Regulatory Mechanisms Controlling Expression of the DAN/TIR Mannoprotein Genes During Anaerobic Remodeling of the Cell Wall in Saccharomyces cerevisiae

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ABSTRACT

The DAN/TIR genes of Saccharomyces cerevisiae encode homologous mannoproteins, some of which are essential for anaerobic growth. Expression of these genes is induced during anaerobiosis in some cases during cold shock. We show that several heme-responsive mechanisms combine to regulate DAN/TIR gene expression. The first mechanism employs two repression factors, Mox1 and Mox2, and an activation factor, Mox4 (for mannoprotein regulation by oxygen). The genes encoding these proteins were identified by selecting for recessive mutants with altered regulation of a dan1::ura3 fusion. MOX4 is identical to UPC2, encoding a binucleate zinc cluster protein controlling expression of an anaerobic sterol transport system. Mox4/Upc2 is required for expression of all the DAN/TIR genes. It appears to act through a consensus sequence termed the AR1 site, as does Mox2. The noninducible mox4Δ allele was epistatic to the constitutive mox1 and mox2 mutations, suggesting that Mox1 and Mox2 modulate activation by Mox4 in a heme-dependent fashion. Heme also regulates expression of some of the DAN/TIR genes through inhibition of expression of MOX4. Indeed, ectopic expression of MOX4 in aerobic cells resulted in partially constitutive expression of DAN1. Heme also regulates expression of some of the DAN/TIR genes through the Rox7 repressor, which also controls expression of the hypoxic gene ANBL. In addition Rox1, another heme-responsive repressor, and the global repressors Tup1 and Ssn6 are also required for full aerobic repression of these genes.

Saccharomyces cerevisiae cells adapt to anaerobic growth by inducing expression of a surprisingly large number of genes (called “anaerobic genes,” Lowry and Zitomer 1984; Zagorec and Labbe-Bois 1986; Hodg et al. 1989; Thorsness et al. 1989; Drgon et al. 1991; Turi and Loper 1992; Zitomer and Lowry 1992; Choi et al. 1996; Donzeau et al. 1996; Evangelista et al. 1996; Sertil et al. 1997; Kwa et al. 1999; ter Linde et al. 1999). Expression of two groups of anaerobic genes is repressed by heme (Lowry and Lieber 1986; Zagorec and Labbe-Bois 1986; Sertil et al. 1997), which is synthesized only in aerobic cells; the “hypoxic genes,” which encode intracellular factors involved in optimizing oxygen utilization; and the DAN/TIR genes, which encode a group of eight cell wall mannoproteins (Donzeau et al. 1996; Sertil et al. 1997). Some of the TIR genes are also induced during cold shock (Kondo and Isouye 1991; Kowalski et al. 1995; Donzeau et al. 1996). Expression of the two heme-repressed regulons is controlled by different mechanisms. The hypoxic genes are regulated by the Rox1 repressor, which is assisted by the ubiquitous Tup1/ Ssn6 repression complex; the mechanism controlling expression of the DAN/TIR genes is described here and includes regulon-specific activation and repression factors.

We find that anaerobic expression of the DAN/TIR genes depends on a common transcriptional activator, Mox4 (for mannoprotein regulation by oxygen). We have found that MOX4 is identical to UPC2 (Crowley et al. 1998), which encodes a binucleate zinc cluster protein involved in the regulation of an anaerobically induced sterol transport system (Lewis et al. 1988). Expression of MOX4 is induced under anaerobic conditions and repressed under aerobic conditions by heme. Mox4 also responds to heme at the functional level, in concert with two repression factors, Mox1 and Mox2. We find that these three factors act through a consensus anaerobic response element (AR1) found at least once in each of the DAN/TIR promoters (Cohen et al. 2001), presumably forming a heme-sensitive regulatory complex. The stringency of regulation of many of the anaerobic genes is striking: repression of DAN1 and other DAN/TIR genes results from the combined action of at

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least six repression factors — Mox1, Mox2, and a newly identified factor, Ros7, along with Ros1, Tup1, and Sn6. Ros7, which, as we report elsewhere, is identical to the Mot3 transcription factor (O. Sertl, R. Kapoor, B. D. Cohen and C. V. Lowry, unpublished results; Grishin et al. 1998; Madison et al. 1998), also represses the hypoxic gene ANB1.

**MATERIALS AND METHODS**

**Plasmids and libraries:** YCpD/U was constructed by amplifying the URA3 gene using the primers AGACGGATCCCTCTGAAAGGCTAGTAAAGGACG and GAGAGAATCTAGGATCCATCTGCACTGACG, which contain 5’ BamHI and SpeI sites, respectively. The PCR product was digested with BamHI and SpeI and ligated to the same sites in YCpD/Z(22) (Cohen et al. 2001), replacing the lacZ open reading frame (ORF) with the URA3 ORF, in frame with the first five codons of DANI.

