Reciprocal Regulation of Anaerobic and Aerobic Cell Wall Mannoprotein Gene Expression in *Saccharomyces cerevisiae*

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The *DAN/TIR* genes encode nine cell wall mannoproteins in *Saccharomyces cerevisiae* which are expressed during anaerobiosis (*DAN1, DAN2, DAN3, DAN4, TIR1, TIR2, TIR3, TIR4,* and *TIP1*). Most are expressed within an hour of an anaerobic shift, but *DAN2* and *DAN3* are expressed after about 3 h. At the same time, *CWP1* and *CWP2*, the genes encoding the major mannoproteins, are down-regulated, suggesting that there is a programmed remodeling of the cell wall in which Cwp1 and Cwp2 are replaced by nine anaerobic counterparts. *TIP1, TIR1, TIR2,* and *TIR4* are also induced during cold shock. Correspondingly, *CWP1* is down-regulated during cold shock. As reported elsewhere, Mox4 is a heme-inhibited activator, and Mot3 is a heme-induced repressor of the *DAN/TIR* genes (but not of *TIP1*). We show that *CWP2* (but not *CWP1*) is controlled by the same factors, but in reverse fashion—primarily by Mot3 (which can function as either an activator or repressor) but also by Mox4, accounting for the reciprocal regulation of the two groups of genes. Disruptions of *TIR1, TIR3,* or *TIR4* prevent anaerobic growth, indicating that each protein is essential for anaerobic adaptation. The Dan/Tir and Cwp proteins are homologous, with the greatest similarities shown within three subgroups: the Dan proteins, the Tip and Tir proteins, and, more distantly, the Cwp proteins. The clustering of homology corresponds to differences in expression: the Tip and Tir proteins are expressed during hypoxia and cold shock, the Dan proteins are more stringently repressed by oxygen and insensitive to cold shock, and the Cwp proteins are oppositely regulated by oxygen and temperature.

The cell wall of *Saccharomyces cerevisiae* is a rigid structure which determines cell morphology and also serves as a protective barrier, providing mechanical protection and enabling selective uptake of macromolecules (1a, 4, 7). A major component of the cell wall is mannoprotein, comprising about 40% of its mass. Mannoproteins are believed to be determinants of cell wall permeability (31), and certain ones are also essential for developmental events such as mating and transition to hyphal growth (18, 24). Some mannoproteins can be extracted from the cell wall with detergent; others are covalently bound but can be released with glucanase (7). Proteins in the latter category have common features, including a region rich in serine and threonine, a glycosylphosphatidylinositol (GPI) anchor attachment signal at C termini (3, 8), and an endoplasmic reticulum localization sequence; several also have a PAU domain. This segment of about 100 amino acids is shared among a group of proteins known as "seripauperins" (29).

The Cwp2 mannoprotein (28) is one of the most abundant proteins of the cell wall and is believed to play a role in its stabilization, along with another homologous constituent, Cwp1 (23, 27, 28). While Cwp1 and Cwp2 are expressed under normal growth conditions, other mannoproteins are expressed in response to environmental stress. The most extensive response is the induction of several homologous mannoproteins during anaerobic growth: Dan1 (26) (for "delayed anaerobic"), Tip1, Tir1, and Tir2 (9, 16, 17, 22). We refer to the genes encoding these proteins as the *DAN/TIR* genes. We find that the *DAN/TIR* group includes genes for five other mannoproteins, designated Dan2, Dan3, Dan4, Tir3, and Tir4 (open reading frames [ORFs] YLR037c, YBR301w, YJR151c, YIL011w, and YOR009w), and that Tir1, Tir3, and Tir4 are required for anaerobic growth. At the same time we show that expression of *CWP1* and *CWP2* is turned off during anaerobiosis. Hence, one group of proteins is replaced by another group, some of which are essential for anaerobic adaptation. In addition to being oxygen regulated a subset of these genes (*TIP1, TIR1, TIR2,* and *TIR4*) are induced during cold shock, while *CWP1* is correspondingly down-regulated. *CWP1, CWP2,* and *TIP1* are also differentially regulated during the cell cycle (2), possibly reflecting an increased demand for cell wall proteins during bud formation. There is also suggestive evidence that expression of *CWP1* and other cell wall components is coregulated (23).

Until recently, little was known about the regulation of mannoprotein expression, beyond expression patterns in response to various signals. We had earlier found that the regulatory coeffector controlling expression of *DAN1* is heme, which functions as an inhibitor of expression in aerobic cells (26). We report elsewhere on a system of regulators which control anaerobic induction and heme repression of *DAN1* and the other *DAN/TIR* genes through a recently identified group of promoter sites (4a). These include principally the Mox4 (or Upc2) activator (1, 5) as well as a group of repressors, Mox1, Mox2, Mot3, and Rox1. We show here that expression of *CWP2* is under the control of some of the same factors, acting in an opposite fashion to induce expression during aerobic growth and to block expression in anaerobic cells.

