing *in vitro* transcription of naked DNA and altering chromatin structure to prevent gene expression [26, 27]. In addition to the hypoxic genes, the Tup1/Ssn6 complex is involved in repression of the **a** mating type genes in yeast α haploid cells through interaction with the α2 repressor [28], the catabolite repressed genes through interaction with the Mig1 repressor [29], and a number of other systems [30–32].

A direct interaction between Rox1 and the Tup1/Ssn6 complex has proven difficult to demonstrate. However, repression by the chimeric constructs in which the repression domain of Rox1 was fused to the Gal4 DNA binding domain could not occur in an *ssn6* deletion mutant, indicating that the Tup1/Ssn6 complex functioned though the Rox1 repression domain [21]. Also, Rox1 DNA binding activity is labile over time and the addition of purified Ssn6, but not Tup1, stabilizes it, providing indirect evidence for a Rox1–Ssn6 interaction [20].

Summary

The known hypoxic functions listed in Table 1 share the common feature that they are oxygen-dependent. Thus, the regulatory scheme has evolved to maximize oxygen utilization rather than to support anaerobic growth. Nonetheless, these functions cover a variety of processes, and, as might be expected, each set of these functions is subject to other, different regulatory signals. Hypoxic repression is super-imposed upon other, diverse regulatory schemes, and the Rox1-repressed genes do not represent a regulon of coordinately expressed genes. The mobilization of the general Tup1/Ssn6 repressor complex is ideally suited

Table 3. Global response genes

Gene	Function/Protein	References
DDR2	Heat shock/DNA damage	[36, 51]
HSP104	Heat shock protein 104	[51]
HSP12	Heat shock protein 12	[52, 53]
TPS2	Trehalose phosphate phosphatase	[54]
GAC1	Putative protein phosphatase Type I regulatory subunit	[51]
ENA1	Na^+, K^+, $Li(^+)$-ATPase	[52]
GDP1	Glycerol-3-phosphate dehydrogenase	[52]
GSY2	Glycogen synthase	[55]
UB14	Polyubiquitin	[51]
CTT1	Catalase	[37]
CYC7	Iso-2-cytochrome c	[33]

to such a system, since it is capable of repressing transcription driven by a variety of different transcriptional activators.

Heme-independent regulation of the Rox3 gene

There are a number of genes that have been cloned from yeast that are induced under oxygen starvation conditions in a heme-independent fashion [12]. Little is known about the function or regulation of most of these. However, one, *ROX3*, has been characterized in some detail. Although its function is still unclear, it plays a role in the response of cells to a variety of stresses [33]. Furthermore, *ROX3* itself is regulated by hypoxia as part of a different general stress response [34].

Rox3 function and the global stress response

Rox3 is a protein of 220 amino acids with no clear homology to any known protein [34]. The protein is localized to the nucleus, but Rox1 has no demonstrable DNA binding activity [34, unpublished results]. Its function is essential; a deletion of the *ROX3* gene is lethal [34]. Only the amino terminal half of the protein is required for growth, but cells carrying such truncations have a variety of phenotypes including temperature sensitivity, sensitivity to high osmotic pressure, and an inability to grow on non-fermentable energy sources [33, 34]. These phenotypes result from a failure of *rox3* mutants to induce functions involved in the global stress response [33].

The global stress response represents the induction of a common set of genes by a variety of starvation or stress conditions including temperature shock, osmotic stress, oxidative stress, low pH, glucose or nitrogen starvation, and DNA damaging agents [reviewed in 35]. The induced genes encode functions that protect the cell against one or more of these conditions, as indicated by the partial list in Table 3. As presented schematically in Figure 3, each stress situation is signaled through a separate signal transduction system that ultimately merges to activate a transcription factor that binds to the sequence 5′CCCCT found in the upstream region of the induced genes [36, 37]. This transcription factor has been identified in yeast extracts, but the cloning of the gene(s) has yet to be reported.