YpD/Z: An integrating plasmid carrying the dan1::lacZ fusion was derived from YpD/Z(22) by excising the fusion fragment with EcoRI and HindIII and ligating to the EcoRI and HindIII sites of YPlac128 (Gietz and Sugino 1988).

A plasmid library containing 5- to 10-kb fragments of the yeast genome carried on a centromeric TRP1 vector was constructed in the pCL6 vector, which was derived from the plasmid YCpGYC3(2.4) (Lowry et al. 1983) by digestion with Smal and MluI, end-filling with Klenow polymerase, and religating the backbone with a Sall linker. For the library a partial Sall digest of DNA isolated from strain D13-1a was ligated to the Sall site of the pCL6 vector. The Sall ends were filled in with dCTP and dTTP and the SallA ends were filled in with dGTP and dATP before ligation to make them cohesive.

Plasmids (AR1), MELI, AR2, MELI, and D6-BST are described elsewhere (Cohen et al. 2001).

MOX4/UPC2 plasmids: pCL6(MOX4), cloned from the plasmid library, contained YDR213w (MOX4) and YCpD/Z(22); the cloned segment included only part of the MOX4 promoter (up to –259). The region from –259 to +2835 was excised with XbaI and NotI and ligated to the Sall and XbaI sites in the polylinker of pUC19 (H.S.) to generate pUC(MOX4); in this derivative of pUC19 the HindIII and Sall sites had been destroyed by end-filling. The same NotI-XbaI fragment was also cloned into the same sites in YCpLac22 to generate YCpMOX4Δ1p. To clone the full promoter region a PCR fragment generated with primers homologous at 865 (CAGAGGATCCATCTGCACTGACG) and at +174 (CTTCTGATCCCTTCTCTTTAGTATCGG) was digested with Pmll (site at –694) and BamHI (site in primer) and ligated to YCpLac22, which had been digested with EcoRI, end-filled, and digested with BamHI; the resulting plasmid, YCpMOX4Δ1p, contains the region from –694 to +174. The segment of MOX4 from +112 to +2835 was excised with AgeI and XbaI and ligated to the same sites in YCpMOX4Δ1p, generating the centromeric expression plasmid YCpMOX4. To construct YCpMOX4::lacZ, a promoter fragment was amplified from genomic DNA with the –865 primer and another primer homologous at +3 (AGACCAGATCCATCTGCACTGACG) containing an AgeI site, GAGACCGGTAGAAAAATAGCTTG

GAAAAAC. A 3’ promoter fragment was amplified using a primer homologous at –349 containing an AgeI site, GAGACCGGTCTTGACCACTTACCGGAAGTG/ (homologous at –349), and the +3 primer. The following fragments were ligated together: the 5’ fragment, digested with EcoRI and AgeI; the 3’ fragment, digested with AgeI and BamHI; and YCpMOX4::lacZ, digested with EcoRI and BamHI. In the resulting construct the sequence beginning at –359 GTAAAG GACG (containing an inverted ARI consensus sequence) is replaced by ACCGGTCTTGTACCACTTACCGGAAGTG. To generate the G888D (upe2-I) (Crowley et al. 1998) and G888A alleles of MOX4 for expression, pUC MOX4 was digested with NdeI and HindIII and ligated to a synthetic segment with the same sequence as the excised segment except for a single nucleotide (nt) substitution at the same position on both strands (+ strand, GAGAATCTCGGAGGTTGCTGATGCA; – strand, TATG CATGCAACCTTGCGGAGTATTTCGTC). The G → A substitution in the + strand (nt +2663) results in the G888D substitution and the C → G substitution in the – strand (nt +2663) results in a G888A substitution. Both alleles were recovered in segregating plasmids isolated from bacterial transformants; the mutant fragments were then excised with AgeI and XbaI and ligated to the same sites in YCpMOX4 to generate YCpMOX4 (upe2-1) and YCpMOX4(G888A).

To construct YCpGAL1::mox4, the MOX4 ORF (–17 to +2765) was excised from pCL6(MOX4) with SpeI and inserted into the end-filled Acc65I and BamHI sites of YCpGAL1/OLE1 (a fusion of the GAL1 promoter to the OLE1 ORF) (S. Mehta, N. E. Abramova, R. D. Anand and C. V. Lowry, unpublished data), placing the MOX4 ORF between –9 of GAL1 and +1379 of OLE1.