MATERIALS AND METHODS

Plasmids and gene disruptions. (i) pBS*TIR1:URA3*. The region containing the *TIR1* gene (−1034 to + 996) was amplified using the primers GAGTCGACA

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AGTATCCAACAGACAGTAGTGCC and GAGAGAATTCATATCTACAA ATATCCCGGC. The PCR product was digested with *Eco*RI and *Sal*I and ligated to pBS-SK [pBluescript SK(+) (Stratagene)] which had been digested with the same enzymes, generating pBS*TIR1*. To generate the disruption construct, the *URA3* gene was inserted into pBS*TIR1*. For this construction, the *URA3* gene from −938 to +1772 was amplified using the primers GAGAGAA TTCATCGATCAACTAACATCACACTTGCTCG and GAGAGTCGACAC GCGTGGAACACAGTGGAGCCTTG and digested with *Bsp*DI (at −938) and *Sma*I (at +882) for insertion into the *Bst*BI (−490) and *Eco*RV(+186) sites in pBS*TIR1*, generating pBS*TIR1:URA3*. The disruption fragment used to transform FY23 cells was excised with *Eco*RI and *Xho*I.

(ii) pBS*TIR3:URA3*. The region containing the *TIR3* gene (−1084 to +728) was amplified using the primers GAGAGTCGACTGCGGAAAATACTTCGT ACC and GAGAGGATCCGCGTTCTTGGAGGTAGCAG. The PCR product was digested with *Bam*HI and *Sal*I and ligated to pBS-SK which had been digested with the same enzymes, generating pBS*TIR3*. To generate the disruption construct, the *URA3* gene was inserted into pBS*TIR3*. For this construction an *Nde*I/*Bsp*DI linker (TAATCGATATCGAT) was inserted into the *Nde*I site (at −149) in pBS*TIR3*. The resulting plasmid was digested with *Nco*I (at +226), end filled with Klenow polymerase, digested with *Bsp*DI, and ligated to the *Bsp*DI/*Sma*I fragment containing *URA3* described above, generating pBS*TIR3: URA3*. The disruption fragment was excised with *Eco*RI and *Xho*I.

(iii) pBS*TIR4:URA3*. The region containing the *TIR4* gene (−993 to +822) was amplified using the primers GAGAGAATTCGTCGACACACGATAAAGTTC TTGAAGAAAG and TGTGACAGCAGAAGAACTAGTAGC. The PCR product was digested with *Spe*I and *Sal*I and ligated to pBS-SK which had been digested with *Spe*I and *Sal*I, generating pBS*TIR4*. This plasmid was digested with *Nco*I (at +290), end filled with Klenow polymerase, digested with *Bst*BI (at −131), and ligated to the *Cla*I-*Sma*I-digested *URA3* fragment, generating pBS*TIR4:URA3*. The disruption fragment was excised with *Not*I and *Xho*I.

Centromeric plasmids containing the *TIR1*, *TIR3*, and *TIR4* genes and their respective native promoters were constructed as follows. For YCp*TIR1*, a fragment containing the *TIR1* gene up to −1034 was excised from pBS*TIR1* with *Eco*RI and *Sal*I and ligated to YCplac22 (12) which had been digested with the same enzymes. For YCp*TIR3*, a region containing the *TIR3* gene (−1084 to +1052) was amplified using the primers GAGAGGATCCGCGTTCTTGGAG GTAGCAG and TGTGGGATCCTTTTCTCGACGGCTGCTAC. The product was digested with *Sal*I and *Bam*HI and ligated to YCplac22 which had been digested with the same enzymes. For YCp*TIR4*, a region containing the *TIR4* gene (−993 to +1651) was amplified using the primers GAGAGAATTCAGT CGACACACGATAAAGTTCTTGAAGAAAG and TCTCGGATCCTCTTCC TGCCCACATTCTG. The product was digested with *Sal*I and *Bam*HI and ligated to YCplac22 which had been digested with the same enzymes.

Strains and growth conditions. Cells of strain FY23 (30) and derivatives or RZ53 (20) were used for all experiments. Aerobic and anaerobic growth conditions in liquid media (yeast-peptone-dextrose [YPD] or synthetic complete medium lacking uracil [SC-ura]) were as described previously (19). Cells were also grown on SDET-ura agar (SC-ura medium containing 0.5% Tween 80 and 10 μg of ergosterol/ml) at 30° under anaerobic conditions in an anaerobic jar containing a BBL anaerobic GasPak. Cells grown to mid-log phase in YPD were subjected to cold shock by shifting to 13°C for 90 min. Cells were harvested and RNA was extracted as described previously (19). For treatment under anaerobic conditions with heme, RZ53 cells were grown as described previously (26). For induction of expression of the *MOX4* gene under the control of galactose, cells carrying YCp*GAL1/MOX4* or the YCp33 (12) vector were grown as described previously (1).

