Thioredoxin Reductase-dependent Inhibition of MCB Cell Cycle Box Activity in Saccharomyces cerevisiae

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Mlu1 cell cycle box (MCB) elements are found near the start site of yeast genes expressed at G1/S. Basal promoters depend on the elements for upstream activating sequence activity. Deletion of MCB elements or MCB reporter genes were found near the start site of yeast genes expressed at G1/S. Basal promoters depend on the elements for upstream activating sequence activity are inactive in ∆swi6 yeast. Yeast were screened for mutations that activated MCB reporter genes in the absence of Swi6. The mutations identified a single complementation group. Functional cloning revealed the mutations were alleles of the trr1 gene encoding thioredoxin reductase. Although deletion of trr1 activated MCB reporter genes, high copy expression did not suppress reporter gene activity. The trr1 mutations strongly (20-fold) stimulated MCB- and SCB (Swi4/Swi6 cell cycle box)-containing reporter genes, but also weakly (3-fold) stimulated reporter genes that lacked these elements. The trr1 mutations did not affect the level or periodicity of three endogenous MCB gene mRNAs (TMP1, RNR1, and SWI4). Deletion of thioredoxin genes TRX1 and TRX2 recapitulated the stimulatory effect of trr1 mutations on MCB reporter gene activity. Conditions expected to oxidize thioredoxin (exposure to H2O2) induced MCB gene expression, whereas conditions expected to conserve thioredoxin (exposure to hydroxyurea) inhibited MCB gene expression. The results suggest that thioredoxin oxidation contributes to MCB element activation and suggest a link between thioredoxin-oxidizing processes such as ribonucleotide reduction and cell cycle-specific gene transcription.

Mlu1 cell cycle boxes or MCBs6 (consensus ACCGCCTNA) are found in the upstream region of budding yeast genes encoding replication enzymes and other proteins preferentially synthesized at the G1/S boundary of the cell cycle (1). Structurally similar elements called Swi4/Swi6 cell cycle boxes or SCBs (consensus CACGAAAA) are found in the upstream region of the HO endonuclease gene, which is also expressed at G1/S (2). Deletion and site-directed mutagenesis has shown that MCB/SCB elements in the upstream regions of TMP1, CDC9, POL1, CLN2, SWI4, and HO are required for efficient gene expression (2–7). Attachment of MCBs or SCBs to basal promoters fused upstream from reporter genes shows that these elements possess G1/S-specific UAS activity (2–4).

Band shift assays using wild type and mutant yeast extracts show that MCBs and SCBs bind a complex containing the transcription factors Swi6 and either Mbp1 or Swi4 (8–11). Band shift assays using purified proteins or in vitro translation products suggest that Swi4 and Mbp1 provide the primary DNA recognition function and that Swi6 enhances the affinity of the complex for its target (10, 12, 13). More recently, another complex that binds SCBs but does not contain Swi6 or Swi4 has been reported (14).

Although the periodicity of MCB-containing genes in vivo and the majority of MCB binding activity in vitro is dependent on an intact MBP1 gene (10), the idea that Mbp1 regulates MCBs whereas Swi4 regulates SCBs may be inaccurate. Overexpression of SWI4 in ∆swi6 yeast activates MCB reporter genes in vivo (15) and binding of Swi4 to SCBs in vitro is efficiently competed by MCB oligonucleotides (12, 13). Thus, Swi4 may have a role in recognizing and regulating both types of cell cycle box elements.

The mechanism linking START and MCB gene induction at G1/S is unresolved. In considering how Swi6 could be activated by START, it has been noted that Swi6 is a phosphoprotein in vivo, contains several potential Cdc28 phosphorylation sites, and can be phosphorylated by human Cdc2 immune complexes in vitro (8). However, site-directed mutations that eliminate all the potential Cdc28 phosphorylation sites in Swi6 do not eliminate MCB gene periodicity (16). SWI4 is maximally expressed at G1/S and contains functional MCB sites in its upstream region (7), suggesting that Swi4 autostimulation may be a key process in inducing MCB gene transcription at G1/S. However, constitutive expression of SWI4 from a heterologous promoter does not eliminate G1/S gene periodicity (17). MCB binding activity measured in band shift assays is mildly periodic, with peak levels at G1/S (4). However, the presence of MCB binding activity, at least as measured in vitro, is not sufficient for MCB gene expression, as MCB binding activity is abundant in α-factor-arrested cells, even though such cells show repressed MCB gene expression (4). In summary, although important cis- and trans-acting elements have been identified, the actual biochemical linkage connecting START and G1/S-specific transcription remains elusive.