**Gene disruptions:** ptpuΔ::URA3: The TUP1 gene was amplified by PCR using primers TCTCGAGATCAAGAAATATATGAAACCAGGAAAGG and TCGGTCGACTAGGAAACCGGACG GTAG, digested with SpeI and SalI, and inserted into the same sites in the plasmid pBluescriptSK+– (S.stragene, La Jolla, CA), generating PBS-tpuD. The TUP1 disruption plasmid was created by inserting the URA3 gene as a BglII-Smal fragment from pNK51 (Alani et al. 1987) into the TUP1 gene at nucleotide positions –145 and +753 in plasmid PBS-tpuD digested with BamHI and Pmll. For the disruption a XbaI-SalI fragment was excised from the resulting plasmid and used for transformation.

**moxΔ::ura3:** To generate a MOX4 disruption construct, the region between the BglII site of MOX4 (+2254) and the Muri site in the TRP1 gene was deleted from YCpMOX4 by digestion with those enzymes and end-filling the BglII site, leaving a 1.6-kb 3’ fragment of MOX4 joined to the vector backbone; this was ligated to the URA3 gene, which had been excised from the plasmid PBS-URA3 as a Musl-Smal fragment, generating pURA-MOX4-3’. pBS-URA3 contains the URA3 gene (–959 to +2772) inserted into the EcoRI and SalI sites of pBS-SK as an EcoRI-SalI-digested PCR fragment generated with primers containing those sites. The region between –460 and +1 of MOX4 was amplified by PCR using primers homologous at those points containing AatII and MunI sites, respectively, digested with those enzymes, and ligated to the same sites in pURAMOX-3’. The resulting construct contained the URA3 gene flanked by 5’ and 3’ segments of MOX4, and this segment was excised with AatII and XbaI for transformation.

**Cell growth and analysis of gene expression:** For β-galactosidase assays (Zitomer et al. 1987) cells were grown to stationary phase overnight in SD medium lacking the appropriate nutrient to maintain selection of the reporter plasmid. Overnight cultures were diluted 1:25 and grown for 2 hr before shifting to anaerobic growth (by bubbling the culture with nitrogen) where indicated or continuing in aerobic growth for 7 hr. For α-galactosidase assays, which were more reproducible than β-galactosidase assays, one colony was picked and grown overnight in SD medium lacking the appropriate nutrient to maintain selection of the reporter plasmid. Overnight cultures were diluted 1:25 and grown for 2 hr before shifting to anaerobic growth (by bubbling the culture with nitrogen) where indicated or continuing in aerobic growth for 7 hr.
Expression of MOX4 under the control of the GALI promoter cells were grown overnight in SD-ura medium, diluted 1:40 into fresh medium, grown for 3 hr in SD-ura, washed twice with SD-ura-raffinose-galactose (1% raffinose, 1% galactose), and grown for 4 hr in that medium before harvesting for RNA extraction.

**Strains and mutant isolation:** Yeast strains used were FY23 (Winston et al. 1995), the nearly isogenic strain FY24, and RZ53 (Lowry and Zitomer 1988). For mutant isolation, FY24 cells were transformed with YlpD/Z (128) and YcpD/U (22) and mutagenized with EMS as described (Rose et al. 1990). Selections for both noninducible and constitutive mutants depended on altered expression of the dan1::ura3 fusion carried on YcpD/U (22). Noninducible mutants were selected in an anaerobic brewer’s jar on SD-trp plates supplemented with 10 μg/ml ergosterol, 0.5% Tween 80, and fluoroorotic acid (FOA; 50 μg/ml); FOA-resistant mutants selected for loss of anaerobic expression of the dan1::ura3 fusion were rescreened for loss of anaerobic expression of the integrated dan1::lacZ fusion (carried on YlpD/Z (128)). Three recessive noninducible isolates were subjected to complementation testing, with two found to be in the moxl group (one other isolate, designated mox5, showed no effect on transcription). For complementation testing, mutant isolates were converted to MATα using the integrating MA7a vector BJc334, courtesy of Joan Curcio (Wadsworth Center, Albany, NY). Diploids resulting from crosses of the MA7a derivatives with the original isolates were tested for heterozygosity by assay of β-galactosidase activity, including β-galactosidase in aerobic cultures (heterozygous diploids were tested in anaerobic culture). Heterozygotes were backcrossed to FY23 carrying the integrated dan1::lacZ fusion (carried in YlpD/Z). Constitutive mutants were screened for constitutive expression of the integrated lacZ fusion (carried in YlpD/Z). Constitutive mutants were screened for constitutive expression of the integrated lacZ fusion (carried in YlpD/Z). Constitutive mutants were rescreened for defective heme production by testing for restoration of repression of the lacZ fusion by addition of heme (25 μg/ml) to aerobic cultures. Complementation between candidate isolates and MA7a derivatives was tested in diploids (heterozygous were β-gal/− in aerobic culture; homozygotes were β-gal+/+). Mutant strains were backcrossed to FY23 carrying the integrated dan1::lacZ fusion and sporulated. Analysis of noninducible or constitutive expression in tetradss indicated that the moxl-1, mox2-2, mox2-1, mox4-1 alleles all segregated 2:2 from the wild-type alleles.

