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Synergistic repression of anaerobic genes by Mot3 and Rox1 in *Saccharomyces cerevisiae*

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ABSTRACT

Two groups of anaerobic genes (genes induced in anaerobic cells and repressed in aerobic cells) are negatively regulated by heme, a metabolite present only in aerobic cells. Members of both groups, the hypoxic genes and the *DAN/TIR/ERG* genes, are jointly repressed under aerobic conditions by two factors. One is Rox1, an HMG protein, and the second, originally designated Rox7, is shown here to be Mot3, a global C2H2 zinc finger regulator. Repression of anaerobic genes results from co-induction of Mot3 and Rox1 in aerobic cells. Repressor synthesis is triggered by heme, which de-represses a mechanism controlling expression of both *MOT3* and *ROX1* in anaerobic cells; it includes Hap1, Tup1, Ssn6 and a fourth unidentified factor. The constitutive expression of various anaerobic genes in aerobic *rox1*Δ or *mot3*Δ cells directly implies that neither factor can repress by itself at endogenous levels and that stringent aerobic repression results from the concerted action of both. Mot3 and Rox1 are not essential components of a single complex, since each can repress independently in the absence of the other, when artificially induced at high levels. Moreover, the two repression mechanisms appear to be distinct: as shown here repression of *ANB1* by Rox1 alone requires Tup1–Ssn6, whereas repression by Mot3 does not. Though artificially high levels of either factor can repress well, the absolute efficiency observed in normal cells when both are present—at much lower levels—demonstrates a novel inhibitory synergy. Evidently, expression levels for the two mutually dependent repressors are calibrated to permit a range of variation in basal aerobic expression at different promoters with differing operator site combinations.

INTRODUCTION

Yeast adapt to the absence of oxygen by expressing several groups of genes. Two of the anaerobic regulons are negatively regulated by heme, which is produced only in the presence of oxygen. The members of one heme-repressed gene group are referred to as 'hypoxic genes' (1,2) and encode intracellular proteins dedicated to more efficient utilization of oxygen. They are all regulated by the Rox1 repressor (3,4), which is induced during aerobic growth by heme(3), resulting in 'aerobic repression' of the regulon. In the absence of oxygen, expression of Rox1 ceases, partly through inhibition by Hap1 (5), and Rox1 is degraded (6), de-repressing transcription. The widely varying efficiency of Rox1 repression of various hypoxic genes depends on the number and fidelity of operator sites (1).

The second group of heme-repressed genes are the *DAN/TIR/ERG* genes, which are transcriptionally activated through a common response element (7) by Mox4/Upc2, a binucleate zinc cluster protein (8,9). Eight of these genes encode the Dan/Tir cell wall proteins (10–12), three of which are essential for anaerobic growth (12); others are involved in sterol synthesis (13) or transport (8). Regulators controlling expression of this group include the oxygen-inhibited Mox4/Upc2 activator, and two repressors bound at neighboring sites responsible for aerobic repression (13). One of these is Rox1 (9). The second, originally designated Rox7, was identified as a repressor of *DAN1* and some of the other *DAN/TIR* genes, as well as of the hypoxic gene *ANB1* (9). As we show here, Rox7 is Mot3. It was previously identified as a zinc finger regulator which functions either as a repressor or activator of a diverse group of genes (14–16), acting through a consensus site (14,15) shown to be important for aerobic repression of *ANB1* (17). Through its versatility as an activator and repressor, Mot3 participates in a cell wall remodeling process (12) in which anaerobic and aerobic cell wall proteins [the CWP proteins (18)] are alternately expressed in response to oxygen.

Anaerobic genes encode proteins serving several different purposes. Presumably, it is advantageous for the regulatory system to permit gene-to-gene variation in basal and induced expression as well as cross-talk by other regulatory pathways.