To search for additional gene products that participate in activating MCB genes following START, we screened for mutations that allowed efficient expression of MCB reporter genes in ∆swi6 yeast. Analysis of the mutations revealed that thioredoxin reductase represses MCB reporter gene expression, and suggests a model whereby thioredoxin oxidation may contribute to increased transcription of certain genes at G1/S.
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**TABLE I**

| Yeast Strains |
|---------------|
| Strain | Genotype | Source/comments |
| SSC18 | MATa ade2 ura3-1 leu2-3,112 trp1-1 his3-11 16 110 | From C. Price, University of Sheffield. |
| EMY60 | MATa ade2-loc ade3a-100 ura3-1 leu2-3,112 trp1-1 his3-11 16 110 lys2-22 HIS3 can1-100 | From M. Johnston, Washington U. |
| EMY66-6D | Same as EMY60 except MATa Dtr1::LYS2 Dtr1::LEU2 | (39), His auxotroph. |
| W303-1a | MATa ade2-1 ura3 leu2-3 trp1-1 his3-11 16 110 can1-100 | (40), Met auxotroph. |
| BY600 | MATa D56::TRP1 ade2 ura3 leu2-3,112 trp1-1 his3-11 16 110 met1 can1-100 ho::LacZ | (21), Met auxotroph. |
| MY1 | MATa D56::TRP1 ade2 ura3 leu2-3 trp1-1 his3-11 16 110 can1-100 | Selected from W303-1a NPD tetrad. |
| MY2 | MATa D56::TRP1 ade2 ura3 leu2-3 trp1-1 his3-11 16 110 can1-100 | Selected from W303-1a NPD tetrad. |
| MY3 | MATa D56::TRP1 ade2 ura3 leu2-3 trp1-1 his3-11 16 110 can1-100 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY4 | MATa D56::TRP1 ade2 ura3 leu2-3 trp1-1 his3-11 16 110 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY10 | same as BY600 except LEU2::MCB/HIS3 | From M. Johnston, Washington U. |
| MY43 | MATa D56::TRP1 ade2 ura3 leu2-3 trp1-1 his3-11 16 110 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY46 | MATa D56::TRP1 ade2 ura3 leu2-3 trp1-1 his3-11 16 110 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY90 | same as MY10 except Dtr1::LYS2 | Selected from W303-1a NPD tetrad. |
| MY157 | same as BY600 except TRR1::LEU2 | Selected from W303-1a NPD tetrad. |
| MY162 | MATa TRR1::LEU2 ade2 leu2::ara3 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY177 | MATa D56::LEU2 ade2 ura3 trp1-1 his3-11 16 110 met1 can1-100 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY182 | MATa D56::LEU2 ade2 ura3-52/ura3 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY191 | MATa D56::LEU2 ade2 ura3-52/ura3 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY196 | MATa D56::LEU2 ade2-101/ara3 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY199 | MATa D56::LEU2 ade2 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY203 | MATa D56::LEU2 ade2 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY213 | MATa D56::LEU2 ade2 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY233 | MATa D56::LEU2 ade2 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY276 | MATa D56::LEU2 ade2 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY281 | MATa D56::LEU2 ade2 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY282 | MATa D56::LEU2 ade2 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |
| MY283 | MATa D56::LEU2 ade2 trp1-1 ho::LacZ | Selected from W303-1a NPD tetrad. |

*Where the presence of ho::LacZ was uncertain, strains were designated as ho::LacZ. NPD, non-parental diploid. In text, strains labeled with suffix “Z” implies strain was transformed with the MCB/LacZ plasmid.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The 2μ-based plasmids containing the MCB/LacZ and mutMCB/LacZ reporter genes were described previously (4), where they were referred to as pMCB87.3 and pMCB87.3 respectively. pMCB/LacZ contains three 5μl sites, separated and flanked by Xbal sites, which are transformed into the host cell and then cut into the vector for transformation or mutagenesis. The plasmid consensus AGCGGCT was mutated to ATCGGATCCTGTTGTGGTGGAG, which destroys UAS activity in vivo and leads to the formation of an inactive plasmid. The 2μ-based vector pRS305 was obtained from M. Johnston, Washington U. (13), and when used for transformation, was transformed into the yeast strain BY600 with a 2μ-based linearized plasmid pLG3 (15). To obtain a linearized plasmid for transformation, the plasmid pLG3 was cut with Bgl II and inserted into pRS305 vector (20). The resulting plasmid was used in subsequent transformations.

**Strains**—Strains are listed in Table I. Except for differences specified in the text or tables, strains were isogenic to W303. To obtain a wild-type strain, the yeast cell was transformed with a wild-type strain of the appropriate strain (obtained from M. Johnston, Washington U.) to MY179 diploid.