Electron microscopy. For electron microscopy, cells of strains FY23, FY23*tir1*Δ, FY23*tir3*Δ, and FY23*tir4*Δ were grown in anaerobic jars as described above, washed from plates in SC medium, pelleted in microcentrifuge tubes. The pellets were high-pressure frozen and fixed by freeze substitution in 1% osmium in acetone at −90°C (72 h), −60°C (48 h), and 4°C (18 h). Cells were washed twice in acetone and embedded in Epon/Araldite. Thin sections were stained with uranyl acetate and lead.

RESULTS

Homology patterns defining the *DAN/TIR* gene family. Several mannoprotein genes have been reported to be induced during anaerobiosis: *TIR1*, *TIR2*, *TIP1*, and *DAN1*. To identify more genes in this group, we searched for ORFs encoding homologous proteins and found five with significant homology
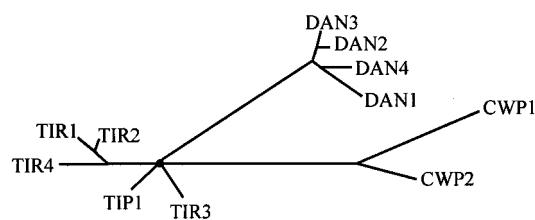

FIG. 1. Homology tree for mannoprotein genes. Puzzle_4 software was used to deduce homology relationships among the *DAN/TIR* and *CWP* genes.

to *TIR1* and *DAN1*: YIL011w, YOR009w, YLR037c, YBR301w, and YJR151c. The hypothetical proteins were subjected to analysis with Puzzle_4 software to deduce a possible lineage tree (Fig. 1). According to this set of comparisons, two of the open reading frames, YIL011w and YOR009w, were relatively more homologous to *TIR1*, and they were designated, respectively, *TIR3* and *TIR4* (we refer here to *TIR1*, *-2*, *-3*, and *-4* and *TIP1* as "*TIR*" genes, for "tip-related" [9]). Three other genes were clustered with *DAN1*: YLR037c, designated *DAN2*; YBR301w, designated *DAN3*; and YJR151c, designated *DAN4*. The *DAN* and *TIR* genes share homology with the seripauperin family of genes in the PAU domain, although unlike most of these proteins, Dan1, Dan4, Tip1, Tir1, Tir3, and Tir4 contain extensive serine-rich domains, presumed to be the site of mannosylation. *DAN2* and *DAN3* are more typical of the PAU group proteins with PAU domains and short serine-threonine rich regions. In addition most of *DAN/TIR* group share homology in GPI anchor domains at the C-terminal end, as well as in N-terminal endoplasmic reticulum localization domains. We noted that the *CWP1* and *CWP2* genes, which encode the major mannoproteins under normal growth conditions, also show weak homology to the PAU domains of the *DAN/TIR* genes.

Differential responses of the *DAN/TIR* genes to hypoxia and temperature, including asynchrony. We found that the mRNAs for all the *DAN/TIR* genes identified in a homology search were induced during anaerobic growth, while under aerobic conditions expression was either weak or undetectable (Fig. 2A). There are differences in expression patterns among subgroups of the *DAN/TIR* genes. First, the *TIR* genes are less stringently repressed by oxygen than the *DAN* genes. Second, as discussed below, four of the *TIR* genes are induced by cold shock while the *DAN* genes are not. Finally, there is asynchrony of expression: *DAN1*, *DAN4*, and the *TIR* genes were maximally induced within 1 h of anaerobiosis, but the appearance of *DAN2* and *DAN3* mRNA was consistently delayed for about two more hours, being maximally induced after 3.5 h (Fig. 2A). This indicated that the genes encoding anaerobic cell wall proteins are programmed for asynchronous expression. As observed earlier for *DAN1*, expression of all of these genes was negatively regulated by heme, e.g., *TIR1* and *TIR4* (Fig. 2C).