**Cloning of MOX4:** The MOX4 gene was cloned by complementation of the moxl mutation with a plasmid library. For selection a construct containing a fusion of the DAN1 promoter to the HIS3 gene was integrated into the moxl-1 strain on YlpDAN1::his3. Cells of this strain were transformed with the pCL6 library and selected for growth on -trp plates. Transformants were pooled and replated on SD-his plates containing Tween 80 and ergosterol and grown under anaerobic conditions. Plasmids recovered from his+ colonies were sequenced and found to contain ORFs YRD213W and YRD214W. The segment containing YRD213W was subcloned into YCplac22, generating YCpMOX4Δp, and was found to complement the moxl mutation.

**RESULTS**

A common activation factor defines the DAN/TIR regulon: Expression of DAN1 is inhibited by heme, which is synthesized only in aerobic cells (Sertil et al. 1997); therefore DAN1 mRNA is detectable only in cells grown under anaerobic conditions. To identify factors involved in the heme-regulated activation of transcription, we sought noninducible mutants with reduced anaerobic expression of DAN1. From a pool of mutagenized ura3 cells carrying a dan1::ura3 fusion plasmid we selected for uracil auxotrophy (resistance to FOA) under anaerobic conditions (see MATERIALS AND METHODS). Two mutants exhibited reduced anaerobic expression of a dan1::lacZ fusion. Both mutations segregated 2:2 from diploid backcrosses to FY23 and fell into one complementation group, designated moxl. Mox4 cells grown under anaerobic conditions contained drastically reduced levels of DAN1 mRNA and of mRNAs of seven other mammoprotein genes normally induced under anaerobic conditions (Figure 1). Thus Mox4 protein is an essential activation factor in expression of the DAN/TIR genes. Mox4 does not appear to have any role in induction of two genes in the hypoxic regulon, ANB1 and HEM13.

Mox4 is Upc2, a zinc cluster transcriptional activator: We cloned MOX4 from a genomic DNA library by complementation of the noninducible moxl phenotype (see MATERIALS AND METHODS), using a dan1::his3 fusion to select for restoration of DAN1 expression. The complementing gene was localized to UPC2 (YDR213w), which encodes a factor regulating expression of a system that transports sterols during anaerobic growth (Crowley et al. 1998). Mox4/Upc2 is a zinc cluster protein homologous to several transcription factors in S. cerevisiae, including Gal4 and Lys14, and to a remarkable degree, Ecm22 (Lussier et al. 1997).

We confirmed the role of Mox4/Upc2 as a transcriptional activator of the DAN/TIR regulon by testing the phenotype of cells carrying a moxl4 allele; loss of Mox4 caused drastically reduced anaerobic expression of the dan1::lacZ fusion as well as of DAN1 mRNA (Figure 2) and several other DAN/TIR mRNAs.

**Identification of repression factors affecting aerobic expression of the DAN/TIR genes:** To identify repressors of anaerobic genes, we sought mutants constitutive for expression of DAN1. Such mutations were expected to cause defective aerobic repression of the dan1::ura3 fusion, permitting growth on SD-ura plates. Using this selection we obtained Ura+ isolates, which also showed elevated aerobic expression of the dan1::lacZ fusion, as well as increased levels of DAN1 mRNA in aerobic cells (Figure 3a). As expected, this group included heme-deficient mutants (see MATERIALS AND METHODS) and flocculent tup1 and ssn6 isolates, which were disregarded. Among the remaining isolates we identified three complementation groups, designated moxl, mox2, and rox7. All were recessive, apparently affecting repression factors, and all three mutations segregated 2:2 in backcrosses to wild type. Identification of Mox1, Mox2, and Rox7 confirmed that there are negative controls
Figure 1.—Expression of anaerobic genes in a mox4 mutant strain. Cells of strain FY24 mox4 (lane 3) and FY24 (lane 2) were grown in YPD under anaerobic conditions for 4.5 hr. A control culture of FY24 was also grown under aerobic conditions (lane 1). Probes generated by random-primer labeling of fragments of the genes indicated were used to probe a Northern blot of RNA derived from these cultures. TIR1 mRNA runs as a double band (arrows), as confirmed by a Northern blot of RNA from cells of FY23 tir1Δ, which showed a band of reduced intensity, corresponding to TIR2 mRNA. TIR2 mRNA (arrow) hybridizes to the TIR1 probe with low efficiency and runs at the same position as the lower TIR1 band, so the result for TIR2 is ambiguous in anaerobic cells. ANB1 (arrow) and TIR51 mRNA both hybridize to the ANB1 probe.