**Diploid**—Strains are listed in Table I. Except for differences specified in the text or tables, strains were isogenic to W303. To obtain a diploid strain, the yeast cell was transformed with a wild-type strain of the appropriate strain (obtained from M. Johnston, Washington U.) to MY179 diploid.
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(MATa ade2 ura3 leu2 trpl his32320 metTr hoLacZ), which itself was derived by crossing YM2061 to MY117 (MATa leu2 ura3 trpl1 metS hoLacZ). MY117 was derived by crossing C537 (MATa ade5 ura3–52 leu2–3 trpl–289) (4) with MY43.

Southern with lys2 mutations were selected by growth on appropriately supplemented minimal sulfate-free YNB agar containing 0.2% aminoadipate, and confirmed by showing that lysine prototrophy was restored by transforming with the LYS2-containing plasmid p8LYS2 (S. Sedgewick, National Institute for Medical Research, London).

Standard yeast genetic techniques were used for tetrad dissections and random spore preparations (22). For lys2 heterozygotes, spores were plated on aminoadipate plates, to select against residual diploid cells. For cells carrying trr1:His3 alleles, it was necessary to plate spores on YEPD.

Mutagenesis and Isolation of trr1 Mutants—Ethylmethanesulfonate (EMS) mutagenesis was done as described by Rose et al. (22). In a pilot experiment, treatment with 30 μg/ml EMS gave optimal results, reducing viability by 50% and increasing the frequency of aminoadipate-resistant clones 25-fold above the control level of 10−6 viable cell. To isolate mutants, 106 MY10Z cells were either treated with 30 μg/ml EMS or mock-treated, and 2 × 106 cells were spread on each of five 10-cm plates containing YNB agar supplemented with adenine, methionine, and 10 mM ATZ. An activated MCB/LacZ reporter gene produces sufficient imidazole dehydrogenase activity to result in ATZ-resistance. By 2.5 days posttreatment, EMS-treated and untreated cells had 50 and 8 ATZ-resistant colonies/plate, respectively. When corrected for the number of viable cells plated in each group (determined by spreading aliquots on histidine-supplemented plates), the frequency of ATZ-resistant cells was 125 × 10−6 in the EMS-treated population and 6 × 10−5 in the mock-treated population. All 250 ATZ-resistant clones from the EMS treatment group were patched to selective plates and assayed for MCB/LacZ reporter gene activation by filter β-galactosidase assay. Sixty-four out of 250 ATZ-resistant colonies gave blue color in the assay, and of these, 30 were randomly selected for further analysis.

Northern and Southern Blot Analyses—RNA was isolated from yeast using glass beads and hot phenol (24). RNA concentration was determined by A260 assuming 1 OD = 40 μg/ml RNA (2–10 μg) was denatured and fractionated by electrophoresis through 1% agarose, 2.2 M formaldehyde gels as described by Lehrach et al. (25) except that 10 mM MOPS, 4 mM sodium acetate, 0.5 mM EDTA was used as buffer. Gels were rinsed 5 min with water, stained 5 min with 1 μg/ml EtBr, rinsed 15 min with water, and blotted overnight to buffer-equilibrated GeneScreen (DuPont) using 10 × SSC (1 × SSC = 0.14 M NaCl, 15 mM sodium citrate, pH 7) as transfer buffer. After UV-cross-linking (1200 J/m2), blots were rinsed in 2 × SSC, 25 mM sodium citrate (pH 7), 2 × SSC, 0.1% SDS, and 0.1 × SSC, 0.1% SDS as transfer buffer. Blots were exposure 1–4 days to x-ray film or PhosphorImager plate. As shown in the fourth column of Table II, all of the diploids assayed for MCB expression, the mutations did not suppress other aspects of a single mutated gene.

RESULTS

Identification of trr1 by Mutations That Activate MCB Reporter Genes—To identify mutations that activated MCB reporter genes in Δswi6 yeast, strain MY10Z, which carried an integrated MCB-dependent His3 reporter gene (MCB/His3) and an episomal MCB-dependent LacZ reporter gene (MCB/ LacZ) was mutagenized with EMS and plated on medium lacking histidine and containing ATZ. Cells that formed colonies on ATZ were assayed for β-galactosidase using a filter assay. Sixty-four out of 250 ATZ-resistant colonies gave blue color. Thirty ATZ-resistant, β-galactosidase-positive mutants were randomly selected for further characterization. The mutants were grown in liquid culture and lysates were assayed quantitatively for β-galactosidase activity. Table II shows β-galactosidase levels in the mutants, arranged in descending order of LacZ activation. As evident from the second column in Table II, β-galactosidase levels varied from 7- to 70-fold over the background levels in Δswi6 parental strain MY10Z.

To assess whether the mutations were recessive, each mutant was mated to Δswi6 yeast strain MY2, and the resulting diploids assayed for β-galactosidase activity. As shown in the third column of Table II, β-galactosidase activity was extinguished in all cases when mutants were mated to nonmutant cells, indicating that all 30 MCB-activating mutations were recessive. When the diploid cells were sporulated, and inheritance of the MCB-activating phenotype was determined by random spore analysis, 50% of the spores showed the mutant phenotype, consistent with the segregation pattern expected for a single mutated gene.