Since expression of the *DAN/TIR* genes depends on the Mox4 activator, we tested the role of Ecm22 protein (ORF YLR228c), which has a high degree of homology to Mox4, especially in a region containing a domain critical to heme regulation. Ecm22 is thought to play a role in cell wall synthe-
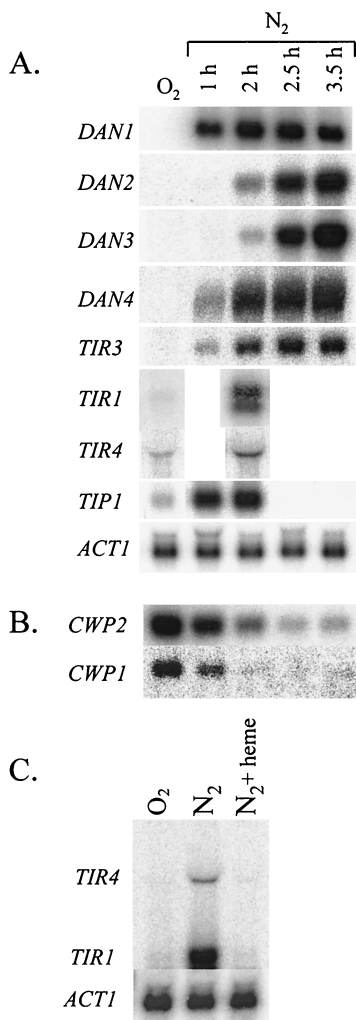
FIG. 2. Expression of *DAN/TIR* and *CWP* genes during anaerobic growth. (A) Cells of strain FY23 were grown under aerobic or anaerobic conditions as described in the text and harvested for RNA extraction at the intervals indicated. Northern blots were probed with *DAN1*, *DAN2*, *DAN3*, *DAN4*, *TIR1*, *TIR3*, *TIR4*, *TIP1*, and *ACT1* as a loading control. (B) The RNA samples used for panel A were probed with *CWP1* and *CWP2*. (C) Cells of strain RZ53 were grown in YPD under aerobic conditions or under anaerobic conditions with and without supplementation by heme (25 μg/ml). The Northern blot was probed with *TIR4* and *TIR1*.

sis, since an *ecm22*:Tn*5* mutation causes sensitivity to cal-cofluor white (21). We found that this allele caused reduced expression of *DAN2* and *DAN3* (data not shown) but not of the other *DAN/TIR* genes, suggesting that Ecm22 is a factor in the induction of these two genes.

Down-regulation of the *CWP* genes during anaerobiosis. During anaerobiosis, expression of *CWP1* and *CWP2* was down-regulated more than 10-fold within 2.5 h (Fig. 2B). In effect, the *CWP1* and *CWP2* mRNAs were replaced by the *DAN/TIR* mRNAs, suggesting that anaerobic adaptation includes extensive remodeling of the cell surface, where a large fraction of mannoproteins are thought to reside. The loss of *CWP1* and *CWP2* mRNAs was not instantaneous, showing a half-life of more than half an hour, after the anaerobic shift.

This contrasts with other more rapidly degraded mRNAs subject to oxygen induction (20, 25). It is unknown whether the slow decrease is due to mRNA stability or to slow cessation of transcription.

Cold shock induction of *TIR1*, *TIR2*, and *TIR4*. The *TIR4* gene was induced during growth at low temperatures (Fig. 3A), as observed earlier for *TIP1*, *TIR1*, and *TIR2*, while the *DAN* genes and *TIR3* remained uninduced (data not shown). Cold shock induction of *TIR4* (and of *TIR1* [data not shown]) depended on the Mox4 activator (Fig. 3C), suggesting that the hypoxic and hypothermic signal pathways converge through this factor. We also observed that expression of *CWP1* mRNA decreased at low temperatures (Fig. 3B) (*CWP2* was unaffected). Hence, the reciprocal expression of the *CWP* and *DAN/TIR* genes in response to oxygen is echoed in response to hypothermia, among subsets of the two gene groups.

To explore further the effect of temperature on induction of *TIP1* and down-regulation of *CWP1*, we examined the expression at different temperatures and found that induction follows a sigmoidal curve, with an inflection at about 16°C (Fig. 3D). Expression of *CWP1* over the same temperature range mirrors that of *TIP1* in reverse, although the temperatures of half-maximal induction (~16°C) and down-regulation (~18°C) of *TIP1* and *CWP1* are not identical.

***TIR1*, *TIR3*, and *TIR4* are required for anaerobic growth.** We found earlier that expression of the *DAN1* gene is not needed for anaerobic growth (26), and it has been reported that disruption of *TIR1* and *TIR2* was also without effect (9). In order to assess the importance of some of the other *DAN/TIR* genes for growth, we generated disruptions of *TIR1*, *TIR3*, and *TIR4*. The *tir1Δ*, *tir3Δ*, and *tir1Δ* strains grew normally on plates under aerobic conditions but not under anaerobic conditions (Fig. 4). When the same strains were transformed with the missing gene carried on a centromeric plasmid, anaerobic growth was restored. These observations indicated that Tir1, Tir3, and Tir4 are necessary for growth without oxygen. Light microscopy of *tir1Δ tir3Δ* and *tir1Δ* cells scraped from anaerobic plates showed that most of the cells were unbudded; a small number (less than 1%) had very small buds. In contrast, a majority of wild-type anaerobic cells were budded. This indicated that anaerobic *tir* knockout cells were able to finish division but not able to restart the cycle, presumably being arrested at G$_1$. Electron microscopy revealed no difference in the density of the outer layer of the cell wall, indicating that there is no gross disruption at least of the pre-existing protein-rich layer caused by loss of the Tir proteins (Fig. 5). None of the three mutant strains showed any growth defect at 15°C.