Figure 2.—Effect of deletion of MOX4 on expression of DAN/TIR genes. Cells of strains FY23 and FY23 mox4 were grown under aerobic and anaerobic conditions. Northern blots were probed with DAN1, DAN4, and TIR4.

that counteract activation by Mox4. The mox1 and mox2 mutations caused increased aerobic expression of DAN1 and several other dan/tir genes, but not of hypoxic genes. In contrast rox7 mutations caused partially constitutive expression of some, but not all, members of both regulons (Figure 3a). The effect of the mox1 and mox2 mutations was much more pronounced on some genes in the regulon than on others (e.g., DAN4 vs. DAN1), suggesting that for some of these genes redundant repression mechanisms might be at work.

**Mox factors act through the AR1 activation sequence:** We report elsewhere that all of the DAN/TIR genes contain at least one AR1 sequence (for “anaerobic response,” TGGTTYAG) and that this site is sufficient to drive expression when fused to a reporter, e.g., in the Figure 1 construct (COHEN et al. 2001). Since all of the DAN/TIR genes are also activated by Mox4, we sought to determine whether it functions at AR1 sites. We compared expression of the (AR1)3/MEL1 construct in a mox4 mutant with expression in the parental strain and found that activation through the AR1 depends on Mox4 (Figure 4a), suggesting that Mox4 binds to the AR1 site. We had also identified a second anaerobic response element in the DAN1, DAN2, and DAN3 promoters, AR2 (COHEN et al. 2001). As shown, expression from AR2/MEL1 was not affected by the mox4 mutation, confirming the specificity of Mox4 for AR1.

Since mox1 and mox2 mutations caused constitutive expression of several DAN/TIR genes, but not of the two hypoxic genes (Figure 3A), it seemed that Mox1 and Mox2 might only regulate genes with AR1 sites. To test this we compared expression from the (AR1)3/
that causes a dominant sterol transport proficiency in aerobic cells (Crowley et al. 1998), possibly by causing constitutive expression of transport proteins normally not expressed during aerobic growth. Thus the gly → asp mutation appeared to pinpoint a regulatory region in Mox4/Upc2, presumably a domain that interacts with an inhibitory ligand or repression factor. To see if this domain participates in repression of the DAN/TIR genes we tested the effect of the G888D substitution and another more conservative one, G888A, on regulation of DAN1, DAN4, and TIR4 expression; both mutations caused increased aerobic expression of DAN1 (Figure 5). As had been observed for the UPC2-1 allele (Crowley et al. 1998), these mutations were dominant when introduced into cells carrying the wild-type MOX4 allele, causing partially constitutive expression. The dominant constitutive phenotype implies the presence of a repression domain in Mox4, possibly one analogous to the Gal80-binding domain in Gal4 (defined by dominant GAL4c mutations). When we analyzed the effect of the G888D mutation on the levels of DAN1 and other DAN/TIR genes we also observed loss of repression, ranging from partial to full (Figure 5). Interestingly, the varying levels of constitutive expression closely paralleled those seen for the same mRNAs in the mox1 and mox2 mutants (Figure 3a); this correlation is consistent with a functional link between Mox1, Mox2, and the regulatory domain on Mox4, although another factor might be the true ligand.

**Regulation of MOX4 expression:** We analyzed expression of the MOX4 gene in FY23 cells to determine whether expression of the DAN/TIR genes is controlled by modulation of Mox4 levels. MOX4 mRNA was 5–10 times more abundant in anaerobic cells than in aerobic cells and also in cells subjected to cold shock (Figure 6a), suggesting that induction of expression of the activator contributes to induction of the DAN/TIR genes under these conditions (see discussion). Expression was partially inhibited by addition of heme to an anaerobic culture of RZ53 cells, indicating that heme is a regulatory co-effector signaling the presence of oxygen, although there may be another signal involved as well (Figure 6b). (RZ53 cells were used in this experiment because they were more sensitive to exogenous heme than FY23 cells.) Expression from a mox4::lacZ reporter fusion was also induced to ∼10 times higher levels in anaerobic cells, indicating that the increase in MOX4 mRNA levels results from transcriptional activation (Table 2). The MOX4 promoter contains an AR1 site, which is positively activated anaerobic gene expression indirectly, by blocking expression or function of Mox1 and Mox2. The fact that a mox4 deletion is epistatic to mox1 and mox2 mutations (Table 1) rules out the latter possibility.