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Thus, repression in aerobic cells ranges from moderate, for some genes, e.g. those whose basal expression is indispensable, like *HEM13* and *OLE1*, to highly stringent, for genes like *DAN1* and *ANB1*. Aerobic repression depends on heme signaling through multiple mechanisms (7,12). We show here that expression of Mot3, like Rox1 (3,5), is induced to inhibitory levels by heme. It acts to de-repress a mechanism which includes Hap1, Tup1–Ssn6 and a fourth unidentified factor. Induction levels for Mot3 and Rox1 appear to be calibrated to establish a range of responses to oxygen levels for each target gene, as determined by the number of operator sites in each promoter.

The requirement for both Mot3 and Rox1 for inhibition of aerobic expression might suggest each is a necessary component of a single complex. Arguing against this, we show that either repressor can almost fully compensate for the lack of the other when artificially expressed at high levels. However, at the low levels present in aerobic cells, Mot3 and Rox1 act synergistically to achieve stringent repression. Though functioning in concert, the two mechanisms are distinct, as shown by the observation that Rox1, but not Mot3, requires the Tup1–Ssn6 co-repressor for its function.

MATERIALS AND METHODS

Strains and plasmids

Strains used were FY23 (19) and RZ53 (3). A *mot3::HIS3* disruption was introduced into FY23 by transformation with a PCR fragment amplified from FY1628 [provided by Fred Winston (15)], using primers homologous at –964 and +1880. This cassette was introduced into FY23*his3*:*DAN1/URA3*, which was obtained by disruption of *HIS3* with a fusion of the *DAN1* promoter to the *URA3* gene. For the construction of the *his3*:*DAN1/URA3* allele, a region of *HIS3* from –560 to +1087 was amplified using primers homologous at those sites (5′: gagagaattcactggaacttggatttatggc; 3′: cctccatggagctcagctgtcgacgtagttgctactgttatttctggc) digested at EcoRI and SalI sites in the primer segments, and inserted into the same sites in pBS-SK (Stratagene), generating pBS-*HIS3*; next the *DAN1/URA3* fusion was excised from YCp*DAN1/URA3* (9) by digestion with EcoRI, end-filling with Klenow, and digestion with SpeI; it was then inserted into the end-filled BstBI site and the NheI site of pBS-*HIS3*, replacing the segment of *HIS3* from –42 to +509, and generating pBS-*his3:DAN1/URA3*. A SpeI–XhoI fragment excised from this plasmid was used to obtain FY23*his3*:*DAN1/URA3* by transformation of FY23 and selection on anaerobic –ura plates (the *DAN1/URA3* fusion is only active in the absence of oxygen). FY23*mot3*Δ(*HIS3*) was obtained by transformation of FY23*his3*:*DAN1/URA3* with the *mot3:HIS3* disruption cassette and selection for the HIS3 marker. *MOT3* disruptants were confirmed by Southern blot, and since these were prototrophic for uracil under aerobic conditions (due to loss of Mot3 repression of *DAN1/URA3*), the *ura3* genotype was regenerated by selection for a spontaneous auxotrophic mutant on FOA. FY23*mot3:KanMx* was generated by transformation of FY23 with a PCR fragment derived from the proprietary *mot3::KanMx* strain derived from derivative of strain BY4741 (Resgen), using the recommended primers (tgaattcatcaagagatttgaaaca and ctccgtctggatttactaaactttg).

FY23*rox1*Δ was generated as described (3), as were FY23*hap1*Δ and FY23*hap2*Δ (9). FY23*tup1*Δ*ssn6*Δ was constructed by serial disruption of *TUP1* and *SSN6*. *TUP1* was disrupted as described (9). FY23*tup1*Δ*ssn6*Δ was constructed by transformation of FY23*tup1*Δ with an XbaI–SphI fragment excised from p*ssn6:LEU2* [obtained from R. Zitomer (6)]. FY23*tup1*Δ*ssn6*Δ isolates were confirmed by Southern blot and the *ura3* genotype was regenerated as described above.