Mot3 is a heme-induced activator of *CWP2* expression. We report elsewhere that each of the *DAN/TIR* genes is activated by Mox 4 and repressed by a mechanism which includes Mox1 and Mox2 (1). We also found that Mot3 (11, 13) mediates heme repression of several of the *DAN/TIR* genes (1) and that it is induced by heme in aerobic cells (O. Sertil et al., submitted for publication). Mot3 has been found to act either positively or negatively at a number of different promoters (13), though it is not known why some promoters are activated and others repressed. We tested for a role of the *DAN/TIR* regulators in control of *CWP* expression and observed a surprisingly large decrease of expression of *CWP2* gene in *mot3Δ* cells (Fig. 6A), indicating that Mot3 activates *CWP2* expression during aerobic
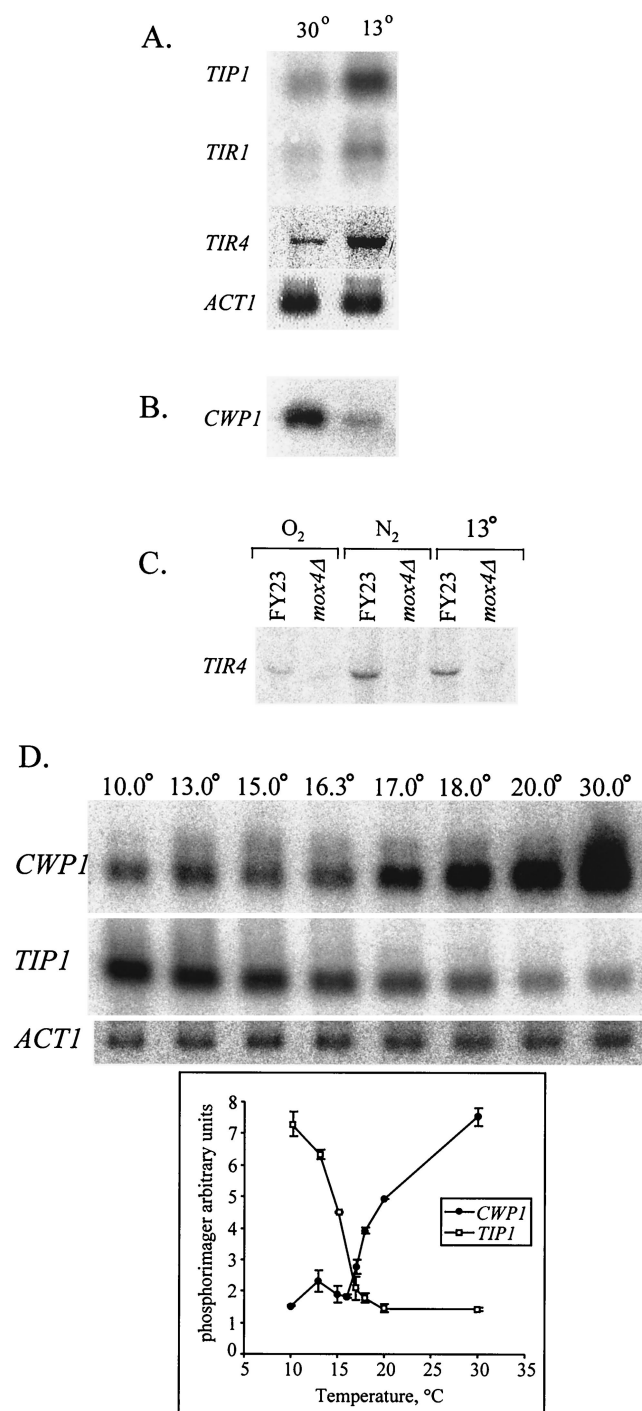
FIG. 3. Regulation of mannoprotein gene expression during cold shock. Cells of strain FY23 were subjected to cold shock as described in Materials and Methods and harvested for RNA extraction. (A) Northern blots were probed with *TIP1*, *TIR1*, and *TIR4*. (B) Cells of strain FY23 and FY23*mox4Δ* were grown under aerobic conditions, under anaerobic conditions, or at 13°C. A Northern blot was probed with *CWP1*. (C) The same blot was probed with *TIR4*. (D) Cells subjected to cold shock at different temperatures were harvested for RNA extraction. Northern blots were probed with *TIP1* and *CWP1*.

growth. Presumably Mot3 acts through three binding sites in the *CWP2* promoter (-668, -500, and -479), which would be expected to be in the high-affinity range, based on an earlier report (13). We found expression of *CWP1* to be unaffected by the *mot3Δ* allele (Fig. 6A).