**Evidence for a regulatory domain in Mox4/Upc2:** The UPC2-1 (MOX4-1) allele is a G888D substitution in MEL1 plasmid in aerobically grown mox2 cells with that in wild-type cells. Expression was well above that in the control cells (Figure 4b), indicating that Mox2 is needed for repression of expression driven from the AR1 sites. At the same time the mox2 mutation caused no increase in aerobic expression from the AR2 reporter plasmid, showing that Mox2 repression is specific for activation from the AR1 site.

**Mox4 acts downstream from Mox1 and Mox2:** Mox4 could be a transcriptional activator of DAN1, or it could activate anaerobic gene expression indirectly, by blocking expression or function of Mox1 and Mox2. The fact that a mox4 deletion is epistatic to mox1 and mox2 mutations (Table 1) rules out the latter possibility.

**Evidence for a regulatory domain in Mox4/Upc2:** The UPC2-1 (MOX4-1) allele is a G888D substitution
els of the activator are sufficient to partially overcome heme inhibition in aerobic cells.

**Rox7 regulates genes from both the hypoxic and DAN/TIR regulons:** Since the hypoxic and DAN/TIR regulons are controlled by distinct mechanisms it was interesting that *rox7* mutations cause substantial constitutive expression of genes from both groups (Figure 3a). Rox1 was thought earlier to be solely responsible for repression of *ANB1*, assisted by the Tup1/Ssn6 corepressor complex. The isolation of several *rox7* mutants with the same phenotype made it unlikely that the constitutive effects on both *DAN1* and *ANB1* expression resulted from mutations affecting more than one locus. To be certain we crossed the *rox7* mutant with the nearly isogenic FY23 and scored segregants in 10 tetrads for constitutive expression of the two genes; each haploid isolate was constitutive for both *ANB1* and *DAN1* (data not shown). As reported elsewhere (O. Sertil, R. Kapoor, B. D. Cohen and C. V. Lowry, unpublished results) we have found that *ROX7* is identical to *MOT3* (Grishin et al. 1998; Madison et al. 1998); it encodes a zinc finger transcription factor that modulates expression of a diverse set of genes. Interestingly, expression of *ROX7/MOT3* is induced by heme under aerobic conditions and repressed by Hap1 under anaerobic conditions, in parallel with *ROX1* (Lowry and Zitomer 1988; Keng 1992; Deckert et al. 1995). In addition, we found that a segment containing high-affinity Mot3 sites is needed for efficient aerobic repression of *DAN1* (Cohen et al. 2001).

**Combinatorial repression of individual DAN/TIR genes by at least six repression factors:** Several yeast genes are stringently controlled by a single repression mechanism, which in many cases is assisted by the Tup1/Ssn6 general repression complex (Keleher et al. 1992). Our identification of three specific factors involved in repression of *DAN1* implied that more than one mechanism is involved in controlling genes of the DAN/TIR regulon. We analyzed the expression of the eight DAN/TIR genes in cells carrying *mox1*, *mox2*, and *rox7* mutants by AR1 and AR2 sites. Cells of strains FY24, FY24 *mox2-1*, and FY24 *mox4-1* were transformed with (AR1)3/*MEL1*, AR2/*MEL1* (a fusion of a 26-bp fragment containing the AR2 site to the *MEL1* fusion), or D0-BST (the promoterless *MEL1* reporter). They were grown as indicated and harvested for α-galactosidase assay. Levels of expression from the D0 vector alone were subtracted from the levels of expression from the AR1 and AR2 site plasmids under the same conditions in each case. (A) FY24 and FY24 *mox4-1* cells transformed with the three plasmids were grown under anaerobic conditions for 8 hr and harvested for α-galactosidase assay. For each strain the difference between expression levels obtained for plasmids containing oligonucleotide inserts [(AR1)3/*MEL1* and AR2/*MEL1*] and levels for the vector plasmid D0-BST were calculated: [(AR1)3/*MEL1* – D0-BST] and [AR2/*MEL1* – D0-BST]. The bar graph shows the average difference in the *mox4* strain for the AR1 and AR2 site plasmids normalized to the corresponding difference in the wild-type strain (set at 100%). Results are shown for four experiments. (B) FY24 and FY24 *mox2-1* cells transformed with plasmids (AR1)3/*MEL1*, AR2/*MEL1*, or D0-BST were grown under aerobic conditions to late log phase and harvested for α-galactosidase assay. The values shown represent the differences [(AR1)3/*MEL1* – D0-BST] and [AR2/*MEL1* – D0-BST] in the *mox2* strain and in the wild-type strain normalized to the corresponding differences for the wild-type strain grown under anaerobic conditions (set at 100%). Two sets of samples showed slightly negative values for the difference between experimental and control values, but these were not significantly different from zero. Results are shown for four experiments.
TABLE 1