In *rox3* mutants, the induction of *CYC7*, the alternate cytochrome c and a global stress response gene, is reduced, and the stress sensitive phenotypes suggest that the stress response in general is deficient [33]. This general deficiency as well as the nuclear location of Rox3 suggests that it interfaces with either the signal transduction pathways and the transcription factor or the basal transcriptional machinery and the transcription factor. As indicated above, Rox3 has no DNA binding activity to either DNA in general or the 5′CCCCT sequence specifically. It should be noted, however, that the inability to induce the stress response in itself should not be lethal under normal growth conditions, so to account for the lethality of a *rox3* deletion mutant, we assume that Rox3 serves additional functions in the cell.

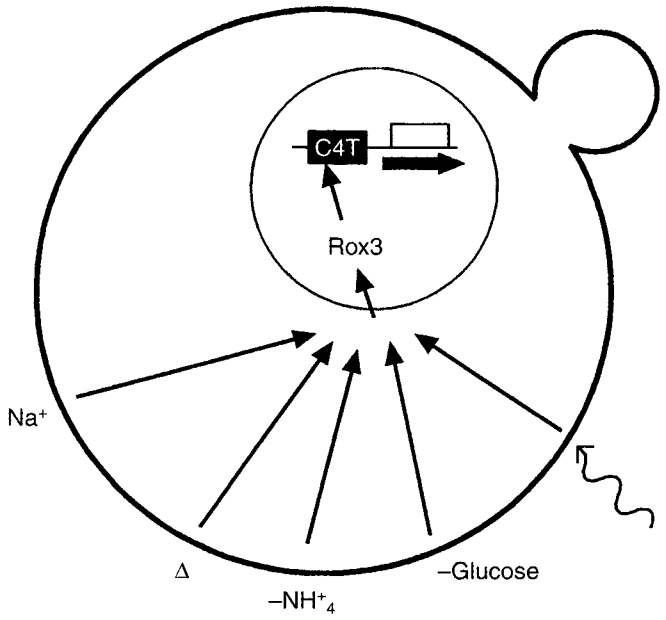

Fig. 3. *Role of Rox3 in the global stress response.* The schematic of a yeast cell indicates the variety of external stresses that can activate the global stress response (Na^+, osmotic stress; Δ, heat shock; $-NH_4$ and -glucose, the respective starvation conditions; the wavy line, DNA damage).

Regulation of ROX3 expression

The expression of the *ROX3* gene is also induced by an overlapping but non-identical set of stresses [33, 34]. *ROX3* mRNA levels increase in response to heat shock and osmotic stress, but not in response to glucose starvation. Also, *ROX3* expression is strongly induced by oxygen starvation. This induction is heme independent. The addition of heme to anaerobically grown cells does not repress *ROX3* induction as it does for the hypoxic genes described in the previous section, and expression is not derepressed in the presence of oxygen in a *rox1* mutant [34, unpublished results].

The *ROX3* upstream region does not contain the 5′CCCCT sequence required for the global stress response, and a deletion analysis of this region suggested that induction was mediated through a sequence $5'GA_{10}GGAA$ that is repeated three times [33]. When a single copy of this sequence was inserted into a heterologous gene, it caused increased expression under osmotic stress, in heat shock, and under oxygen starvation conditions. Two copies resulted in even stronger induction, but a single copy of the sequence $5'GA_9GGAA$, lacking a single A/T base pair, had no activity [33].

A number of questions remain. First, what is the function of this hypoxic or anaerobic induction of *ROX3* expression? Are there a

subset of the global stress response genes that are induced under oxygen limiting conditions? If so, do they differ in function from the hypoxic genes listed in Table 1? Second, what is the mechanism of stress induction of *ROX3*, and what is the mechanism of the heme-independent oxygen sensing? Whatever the answers are, it is clear that this system represents a completely different mechanism than the heme-dependent Rox1 repression.

Summary

The two systems described above represent different mechanisms for the yeast response to oxygen limitation. Neither appears to parallel the HIF-1 hypoxia sensing system in mammals. The Rox1 heme-dependent system differs because it involves repression by a very different kind of DNA binding protein. *ROX3* regulation differs in that it represents a more general stress response. Whether there are parallel oxygen sensing systems in yeast and humans is still an open question.

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