Regulation of *CWP2* by Mox4. Although expression of *CWP2* was reduced by 70% in a *mot3Δ* strain, there was still a difference between aerobic and anaerobic expression, indicating that other factors might be involved in mediating induction. One such factor may be Mox4 or a factor associated with it, since overexpression of *MOX4* in aerobic cells under the control of the *GAL1* promoter caused a decrease in *CWP2* expression (Fig. 6B), along with an increase in *DAN/TIR* gene expression (data not shown), as observed earlier (1). In addition, *mox4Δ* cells consistently showed a significant increase in anaerobic *CWP2* expression (Fig. 6C) along with the expected loss of expression of the *DAN/TIR* genes. Hence, Mot3 and Mox4 exert opposite effects on the *DAN/TIR* and *CWP2* promoters. *MOT3* expression was not affected by the *mox4Δ* phenotype (data not shown). Neither the loss nor the overexpression of *MOX4* had any effect on the expression of *CWP1*.

Effect of other transcriptional regulators on the expression of *CWP* genes. We also tested the effect of the global repressors Tup1 and Ssn6 on the expression of *CWP1* and *CWP2*. Anaerobic expression of *CWP2* was increased modestly—about twofold—by the loss of Tup1 and Ssn6. *CWP2* expression may increase in anaerobic *tupΔ ssn6Δ* cells because these factors normally help repress *MOT3* expression in anaerobic cells (Sertil et al., submitted). In contrast, expression of *CWP1* was strongly increased in *tupΔ ssn6Δ* cells both aerobically and anaerobically, reaching levels far higher than the maximum observed during aerobic growth. This suggests that Tup1 and Ssn6 regulate *CWP1* expression, by assisting an unidentified promoter-specific repressor. Since this superinduction is observed in aerobic cells, the hypothetical repressor must exert some effect even in the presence of oxygen, and it may be the target of an unknown derepressing signal. The existence of such signals is implied by increased expression of *CWP1* in response to cell cycle signals (2) and to loss of cell wall integrity (23). Given the complexity of the inducing signals for *CWP1* it is possible that the effect of the *tupΔ ssn6Δ* mutations is indirect, i.e., that increased expression is caused by the same alterations in cell wall structure which cause the flocculence characteristic of *tup1* and *ssn6* mutants.

DISCUSSION

We have shown that *TIR1*, *TIR2*, *TIP1*, and *DAN1* are members of a larger group of homologous genes encoding mannoproteins involved in anaerobic adaptation. *DAN2*, *DAN3*, *DAN4*, *TIR3*, and *TIR4* are all induced during hypoxia, but their expression is asynchronous, with expression of *DAN2* and *DAN3* being delayed for about 2 h after expression of the other *DAN/TIR* genes. We also found that the genes encoding the two major cell wall mannoproteins, *CWP2* and *CWP1*, are down-regulated during anaerobic adaptation. In effect, it appears that oxygen deprivation results in extensive programmed remodeling of the cell wall, with Cwp1 and Cwp2 being replaced by the Dan/Tir proteins. Whether the latter proteins
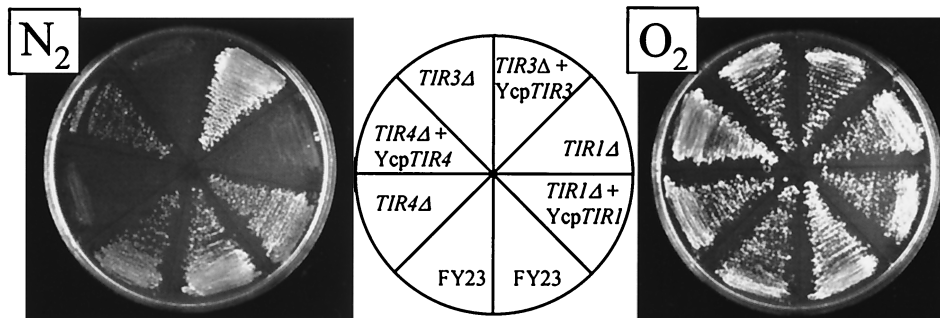
FIG. 4. Growth of strains carrying disruptions of *TIR* genes. Strains FY23, FY23*tir1*Δ, FY23*tir3*Δ, and FY23*tir4*Δ transformed with the indicated plasmids were grown under aerobic and anaerobic conditions on SDET-ura plates.

substitute functionally for Cwp1 and Cwp2 remains to be determined.