To begin to assign the mutations to complementation groups, a MATa derivative of strain MY74, obtained by sporulating a MY74 × MY2 diploid, was mated to all 30 of the original mutants and the resulting diploids assayed for β-galactosidase. As shown in the fourth column of Table II, all of the diploids showed high β-galactosidase activity. The failure of any diploids to show the low β-galactosidase activity characteristic of nonmutant cells indicated that all 30 mutations were in the same complementation group. In the fifth column of Table II, each mutant allele is identified by a number indicating its relative placement in the array of phenotypes. The various alleles gave varying degrees of MCB reporter gene activation, suggesting that at least the more poorly activating mutations were not null alleles. Although they restored MCB reporter gene expression, the mutations did not suppress other aspects.
of the swi6 phenotype. None of the mutations listed in Table II corrected the abnormal morphology of Δswi6 cells, and at least the 1–21 mutation (the only one tested) did not suppress the synthetic lethality of swi6 swi6 double mutations (data not shown).

Strains with the more strongly activating mutations grew significantly slower than parental cells. Strain MY90 grew with a generation time nearly equivalent to nonmutant parental cells. Therefore MY90 and strains derived from MY90 that carried the 1–21 allele were used in further characterization of the MCB-activating mutation.

The wild type allele of the gene identified by the mutations was cloned by complementation. Strain MY43Z, a derivative of primary isolate MY74. The data show all mutations were recessive and allelic. The alleles were numbered according to how strongly they activated MCB/LacZ expression. Strain genotypes were identical to that listed for MY90 in Table I, except that MY66, 82 and 100 had converted to Met prototrophy during isolation. Strain MY10, the Δswi6 parental cell used for mutagenesis, was included as a negative control. MY10 transformed with a SWI6 allele were used in further characterization of the MCB/LacZ reporter gene activity obtained in the presence of Swi6 protein.

| Strain name | Primary isolate | After mating to nonmutant strain MY2 | After mating to mutant strain MY40a | Allele no. |
|-------------|-----------------|-------------------------------------|-----------------------------------|-----------|
| MY292       | 1218            | 11                                  | 1073                              | 1–1       |
| MY272       | 786             | 11                                  | 204                               | 1–2       |
| MY100       | 723             | 28                                  | 458                               | 1–3       |
| MY66        | 699             | 16                                  | 272                               | 1–4       |
| MY80        | 655             | 17                                  | 369                               | 1–5       |
| MY73        | 351             | 10                                  | 274                               | 1–6       |
| MY87        | 551             | 11                                  | 680                               | 1–7       |
| MY101       | 502             | 11                                  | 600                               | 1–8       |
| MY50        | 493             | 25                                  | 312                               | 1–9       |
| MY91        | 478             | 15                                  | 239                               | 1–10      |
| MY68        | 472             | 15                                  | 282                               | 1–11      |
| MY71        | 469             | 15                                  | 254                               | 1–12      |
| MY79        | 464             | 13                                  | 825                               | 1–13      |
| MY95        | 409             | 9                                   | 256                               | 1–14      |
| MY65        | 361             | 15                                  | 296                               | 1–15      |
| MY81        | 357             | 10                                  | 306                               | 1–16      |
| MY92        | 336             | 13                                  | 507                               | 1–17      |
| MY51        | 326             | 14                                  | 311                               | 1–18      |
| MY70        | 292             | 14                                  | 512                               | 1–19      |
| MY54        | 288             | 24                                  | 292                               | 1–20      |
| MY90        | 281             | 8                                   | 541                               | 1–21      |
| MY78        | 245             | 14                                  | 204                               | 1–22      |
| MY59        | 234             | 14                                  | 235                               | 1–23      |
| MY93        | 216             | 11                                  | 253                               | 1–24      |
| MY60        | 216             | 22                                  | 887                               | 1–25      |
| MY74        | 163             | 10                                  | 136                               | 1–26      |
| MY57        | 138             | 13                                  | 410                               | 1–27      |
| MY61        | 132             | 16                                  | 407                               | 1–28      |
| MY62        | 128             | 13                                  | 332                               | 1–29      |
| MY53        | 122             | 16                                  | 216                               | 1–30      |
| MY10        | 18              | 12                                  | 7                                 | Wild type |
| MY100       | 1140            | ND                                  | ND                                | Wild type |

+pBD177(SWI6)

* Mutants carrying the MCB/LacZ reporter gene were assayed for β-galactosidase activity either directly, or after mating to MY2 (a Δswi6 nonmutant strain) or to MY74a (a MATa by2 derivative of primary isolate MY74). The data show all mutations were recessive and allelic. Strain MY10, the Δswi6 parental cell used for mutagenesis, was included as a negative control. MY10 transformed with a SWI6 plasmid was used as a positive control to show the level of MCB/LacZ reporter gene activity obtained in the presence of Swi6 protein.
formed into MY43Z yeast. Only in yeast transformed with the latter subclone was β-galactosidase activity extinguished, proving that TRR1 possessed the complementing activity.