Epistasis of mox4Δ allele

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>β-Galactosidase activity</th>
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<tbody>
<tr>
<td>FY24</td>
<td>wt</td>
<td>1.13 ± 0.04</td>
</tr>
<tr>
<td>BC101</td>
<td>mox1-1</td>
<td>5.41 ± 0.14</td>
</tr>
<tr>
<td>BC202</td>
<td>mox2-2</td>
<td>3.6 ± 0.12</td>
</tr>
<tr>
<td>NA400</td>
<td>mox4Δ</td>
<td>0.012 ± 0.001</td>
</tr>
<tr>
<td>NA401</td>
<td>mox1 mox4Δ</td>
<td>0.015 ± 0.004</td>
</tr>
<tr>
<td>NA402</td>
<td>mox2 mox4Δ</td>
<td>0.022 ± 0.002</td>
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Cells of strain FY24 and various mutant derivatives, all carrying an integrated dan1::lacZ fusion (Yipdan1::lacZ), were grown under aerobic conditions and harvested for β-galactosidase assay.

...and also examined the effect of tup1Δ, ssn6Δ, and rox1Δ mutations. Each of these mutations caused defects in aerobic repression of some or all of the DAN/TIR genes (Figure 3). This indicated that each of these repression factors involved, Mox1, Mox2, Rox7, Rox1, Tup1, and Ssn6, act in combinatorial fashion to maintain aerobic repression. However, the relative importance of each repression factor differed from gene to gene:

1. The mox1 and mox2 mutations caused almost fully constitutive expression of TIR1, TIR3, TIR4, and DAN4; a slight effect on DAN1 and DAN3; and no detectable effect on DAN2. The effects of the two mutations varied in parallel, suggesting that Mox1 and Mox2 act together.
2. The rox7 mutation caused varying degrees of constitutive expression of DAN1, DAN4, TIR1, TIR3, and TIR4, as well as constitutive expression of ANB1, but had no effect on expression of DAN2 or DAN3.
3. The effects of tup1 and ssn6 mutations also varied greatly both from gene to gene and relative to each other; notably DAN3, TIR2, and TIR4 showed a particularly strong dependence on Tup1 for repression.
4. Finally, the rox1 deletion caused a mild loss of repression of some of the dan/tir genes, contrasting with fully constitutive expression of the hypoxic genes, which are defined by their response to Rox1 (Lowry et al. 1990).

DISCUSSION

We have identified a set of factors that regulate and define the DAN/TIR regulon. Mox4 is an essential activator of this regulon, and Mox1 and Mox2 are repression factors. An epistasis test shows that Mox4 functions downstream of Mox1 and Mox2. Mox4, a zinc cluster protein homologous to known transcriptional activators, presumably activates transcription, subject to inhibition by Mox1, Mox2, and possibly other factors we haven’t identified. A possible mechanism of regulation is suggested by mutations in a C-terminal domain of Mox4, which could cause constitutive activation of the DAN/TIR genes. (A) FY23 mox4Δ cells carrying plasmid YCpmox4 or derivatives were grown aerobically or anaerobically in SD-trp medium and harvested for RNA analysis. Northern blots were probed with DAN1, DAN4, and TIR4. (B) FY23 mox4Δ cells carrying an integrated dan1::lacZ fusion were transformed with YCpmox4, YCpmox4(G888D), or YCpmox4(G888A), grown under aerobic conditions to late log phase in SD-trp medium, and harvested for β-galactosidase assay.
TABLE 2

<table>
<thead>
<tr>
<th>Plasmid fusion</th>
<th>β-Galactosidase activity in aerobic cells</th>
<th>β-Galactosidase activity in anaerobic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>mox4::lacZ</td>
<td>0.88 ± 0.03</td>
<td>11.2 ± 2.3*</td>
</tr>
<tr>
<td>mox4ΔAR1::lacZ</td>
<td>1.73 ± 0.09</td>
<td>3.99 ± 0.81</td>
</tr>
</tbody>
</table>

Cells transformed with YCp-mox4::lacZ or YCp-mox4ΔAR1::lacZ were grown under anaerobic and aerobic conditions and harvested for β-galactosidase assay.

*Shown here is the average of two independent experiments. Anaerobic levels of lacZ vary from one experiment to another due to variations in lag time before enzyme synthesis. Values of β-galactosidase activity in individual experiments showed lower standard deviations, being 13.21 ± 1.14 and 9.26 ± 0.11.

Figure 6.—Regulation of MOX4 expression. (A) FY23 cells were grown under aerobic or anaerobic conditions or under cold shock conditions (90 min at 13°C). A Northern blot was probed with MOX4. (B) RZ53 cells were grown under aerobic conditions or under anaerobic conditions with or without addition of heme (25 μg/ml). Northern blots were probed with MOX4 and ROX1. (C) FY23 cells transformed with YCp-gall1::mox4 or the vector YCpplac33 were grown under aerobic conditions in SC-ura-raffinose-galactose. Northern blots were probed with DAN1 and MOX4.