YEp*GAL1/ROX1* was constructed by cloning an XbaI–HindIII fragment containing *ROX1* (–433 to +1592) from the plasmid clone (3) into the same sites in pBS-M13+ (Stratagene). The EcoRI–BglII fragment from plasmid YCpGZ-15-Bg (1) containing the region from –830 to –268 of the *GAL1* promoter was inserted 5′ to the *ROX1* gene between the EcoRI and BamHI sites of the pBS-M13+ polylinker. The resulting fusion joined the *ROX1* gene at –433 to the *GAL1* promoter at –268; it was excised with EcoRI and HindIII and inserted into the same sites of the 2μ vector, YEpLac195 (20), generating YEp*GAL1/ROX1*. YCp*GAL1/MOT3* was constructed in two steps: first a plasmid containing the *GAL1* promoter region (–820 to –9) was constructed by inserting a PCR fragment amplified from the *GAL1* promoter with primers homologous at those sites (5′: gagaggtaccgaattcgacaggttatcagcaac; 3′: gagaggatcctctccttgacgttaaagtatagagg); the fragment was digested at EcoRI and BamHI sites in the primer segments and inserted into YCpLac33 (20) at the same sites, generating YCpp*GAL1*. Next a fragment (–1 to +2389) carrying the *MOT3* ORF (which extends from +1 to +1971) was amplified by PCR using primers homologous at those sites (5′: gagaggatccacaataatgaatgcggaccatcac; 3′: gaaaatctgtcccctagcg); it was digested at a *Bam*HI site contained in the 5′ primer segment and at an EcoRV site at +2227 and ligated to YCpp*GAL1* which had been linearized with HindIII, end-filled, and digested with BamHI. A *MOT3/lacZ* fusion plasmid was constructed in two steps: first the *MOT3* promoter region (–978 to +5) was amplified using primers (5′: tttacttcattcatgcttacagag; 3′: gagaggatcctcgttcattgctcaaatatgatatgtc) homologous at those sites, and cloned into the TA cloning vector pCRII (Invitrogen). It was then excised by digestion at an EcoRV site in the polylinker and a BamHI site in the 3′ primer segment, and ligated to the BamHI site and the end-filled EcoRI site of YCp*DAN1/lacZ* (9); this replaced the *DAN1* promoter fragment with the *MOT3* promoter region, generating YCp*MOT3/lacZ*.

Cloning *ROX7*

ROX7 was cloned by complementation of the *rox7-1* mutation in FY23MS*rox7-1*, which carries integrated *DAN1–URA3* and *DAN1–lacZ* fusions (9) and which is prototrophic for uracil due to the constitutive phenotype of the recessive *rox7* allele. FY23MS*rox7-1* was transformed with a centromeric library (9), and cells transformed with the wild-type *ROX7* gene were selected on FOA plates for loss of uracil prototrophy resulting from complementation.

Cell growth and analysis of gene expression

Cells were grown under aerobic or anaerobic conditions in YPD or SC media. Anaerobic cultures were bubbled with high purity nitrogen. Anaerobic cultures for RNA analysis were harvested in late log phase after 90 min of anaerobic growth.

RNA was extracted and analyzed as described (21). Anaerobic cultures for lacZ assay were harvested after 7 h and the activity quantified as described (22). For expression of *MOT3* and *ROX1* FY23 cells carrying YCp*GAL1/MOT3* or YCp*GAL1/ROX1* were grown in SD-ura overnight, diluted 40-fold into SD-ura, grown for 3 h into log phase, washed twice in SD-ura-raffinose (1%)–galactose (1%) and grown for 4 h in the same medium before harvesting for RNA analysis.

RESULTS

ROX7 identified as *MOT3*

The *ROX7* locus was defined by recessive mutations in one of three complementation groups (9) selected for constitutive expression of *DAN1*, which is normally repressed during aerobic growth. *rox7* mutations also de-repressed the hypoxic gene *ANB1*. We cloned *ROX7* from a centromeric library by complementation in cells carrying an integrated *DAN1–URA3* fusion (see Materials and Methods). Complementing plasmids carried the YMR070W open-reading frame, previously identified as *MOT3*, encoding a zinc finger protein that regulates expression of a number of different genes (14,15), but which had not been associated with oxygen regulation.