To establish that TRR1 and the gene identified in the mutant screen were allelic, the chromosomal TRR1 locus was non-disruptively tagged in a nonmutant Δswi6 strain by insertion of a LEU2-marked plasmid. Integration at the TRR1 locus was confirmed by Southern blot analysis. The TRR1:LEU2-tagged strain was mated to mutant strain MY43 and inheritance of the Leu+ and β-galactosidase+ phenotype was monitored. In 16 dissected tetrads and 43 random spores, the Leu+ and β-galactosidase+ phenotype were always inherited reciprocally, thus establishing that TRR1 and the mutation responsible for LacZ reporter gene activation were allelic.

To determine the effect of disrupting TRR1 on cell viability and on MCB reporter gene expression, the TRR1 coding region was transplanted by HIS3 in a diploid strain. Disruption of the TRR1 gene was confirmed by Southern blot analysis. When the heterozygous Δtrr1:HIS3 deletion mutant (MY196) was sporulated and asci were dissected, most tetrads yielded only two or three colonies, two of which were large and His+. In those tetrads that gave four colonies, two were large and His+ and two were small and His+. We concluded that disruption of TRR1 resulted in poor viability, and that in those Δtrr1:HIS3 disruptants that managed to survive, the growth rate was significantly slower than in TRR1 cells. The same pattern was obtained when Δtrr1:HIS3 segregants were backcrossed several times to W303-1. Thus, poor viability and slow growth was intrinsic to the disruption of TRR1 and not due to the segregation of other potential polymorphisms in the diploid used for the gene disruption. The viability problem was even more evident when spores were plated on supplemented minimal plates. When random spores were directly plated on supplemented minimal plates, no Δtrr1:HIS3 spores formed colonies. When the experiment was repeated but spores were allowed to form colonies on YEPD plates and then were replica-plated to supplemented minimal plates, some small Δtrr1:HIS3 colonies were evident, but at much lower frequency than the 50% expected. The results suggested that Δtrr1:HIS3 mutants have trouble germinating on YEPD plates and cannot germinate at all on supplemented minimal medium.

When a dissected tetrad that yielded four viable spores was analyzed to quantitate the effect of deleting TRR1 on cell growth rate, the Δtrr1:HIS3 segregants grew with an average doubling time of 3.1 h, which was 70% longer than the 1.8-h doubling time of TRR1 segregants.

The viability of Δtrr1 null mutants allowed us to test the effect of TRR1 disruption on MCB reporter gene activity. A Δtrr1:HIS3 disruptant (MY199) was mated to Δswi6:TRP1 strain MY2Z, and segregants with the four expected haplotypes were isolated. As shown in Table III, Δtrr1:HIS3 Δswi6:TRP1 segregants showed 50-fold higher β-galactosidase levels than their TRR1 Δswi6 counterparts. An effect of deleting TRR1 was also observed in SW16 cells, where Δtrr1:HIS3 segregants showed 2.5-fold higher β-galactosidase activity than their TRR1 counterparts. In Δswi6 cells, the 50-fold effect of the Δtrr1 deletion mutation on MCB reporter gene expression was roughly equivalent to that of the strongest activating trr1 alleles isolated in the mutant screen (Table II).

As loss of function trr1 mutations restored MCB reporter gene activity in Δswi6 yeast, it suggested that Trr1 protein functions as an inhibitor of MCB element activity. We therefore investigated whether introduction of the TRR1 gene on a high copy plasmid suppressed MCB reporter gene activity in SW16+ yeast. It did not. Wild type yeast transformed with a high copy TRR1 plasmid showed the same level of reporter gene activity as wild type yeast transformed with a control plasmid (data not shown). We concluded that a single normal copy of the TRR1 gene produced sufficient Trr1 protein to maximally suppress reporter gene activity.