MOX4 genes, as well as of components of a sterol transport system (Crowley et al. 1998). Mox 4 is highly homologous to Ecm22, which may also regulate factors involved in cell wall synthesis, since ecm22 mutants are sensitive to cell wall-specific drugs (Lussier et al. 1997).

We obtained circumstantial evidence for a link between Mox2 and Mox4 by showing that mox2 and mox4 mutations affect expression of a MEL1 reporter driven by synthetic AR1 sites, but not of an AR2 site reporter or of anaerobic genes that do not contain an AR1 [ANB1, HEM13 (Figure 1), and OLE1 (not shown)]. The Mox2 and Mox4 regulators may function independently, even though they operate through the same segment. For example Mox1 and Mox2 could be repressors that bind to AR1 and prevent binding by Mox4. However, the presence of an inhibitory domain in the activator suggests that Mox2 (and presumably Mox1) interacts directly with AR1, via an interaction with Mox4. The simplest model for transcriptional regulation through the AR1 site is probably one in which an activator and a repressor (presumably Mox4, Mox1, and Mox2, or factors dependent upon them) form a heme-sensitive regulatory complex analogous to the galactose-sensitive Gal4:Gal80 heterodimer. Another way in which oxygen regulates through the Mox system is through regulation of MOX4 expression; this includes a modest effect of heme. Whether the induction of MOX4 plays a significant regulatory role during hypoxic and cold shock induction is not clear, since most of the DAN/TIR genes are induced simultaneously with MOX4 rather than after a lag; it may be that induction of MOX4 is a more important factor in the delayed expression of DAN2 and DAN3.

The Mox factors are part of a complex regulatory mechanism. The DAN1 promoter is a mosaic of positive and negative regulatory elements (Cohen et al. 2001) targeted in combinatorial fashion by various complexes that activate transcription during anaerobiosis and repress it during aerobic growth. Hence distinct complexes forming at the AR1 and the AR2 sites each participate in transcriptional activation of DAN1, presenting an unusual situation in which two different mechanisms responding to the same signal activate the same promoter. Repression is also combinatorial, achieved by an ensemble of mechanisms: Mox1, Mox2, Mot3, Rox1, and Tup1/Ssn6. The fact that each of the repressor mutations causes a loss of oxygen repression indicates that each repression factor mediates inhibition by heme. The pattern of effects caused by six repressor mutations on the expression of eight genes is more variable than might be expected in response to a common signal. Possibly the full set of repressors act together on some of the DAN/TIR genes, but only a subset of them regulate others, e.g., DAN2. Also, for some genes repression may be redundant; i.e., the whole group of repressors may
be so efficient that loss of only one of them has little effect.

The fact that there are several independent mechanisms mediating regulation by heme may represent convergent evolution. From this viewpoint, at each step in the evolution of a complex promoter like that of \( \text{DAN1} \), binding sites for one repressor or another appeared randomly, incrementally moving the gene toward optimal regulation; a similar process would have recruited the activation mechanisms acting through the \( \text{AR1} \) and \( \text{AR2} \) sites. However, it is possible that these regulatory mechanisms are not truly equivalent. For example, the aerobic repression mechanisms that control \( \text{TIR1} \) and \( \text{TIR4} \) give way when these genes are induced by cold shock (in aerobic cultures), and they may have been engineered to accommodate responses to stresses other than hypoxia.

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**LITERATURE CITED**


Evangelista, C. C., Jr., A. M. Rodriguez Torres, M. P. Limbach and R. S. Zitomer, 1996 \( \text{RoX3} \) and \( \text{Ris1} \) function in the global stress response pathway in baker’s yeast. Genetics 142: 1083–1093.


Keng, T., 1992 \( \text{HAP1} \) and \( \text{ROX1} \) form a regulatory pathway in the repression of \( \text{HEM3} \) transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 12: 2616–2623.

Kondo, K., and M. Inouye, 1991 \( \text{TIP1} \), a cold shock inducible gene of *Saccharomyces cerevisiae*. J. Biol. Chem. 266: 17537–17544.


Lowry, C. V., and R. S. Zitomer, 1988 \( \text{ROX1} \) encodes a heme-induced repression factor regulating \( \text{ANB1} \) and \( \text{CYC7} \) of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 8: 4651–4658.


Sethi, O., B. D. Cohen, R. J. D. Davies and C. V. Lowry, 1997 The \( \text{DAN1} \) gene of *S. cerevisiae* is regulated in parallel with the hypoxic genes, but by a different mechanism. Gene 192: 190–205.


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