Synergistic aerobic repression of *ANB1* and *DAN1* by Mot3 and Rox1

As expected from the constitutive phenotype of the *rox7* alleles, disruption of *MOT3* caused partial loss of repression of *ANB1* and *DAN1* (Fig. 1), indicating that Mot3 functions along with Rox1 as an aerobic repressor of both genes. We focused on the roles of the two repressors on *ANB1* expression, since the regulation of *DAN1* is complicated by the contributions of two other oxygen-sensitive factors (7,9) and shows a lesser dependence on Rox1 and Mot3. Disruption of *ROX1* caused an apparent full loss of repression of the *ANB1* gene: expression in the *rox1*Δ strain was equal under aerobic and anaerobic conditions (Fig. 1); moreover, expression in aerobic *rox1*Δ cells was equal to that in anaerobic *mot3*Δ*rox1*Δ cells (Fig. 1B), representing the fully de-repressed output of the promoter. Hence, Mot3 has no repression function on its own under these conditions, and even though Rox1 represses weakly by itself, the efficient repression observed in normal aerobic cells occurs only when the two proteins function in concert, i.e. 'synergistically'. Negative synergy is a less well defined concept than positive, but a reasonable index for synergisic inhibition is a comparison of the residual activities when one or both repressors are present. These were determined to be 100 or 20–40%, respectively, with Mot3 or Rox1 alone, versus <1% when both are functioning. Hence, the residual expression with both repressors present is much less than that with either one alone, i.e. the combined inhibitory effects are clearly more than multiplicative (see Discussion).

Differences in the expression of *DAN1* and *ANB1* in these mutants reveals a variable degree of participation in repression by the two factors at different promoters. The full loss of repression of *ANB1* in the *rox1*Δ strain compared with partial loss in the *mot3*Δ strain implies that Rox1 plays a more important role than Mot3 at this promoter, presumably

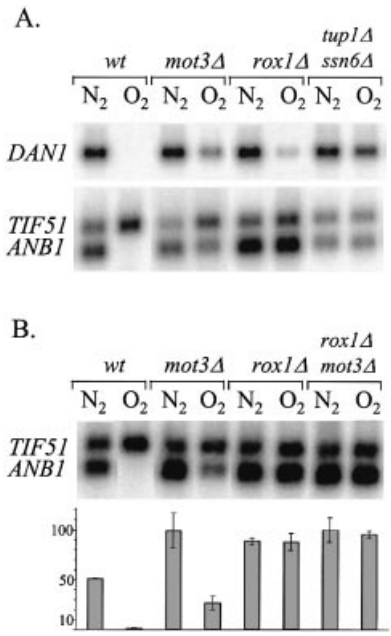

Figure 1. Effect of regulatory mutations on expression of anaerobic genes. (**A**) Strains of FY23 carrying disruptions of *MOT3* (*mot3::HIS3*), *ROX1*, or a double disruption of *TUP1* and *SSN6* were grown under aerobic and anaerobic conditions as described in Materials and Methods; RNA was extracted and subjected to northern blot analysis using probes prepared from the *DAN1*, *ANB1* and *ROX1* genes. The *ANB1* probe hybridizes to *ANB1* mRNA and also to the homologous *TIF51* transcript. (**B**) Cells of strains FY23, FY23*mot3*Δ(*mot3::HIS3*), FY23*rox1*Δ and FY23*rox1*Δ*mot3*Δ-(*mot3::Kan-Mx*) grown under the same conditions and analyzed as in (A). Duplicate northern blots were quantified by phosphorimager analysis with values (and standard deviations) for *ANB1* mRNA levels shown in units of percentage of the maximum value in the sample set (anaerobic FY23*rox1*Δ*mot3*Δ cells). The value for aerobic expression of *ANB1* in wild-type cells is statistically indistinguishable from zero, at the limit both of phosphorimager quantification and direct visualization.

reflecting the preponderance of Rox1 sites (1). In contrast, aerobic repression of *DAN1* was more affected by loss of Mot3 than of Rox1, also consistent with the relative numbers of Mot3 and Rox1 sites (1,7). The greater overall sensitivity of *ANB1* to both *rox1* and *mot3* mutations compared with *DAN1* may reflect the fact that *DAN1* is also repressed by at least two other oxygen-sensitive mechanisms in aerobic cells (7,9). The results show constitutive expression of both genes in the absence of the Tup1–Ssn6 complex. The fact that expression in anaerobic *tup1*Δ*ssn6*Δ cells is lower than in wild type (Fig. 1A) may result from de-repression of *ROX1*or *MOT3*, which are both regulated by the heterodimer (6, and see below); the same could hold for the relatively lower expression in aerobic *tup1*Δ*ssn6*Δcells compared with *rox1*Δ cells (see Discussion).