Specificity of trr1 Effect on MCB and SCB Activation—Using genetic screens similar to ours, a number of global repressors of transcription have been isolated in yeast (28). A characteristic of global transcriptional repressors is that recessive mutations in the encoding gene result in elevated expression from UAS-less basal promoters such as the Δ178CYC1 promoter. To determine the effect on MCB elements, the Δtrr1:HIS3 Δswi6:TRP1 (MY203) strain was mated to mutant strain MY43 and inheritance of the Leu+ and TRP1 gene was scored. Table IV shows that a single normal copy of the TRR1 gene produced sufficient Trr1 protein to maximally suppress reporter gene activity. A Δtrr1:HIS3 Δswi6:TRP1 (MY209) strain was mated to mutant strain MY43 and inheritance of the Leu+ and TRP1 gene was scored. Table IV shows that a single normal copy of the TRR1 gene produced sufficient Trr1 protein to maximally suppress reporter gene activity.
FIG. 1. Effect of trr1–21 mutation on endogenous MCB gene periodicity in elutrially synchronized cells. Diploid strains MY182 (trr1–21) or MY191 (TRR1) were fractionated by elutriation, and small early G1 cells were inoculated into YEPD and incubated at 30°C. At indicated times, the budding index was determined by scoring 200 cells (A), or RNA was prepared for Northern blot analysis (B). Blots were sequentially hybridized with radiolabeled TMP1, LEU2, RNR1, SWI4, and SWI6 DNA probes. All cells carried the TMP1-encoding 2μ plasmid pEM54, which uses LEU2 as the selectable marker (3). Message produced from the nonperiodically expressed LEU2 gene and SWI6 gene were analyzed to control for plasmid copy number and RNA loading. C, TMP1 mRNA levels, normalized to LEU2 mRNA levels, and RNR1 and SWI4 mRNA levels, normalized to SWI6 mRNA levels, were quantitated and plotted as a function of time after inoculation.

Characterization of MCB Gene mRNA Levels in trr1 Yeast—Having established that trr1 mutations elevated MCB reporter gene activity, we next investigated whether the mutations affected expression of endogenous MCB-containing genes. The TMP1 gene encoding thymidylate synthase is expressed maximally at G1/S and has the best characterized MCB element region (3). TMP1 mRNA was not detectable by the Northern blot method, so a RNase protection assay was used to measure TMP1 mRNA in cells with or without trr1 mutations (data not shown). Inclusion of a standard curve generated using synthetic TMP1 pseudo mRNA allowed absolute quantitation of TMP1 mRNA levels. Assuming 1 pg of total RNA/yeast cell, 5 × 10^6 exponentially growing wild type yeast (strain MY224) or Δswi6 yeast (strains MY1, 10, 216, and 217) yielded about 500 amol of TMP1 mRNA, which was equivalent to about 10 copies of TMP1 message/cell. TMP1 mRNA levels were unchanged in several trr1 mutants selected in the mutant screen (strains MY100, 68, 95, 90, 61, and 62) and in several Δtrr1 deletion mutants (strains MY210, 213, and 214). Northern blots assays were used to determine the activity of three other G1/S-specific, MCB-containing genes: RNR1, CDC9, and SWI4. As with TMP1, no significant change in the level of expression of these genes was associated with the trr1 mutations (data not shown).

The trr1 mutations similarly did not affect the level of expression of five nonperiodic genes: SWI6, BRY1(SGN7), LEU2, URA3, and P1, or the S phase-specific H2A gene (data not shown). Thus, at least as measured in asynchronously growing cells, trr1 mutations that strongly and specifically activated MCB-containing reporter genes did not activate several MCB-containing endogenous genes.

Although the trr1 mutations did not affect endogenous MCB gene mRNA levels, neither did deletion of SWI6, a gene known to encode an important MCB element regulator. Previous studies have also reported little effect of SWI6 deletion on asynchronous cell CDC9, RNR1, and TMP1 mRNA levels (8, 9). However, in these previous studies, deletion of SWI6 did disrupt the normal periodicity of these transcripts. We therefore investigated whether the trr1 mutations affected endogenous

determine whether trr1 mutations enhanced transcription from a CYC1 promoter lacking a functional UAS, the effect of the trr1–21 mutation on expression of a UAS-less Δ178CYC1/LacZ gene or a mutant MCB/LacZ gene, in which the upstream MCB elements were mutated to ACTaGT, was determined. In addition, the effect of the trr1–21 mutation on the expression of a Δ312CYC1/LacZ reporter gene carrying the native CYC1 UAS and on the expression of a SCB/LacZ reporter gene (pBd1390) (29) carrying three upstream SCB elements (consensus CACG-CAAAA) derived from the HO gene was determined. As shown in the top rows of Table IV, the trr1–21 mutation elevated β-galactosidase activity 12–25-fold in cells carrying either the MCB/LacZ or SCB/LacZ reporter gene. In contrast, the trr1–21 mutation elevated β-galactosidase activity only 2–4-fold in cells carrying the Δ312CYC1/LacZ, Δ178CYC1/ LacZ, or mutMCB/LacZ reporter gene. Strains BY600 and MY43 have an integrated ho:LacZ gene. Although this gene is silent in Δswi6 yeast (2), it was possible that the gene was activated in trr1–21 yeast and was contributing to observed β-galactosidase activity. To control for this possibility, β-galactosidase levels were also assayed in cells transformed with a vector (YEp195) lacking any LacZ reporter gene. As shown in the right column of Table IV, β-galactosidase activity in YEp195 transformants was low and unchanging, which indicated that the trr1–21 mutation had no effect on expression of the integrated ho:LacZ gene.