An analogous substitution process seems to occur when cells are subjected to cold shock, as *TIR1*, *TIR2*, *TIR4*, and *TIP1* are induced and *CWP1* is down-regulated. In attempting to perceive a rationale for the replacement of one group of cell wall proteins by another during hypoxia and cold shock, one possibility is that the adaptation event is related to membrane fluidity. Cells subjected to these two seemingly unrelated environmental stresses both experience reduction in membrane fluidity—during hypoxia as a result of depletion of unsaturated fatty acids and during hypothermia as a result of membrane phase transition. The cell wall proteins transit the membrane during cell wall assembly; this process may be affected by membrane properties, and conceivably, alternate protein forms or variations in the mechanisms of secretion may accommodate differences in fluidity. Another possibility is that some of these proteins play a role in the transport of sterols, as suggested by the fact that the Mox4 regulator, which controls *DAN/TIR* gene expression, also controls expression of factors involved in this process (5). Clearly, the special function of the anaerobic cell wall proteins will be more obvious when the function of cell wall proteins and the mechanisms of cell wall assembly are better understood.

We observed that the critical induction temperature both for expression of *TIP1* and down-regulation of *CWP1* was 16 to

18°C. Hence, induction and down-regulation are half-maximal at a temperature in the range within which phase transition might be expected to occur (10). Another observation suggesting that membrane fluidity is a factor in hypoxic adaptation was that unsaturated fatty acids repress hypoxic and cold shock-induced expression of *TIP1*. Whether changes in membrane fluidity actually signal low-temperature induction of any of several genes showing this response remains to be determined. Clearly, not all cold shock-induced genes are activated by the same factors. We showed here that cold shock induction of *TIR1* and *TIR4* requires Mox4, whereas induction of *OLE1* and *TIP1* was not affected by the *mox4*Δ allele (data not shown). Hence, if there is a common signal pathway it must diverge at the level of transcriptional regulators.

We noted a correspondence of homology and expression patterns, suggesting that there might be a functional basis for the difference in the patterns of regulation of these genes: (i) *TIP1* and the *TIR* genes are clustered in a homology tree and are less stringently repressed by oxygen than the *DAN* genes; four *TIR* genes—*TIP1*, *TIR1*, *TIR2*, and *TIR4*—are induced by cold shock; (ii) the four *DAN* genes are also clustered and are stringently regulated and not induced by cold shock; and (iii) the two *CWP* genes, weakly homologous to the *DAN/TIR* genes, are quite similar and are induced by oxygen.

In general, the function of mannoproteins is not well understood, though there is evidence that Cwp1 and Cwp2 help maintain cell wall integrity, and a fatty acid esterase activity has been tentatively attributed to Tip1 (14). As a first step in deducing the role of the Dan/Tir proteins, we tested the effect of deleting *TIR1*, *TIR2*, or *TIR4* and found that each is essential for anaerobic growth. This observation was at variance with an earlier report for *TIR1* and *TIR2* (9), possibly because of strain differences or differences in the degree of hypoxia achieved in the growth chamber. Anaerobically grown *tir*Δ cells were unbudded, presumably becoming arrested in G_1 after onset of anaerobiosis. The clear growth phenotype of these mutants will facilitate structure-function analysis of the Tir proteins, including the role of the PAU domain.

We have reported elsewhere that a network of regulators is responsible for the coordinate regulation of the *DAN/TIR* genes. This includes anaerobic induction by the Mox 4 transcriptional activator and aerobic repression by the Mox1 and Mox2 repressors. Some of the *DAN/TIR* genes are also repressed during aerobic growth by Mot3, a repressor which is
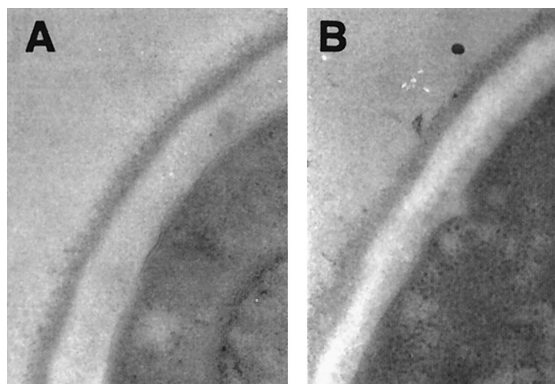