Mot3 function is independent of oxygen

The role of Mot3 in aerobic repression suggested that it might be converted to a repressing form in the presence of oxygen (or heme). To test this we monitored expression of *DAN1* and *ANB1* in anaerobic cells expressing *MOT3* under galactose control. Both genes were repressed in cells expressing high levels of Mot3 (Fig. 2), implying that inhibition of transcription by the repressor is concentration dependent and not the result of a conformational change occurring as a result of the

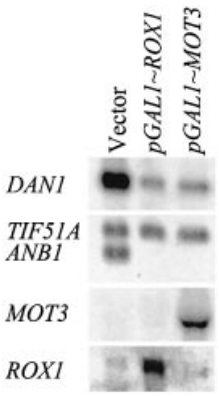

Figure 2. Over-expression of *MOT3* represses *DAN1* and *ANB1* in anaerobic cells. Cells of strains FY23, FY23*mot3*Δ and FY23*rox1*Δ transformed as indicated with YEp*GAL1/ROX1*, YCp*GAL1/MOT3* or YCpLac33(vector) were pre-grown in SD(–ura) medium to early log phase, pelleted, washed twice with SD-raf/gal(–ura) medium, grown in that medium to mid-log phase (2 h), then shifted to anaerobic growth for 6 h before harvesting for RNA extraction. Northern blots were probed with *DAN1*, *ANB1*, *ROX1* and *MOT3*.

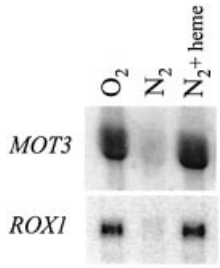

Figure 3. Regulation of *MOT3* expression by oxygen and heme. RZ53 cells were grown under aerobic conditions and under anaerobic conditions with and without supplementation with heme at 25 μg/ml; heme was added to the culture 40 min before shifting to anaerobic growth. Northern blots were probed with *MOT3* and *ROX1*.

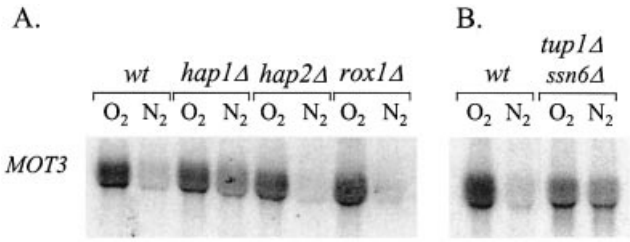

Figure 4. Role of Hap1, Hap2, Tup1 and Ssn6 in regulation of *MOT3*. (**A**) FY23 cells carrying disruptions of *HAP1, HAP2* and *ROX1* were grown under aerobic and anaerobic conditions. (**B**) FY23 cells carrying a double disruption of *TUP1* and *SSN6* were grown under aerobic and anaerobic conditions. Northern blots were probed with *MOT3*.

presence of oxygen and/or heme. The same was earlier demonstrated for Rox1 (4).

Induction of *MOT3* by heme and repression by Hap1 and Tup1–Ssn6

Concentration-dependent repression by Mot3 suggested that heme inhibits expression by inducing *MOT3* expression. This was confirmed by the observation first, that *MOT3* mRNA is down-regulated in anaerobic cells (Fig. 3) and second, that expression is de-repressed by heme, in parallel with *ROX1* (3).