The specificity of TRR1 involvement in MCB activation was also tested in Δtrr1 null mutants. As shown in the lower part of Table IV, the Δtrr1:HIS3 mutation elevated β-galactosidase 17- to 20-fold in cells carrying the MCB/LacZ or SCB/LacZ reporter gene but only 3-fold in cells carrying the mutMCB/LacZ, Δ178/ LacZ or Δ312/LacZ reporter genes. In summary, the data in Table IV indicated that trr1 mutations weakly and nonspecifically activated all reporter genes utilizing the basal CYC1 promoter. However, the trr1 mutations gave an additional, specific, 5-fold activation of reporter gene expression if the basal CYC1 promoter was provided with upstream MCB or SCB elements.
Thioredoxin Reductase Inhibition of MCB Activity

Fig. 2. Analysis of trr1−21 effect on endogenous MCB gene periodicity in cdc15-synchronized yeast. Strains MY283 (TRR1) or MY282 (trr1−21), both of which carried a cdc15 mutation, were arrested at M/G1 by a 3-h incubation at the nonpermissive temperature. At indicated times after release to the nonpermissive temperature, RNA was isolated and analyzed by Northern blot method. Panel A shows blots probed for the G1/S-specific RNR1 transcript. Lanes designated A show RNA from asynchronously growing cells harvested prior to arrest at the nonpermissive temperature. BE, bud emergence. Panel B shows RNR1 mRNA levels normalized to the nonperiodic P1 transcript.

MCB gene periodicity. Strains carrying the trr1−21 allele were selected for study because they grew at wild type rates. Strains carrying the more strongly activating alleles listed in Table II or strains with the Δtrr1 disruption mutation grew slowly, and therefore, perturbations in expression patterns would be more difficult to interpret.) As Δswi6 cells were difficult to synchronize and were already known to show altered DNA synthesis gene periodicity (8, 9), we investigated the effect of trr1−21 on DNA synthesis gene periodicity in SWI6 cells. Cells were synchronized using centrifugal elutriation (4). To improve the size homogeneity of the population, diploid cells were used. Cells that were either trr1−21/trr1−21 homozygotes or TRR1/trr1−21 heterozygotes were analyzed. To facilitate measurement of TMP1 mRNA, both cell types carried the high copy TOM1-encoding plasmid pEM54. Synchrony was monitored by determining the budding index.

As shown in Fig. 1A, bud emergence in both populations began about 60 min after elutriation. Northern blot analysis of RNA from the synchronized cells (Fig. 1B) showed that mRNA levels expressed from three different MCB genes (TMP1, RNR1, and SWI4) remained periodic in the trr1−21 mutant. However, in contrast to the simultaneous onset of budding, peak MCB gene mRNA levels in trr1−21 cells occurred about 15 min earlier than in TRR1 cells. Fig. 1C shows TMP1 mRNA levels, normalized to the nonperiodic LEU2 message, and RNR1 and SWI4 mRNA levels, normalized to the nonperiodic SWI6 message.

The effect of trr1 mutations on MCB gene periodicity was also investigated in cells synchronized by release from a cdc15 block. Fig. 2 shows RNR1 mRNA in cdc15-synchronized trr1−21 or wild type yeast. In both populations bud emergence began 48 min after release from the nonpermissive temperature. As was previously observed for elutrially synchronized yeast, RNR1 mRNA remained periodic in cdc15-synchronized trr1−21 yeast.

We also investigated whether introduction of TRR1 on a high copy plasmid affected endogenous MCB gene periodicity. It did not. The G1/S-specific pattern of RNR1 expression was remarkably similar in cells transformed with a high copy TRR1 plasmid and cells transformed with a control plasmid. The results suggested that overproduction of thioredoxin reductase did not alter MCB gene periodicity.

Mechanism of trr1 Effect on MCB Element Activity—Despite its name, the enzyme thioredoxin reductase may reduce substrates other than thioredoxin. For example, in E. coli, mutations in the TrxB gene encoding thioredoxin reductase, but not the TrxA gene encoding thioredoxin, allow the accumulation of active alkaline phosphatase in the cytosol (30), suggesting that thioredoxin reductase can influence the structure of certain cytosolic proteins by a thioredoxin-independent mechanism. To determine whether trr1 mutations in yeast activated MCB element activity by a thioredoxin-dependent mechanism, MCB reporter gene activity was determined in Δswi6 strains lacking one or both of the S. cerevisiae thioredoxin genes TRX1 and TRX2. As shown in Table V, Δswi6 yeast in which both thioredoxin genes were deleted showed 50-fold higher β-galactosidase activity than Δswi6 yeast in which the thioredoxin genes were intact. Table V also shows that TRX1 and TRX2 were not equivalent in their ability to repress MCB reporter gene activity. Strains in which only TRX2 was deleted showed the same low β-galactosidase activity as Δswi6 cells. In contrast, strains in which only TRX1 was deleted showed substantially higher β-galactosidase activity, although not nearly as high an activity as in cells in which both thioredoxin genes were deleted. The results in Table V show that deleting both thioredoxin genes gave about the same level of MCB/LacZ reporter gene activity as deleting TRR1. Thus, we concluded that the negative effect of thioredoxin reductase on MCB element activity was mediated through thioredoxin.