FIG. 5. Electron microscopy images of cell walls in anaerobic cells. Cells of strain FY23 (A) and FY23*tir3*Δ (B) were grown in an anaerobic jar for 36 h and processed for electron microscopy as described in Materials and Methods.
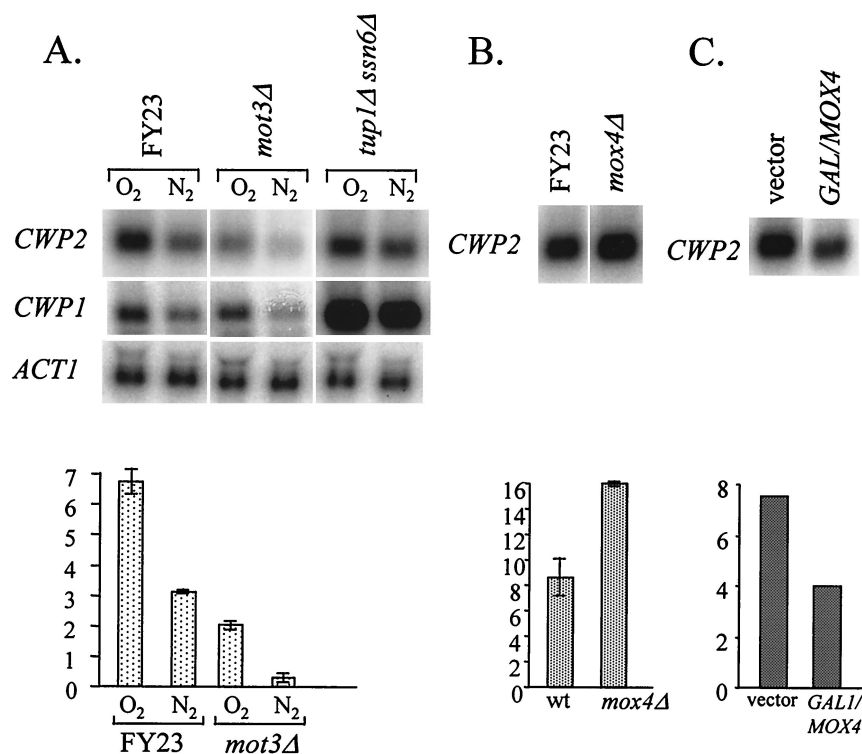
FIG. 6. Regulatory factors controlling expression of *CWP1* and *CWP2*. (A) Cells of strains FY23, FY23*mot3*Δ, and FY23*tup1*Δ*ssn6*Δ were grown in YPD under aerobic and anaerobic conditions and harvested for RNA extraction. Northern blots were probed with *CWP1*, *CWP2*, and *ACT1*. Phosphorimager data (in arbitrary units) for relative intensities are shown at the bottom. (B) Cells of strains FY23 and FY23*mox4*Δ were grown under anaerobic conditions in YPD. Phosphorimager data are shown. (C) Cells of strain FY23 carrying YCp*GAL1*/*MOX4* or the YCplac33 vector were grown at 30°C for 4 h under aerobic conditions in SC-galactose-raffinose medium (1) and harvested for RNA extraction. Data from an autoradiographic scan are shown.

induced by heme and repressed in anaerobic cells by Hap1 (Sertil et al., submitted), in parallel with the Rox1 repressor (6, 15, 20). Surprisingly, mutations affecting Mot3 and Mox4 also affected expression of *CWP2* but in a manner opposite to their effect on *DAN/TIR* expression, helping to account for induction of *CWP2* in aerobic cells. We have concluded that Mot3, which is known to function as an activator or repressor of different genes through the same binding sites (13), is an important activator of *CWP2*, presumably through the three Mot3 sites in the promoter. It is interesting that the versatility of Mot3 allows it to mediate both positive and negative regulation by heme. Conversely, Mox4, the activator of the *DAN/TIR* genes, plays a significant role in repressing *CWP2*, though the mechanistic basis of this reciprocal effect is unknown. We also observed that the Tup1-Ssn6 repressor complex contributes to repression of anaerobic *CWP2* expression, possibly by virtue of its role in repression of *MOT3* (Sertil et al., submitted). *CWP2* was earlier observed to be regulated during the cell cycle, as would be expected for a gene product associated with bud formation. Interestingly, analysis of genes regulated during the cell cycle revealed that expression of *MOT3* is also cyclical, suggesting that fluctuations in Mot3 may account for cell cycle regulation of *CWP2*.

Regulation of *CWP1* is still not well understood, except for a descriptive list of signals affecting its expression, either positively (induction during the cell cycle or when cell wall synthesis is disrupted) or negatively, as shown here during hypoxic or hypothermal stress. Although *CWP1* appears to be regulated by oxygen in parallel with *CWP2*, *mot3* and *mox4* mutations do not affect its expression, indicating that it is controlled by a different regulatory pathway. However, we did observe constitutive expression of *CWP1* in a *tup1*Δ *ssn6*Δ strain during hypoxia, indicating that expression in that state is normally blocked by a repressor associated with Tup1-Ssn6. It is worth noting that even though *TIP1* is regulated in parallel with the *TIR* genes, it is also controlled by a different pathway, showing no dependence on Mox4 or Mox1, Mox2, or Mot3 for repression by oxygen or induction by cold shock (data not shown). Work in this area has demonstrated that several distinct but interconnected mechanisms are deployed in yeast to achieve essentially the same effect, i.e., regulation by oxygen, targeting genes in several regulons.

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