We tested factors known to mediate heme regulation for involvement in induction of *MOT3*. Neither Hap1 nor Hap2 were required for expression (Fig. 4A). However, rather than activating aerobic *MOT3* expression, Hap1 functions by repressing in the absence of heme, as indicated by higher levels of *MOT3* mRNA in *hap1*Δ cells during anaerobic growth. Heme regulation of *MOT3* is not attributable solely to Hap1, since *MOT3* mRNA levels in the *hap1*Δ strain were still higher in the presence of oxygen. This implicates another unknown oxygen-sensitive factor in the regulatory pathway. Anaerobic repression of *MOT3* also depends on Tup1–Ssn6 more than on Hap1, as indicated by the presence of de-repressed levels of mRNA in anaerobic *tup1*Δ*ssn6*Δ cells (Fig. 4B). Whether the co-repressor complex is recruited by Hap1 and/or another repressor is not clear. Thus, *MOT3* and *ROX1* expression are co-regulated in four respects: heme induction, repression of basal expression by Hap1, regulation by a second heme-responsive system, and repression by Tup1–Ssn6.

We tested for auto-repression of *MOT3* analogous to that observed for *ROX1* (6), but saw no increase in expression of the *MOT3-lacZ* reporter in the *mot3*Δ strain (data not shown). We also observed that neither factor regulates expression of the other (Fig. 4A and data not shown).

Rox1 requires Tup1–Ssn6, but Mot3 functions through another mechanism

Tup1 and Ssn6 are co-repressors of the hypoxic genes (23–25) and of the *DAN/TIR/ERG* genes (9) (see Fig. 1). It has been assumed that the heterodimer is recruited by Rox1, as with other repressors (26,27). If Tup1–Ssn6 is required for Rox1 function, loss of either the repressor or the co-repressor would be expected to cause similar levels of constitutive expression. However, it has been repeatedly observed that constitutive expression of *ANB1* in aerobic *tup1*Δ*ssn6* cells is significantly lower than in *rox1*Δ cells (Fig. 1). This difference doesn't mean that Rox1 and Tup1–Ssn6 don't interact, since Rox1 might be able to repress inefficiently by itself. Another explanation might be that the weaker Mot3 repressor, rather than Rox1, recruits the heterodimer. To test for these interactions, we made use of the observation that Gal4-driven expression of either factor causes efficient repression of *ANB1* and *DAN1* in wild-type anaerobic cells. In a *tup1*Δ*ssn6* strain, Gal4-induced expression of *MOT3* still caused efficient repression of *ANB1*, but expression of *ROX1* did not (Fig. 5). Hence, Rox1 requires the Tup1–Ssn6 co-repressor in this context, while Mot3 evidently functions through a different mechanism.

Mot3 and Rox1 are independent synergizing repressors

The failure of Rox1 and Mot3 to repress by themselves is in striking contrast to the highly stringent repression observed when both factors are present (Fig. 1). The mutual dependence of Mot3 and Rox1 could arise either because both are essential parts of a single mechanism (e.g. Tup1–Ssn6) or because they act independently in a synergistic fashion. We tested whether either factor is sufficient when over-expressed, starting from the observation that Gal4-driven expression of either *MOT3* or

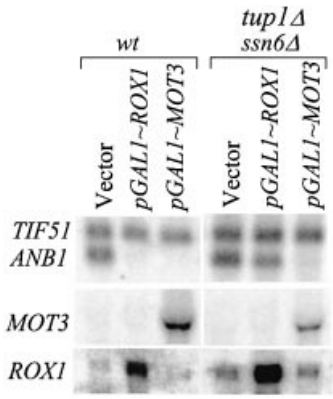

Figure 5. Role of Tup1–Ssn6 in repression by *MOT3* and *ROX1*. FY23 cells and *tup1*Δ*ssn6*Δ derivatives carrying the indicated plasmids were grown anaerobically, as described for Figure 2. Northern blots were probed with *ANB1*, *ROX1* and *MOT3*.

ROX1 in anaerobic cells resulted in efficient repression of both *DAN1* and *ANB1* (Fig. 2). This observation had implied that each factor is mechanistically sufficient for repression, since under these conditions high levels of Mot3 were paired with very low (uninduced) levels of Rox1, or vice versa. To rule out the possibility that low levels of the non-induced factor assisted the over-expressed one, we repeated the experiment in *mot3*Δ and *rox1*Δ mutants. In both cases, expression of *ANB1* was still almost fully inhibited, in both aerobic and anaerobic cells (Fig. 6), each repressor compensating for the absence of the other. Thus, either one of the two independent repression mechanisms is effective at high operator occupancy. It was clear, however, that repression is significantly more efficient in aerobic wild-type cells with both factors present (Figs 1 and 6B), even at their relatively low endogenous levels. Hence, even though both Mot3 and Rox1 can repress independently at artificially high levels, stringent regulation in aerobic cells depends on a synergistic interaction.

DISCUSSION

We have identified Mot3 as a repressor that functions synergistically with Rox1 to suppress aerobic expression of genes in two different anaerobic regulons. Mot3 and Rox1 are part of a complex regulatory network which mediates heme regulation through several different pathways (Fig. 7). Remarkably, heme regulation of the *DAN/TIR* genes, exemplified by *DAN1*, depends on four independent mechanisms, functioning at four different promoter sites: the Tup1–Ssn6-dependent Rox1 mechanism; the Tup1–Ssn6-independent Mot3 mechanism; an inhibitory mechanism which operates at the 'AR1' site through a regulatory domain in the oxygen-regulated Mox4/Upc2 activator (8,9); and a fourth anaerobic induction mechanism involving an unidentified activator operating at a second anaerobic response element (AR2) (7). Regulation through the AR1 and AR2 sites by heme is not highly stringent, and the combined action of Mot3 and Rox1 bound to their respective operators serves to enhance aerobic repression, reducing basal expression to undetectable levels. In the simpler *ANB1* promoter, Mot3 and Rox1 appear to be

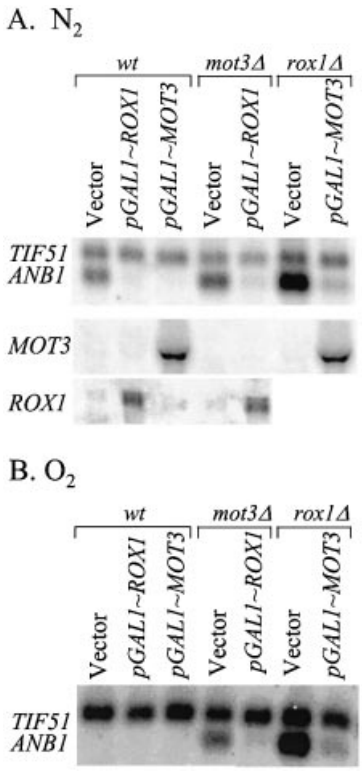

Figure 6. Independent repression by Mot3 and Rox1. Strains described in Figure 2 were grown as described. (**A**) Anaerobic growth. (**B**) Aerobic growth.

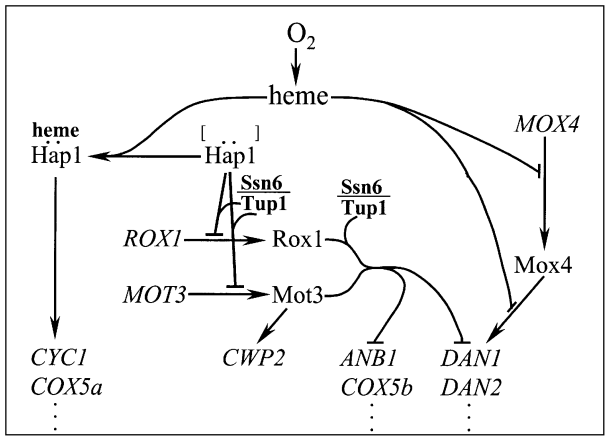

Figure 7. The heme regulatory network controlling expression of anaerobic and aerobic genes. The scheme is consistent with evidence on the interlocked regulatory pathways controlling expression of aerobic and anaerobic genes, as described here and elsewhere (3,5,9,12). Heme regulates the anaerobic genes via several mechanisms. It de-represses Hap1-inhibited expression of the Mot3 and Rox1 repressors, which in turn block expression of several anaerobic genes during aerobic growth. A subset of anaerobic genes are activated by Mox4/Upc2, whose activation function and expression are both inhibited by heme during aerobic growth. In addition, there is a second unidentified oxygen-inhibited factor operating through the AR2 site in the *DAN1* promoter (7). Heme also induces expression of a large group of genes (e.g. *CYC1* and *SOD1*), via Hap1 in its alternate role as a heme-dependent activator. Finally, Mot3, known to function as either an activator or a repressor (14), also activates expression of the aerobically induced *CWP2* mannoprotein gene (12).

solely responsible for heme regulation, providing a useful model for their possible interaction.

The mutual dependence of Mot3 and Rox1 was originally inferred from the strongly constitutive expression of *ANB1* in *rox1* and *mot3* mutants, which implies that neither factor can repress on its own at endogenous levels. We have demonstrated that the highly stringent repression of the *ANB1* gene results from true synergy between the two repressors, i.e. through concerted action by independent mechanisms, rather than through obligatory co-participation in a single mechanism. The independent function of Mot3 and Rox1 was shown by the fact that over-expression of either can compensate for lack of the other. Strikingly, however, even when titrated by high levels of either Mot3 or Rox1, single-factor repression is still less stringent than in cells containing low levels of both repressors. One possible explanation for synergistic co-dependence might be cooperative binding, though *in vitro* studies have indicated that this does not occur on naked DNA (17). Perhaps more likely is the possibility that the two factors act through different targets in the PolII initiation complex, to produce concerted inhibition analogous to numerous examples of synergistic activation. A two-target model is strengthened by the indication that Mot3 and Rox1 utilize different mechanisms, with only the latter requiring Tup1–Ssn6 for its function.

This regulatory system has solved the problem of allowing some anaerobic genes to be expressed at low or moderate levels in aerobic cells while others are stringently repressed. Apparently, during evolution of the regulatory pathway, the levels of Mot3 and Rox1 have been calibrated in such a way that on some promoters they are able to fully repress, and at others, less so, depending on the number and quality of operator sites [which vary considerably (1)], and possibly on the type and strength of activation mechanism driving expression of each gene.

The response to oxygen depends on the fact, established here for *MOT3* and earlier for *ROX1* (3,5,6), that expression of the repressors is de-repressed from an inhibited state by heme. This occurs, at least in part, through a regulatory system which controls both genes, with repression in anaerobic cells being fully dependent on Tup1–Ssn6, and partly dependent on the heme-unbound form of Hap1 (5,6). A second factor which appears to play a role in repression of *ROX1* and *MOT3* has not been identified, nor have factors responsible for activation under aerobic conditions.

We have concluded that the Rox1 repression mechanism requires the participation of the Tup1–Ssn6 co-repression complex as originally suggested (23), and that Mot3 functions in a different way. This is based on the observation that repression of *ANB1* caused by over-expression of *ROX1* in anaerobic cells is lost in a *tup1*Δ*ssn6*Δ strain, whereas repression resulting from over-expression of *MOT3* is still in effect. However, the role of Tup1–Ssn6 is not clear. For example, though expression of *ANB1* under anaerobic and aerobic conditions is equal in the absence of the heterodimer (see Fig. 1A), the mRNA level is consistently lower than in anaerobic wild-type cells or in aerobic *rox1*Δ cells. The de-repression of *ROX1* in *tup1*Δ*ssn6*Δ cells may cause reduced expression of *ANB1*, though this seems unlikely in view of the dependence of Rox1 on Tup1–Ssn6 (Fig. 5). More likely, reduced expression of *ANB1* results from de-repressed expression of Mot3 (in glucose cultures) (Fig. 4). In addition, the absence of Tup1–Ssn6 may lead to reduced expression through some other perturbation of the regulatory circuits in this system. The genetic interaction of Rox1 and Tup1–Ssn6 suggests that Rox1 recruits Tup1–Ssn6 to the promoter during aerobic growth. However, recent evidence of constitutive binding of Tup1–Ssn6 to the *ANB1* promoter (28) suggests that the functional relationship between Rox1 and Tup1–Ssn6 may not be one of recruitment.

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