MCB-controlled genes are activated shortly after START and inactivated in mid-S phase (3, 4). Thus, MCB reporter gene activation in trr1 mutants could be a consequence of disproportional expansion of a cell cycle compartment during which MCB elements are active. For example, if trr1 mutations delayed cytokinesis by prolonging S or G2, daughter cells would be larger and pass through early G1 more quickly. The result would be that more cells would be in the S/G2 phases of the cell cycle than in G1 phase. To investigate the possibility that trr1 mutations disproportionately affected the duration of cell cycle compartments, exponentially growing TRR1 wild type and trr1−21 mutant cells were stained with propidium iodide and analyzed by flow cytometry. As shown in Fig. 3A, in both a SWI6 background (compare top two panels) or Δswi6 back-
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Fig. 3. Flow cytometric analysis of trr1–21 effect on cell cycle distribution and cell size. Exponentially growing yeast with the indicated relevant genotypes were stained with propidium iodide and analyzed for DNA content by fluorescence (F) or cross-sectional area by forward angle light scatter (FALS). The percent of cells with a G2 DNA content is indicated for each population.

If trr1 mutations delayed cytokinesis without affecting mass accumulation, trr1 cells should be larger than TRR1 cells. Forward angle light scattering (FALS) is a measure of the cross-sectional area of an object as it passes the flow cytometer interrogation point. FALS values thus were used to assess any effect of trr1–21 on cell size. As shown in Fig. 3B, the trr1–21 mutation did not affect the modal FALS value of either SWI6 cells (compare top two panels) or Δswi6 cells (compare bottom two panels), indicating that trr1–21 did not increase average cell size. As expected, deletion of SWI6 did increase the modal FALS value (compare top two panels with bottom two panels), consistent with the microscopic observation that Δswi6 cells are larger than SWI6 cells (2).

Based on DNA content and FALS data, the trr1–21 allele did not alter the relative distribution of cells within the cell cycle and did not increase cell size. The trr1–21 mutant was initially selected for characterization because it had a doubling time that was only slightly longer than TRR1 parental cells (2.5 h for MY90 versus 2.2 h for MY10). Other trr1 alleles, that more strongly activated MCB reporter gene expression, resulted in significantly longer doubling times. However, flow cytometric analysis showed that even the most strongly activating trr1 alleles listed in Table II, as well as the Δtrr1 null mutation, caused no shift in the relative proportion of G1 and S/G2 cells (data not shown). We concluded that even in the more slowly growing trr1 mutants, which showed high levels of MCB reporter gene activation, all phases of the cell cycle were expanded equally.

As the thioredoxin reductase/thioredoxin system presumably functions to balance oxidative processes in the cell, we investigated whether trr1 mutations resulted in hypersensitivity to the oxidant H2O2. To assess H2O2 sensitivity, exponentially growing cells were spread on YEPD plates, and a drop of H2O2 was introduced at the center of the plate. After allowing cells to grow for 12–24 h, the diameter of the zone in which growth was suppressed, the halo, was determined. In all yeast strains tested in which the TRR1 gene was intact, halos 3.5–4.5 centimeters in diameter were obtained. In all strains tested in which the TRR1 gene was deleted or mutated, halos 5.5–6.5 centimeters were obtained. An example of the difference in halo diameter between trr1 mutants and wild type cells is shown in Fig. 4. The greater diameter of the halos in trr1 mutants was consistent with the idea that the mutations resulted in greater sensitivity to oxidizing conditions.

The H2O2 halo assays provided further insight into the mechanism by which trr1 mutations activated MCBs. As yeast possess a thioredoxin-dependent peroxidase (27), exposure to H2O2 might be expected to oxidize thioredoxin and allow MCB reporter gene expression in Δswi6 yeast. To test whether H2O2 allowed MCB reporter gene activation in Δswi6 yeast, cells that had been grown in H2O2 halo assays were replica-plated to filters and assayed for β-galactosidase activity. Results of such an assay are shown in Fig. 4. The left-hand panels show control cells not exposed to H2O2. As expected, β-galactosidase activity was high in Δswi6 Δtrr1 cells (strong blue color) and low in Δswi6 cells. The right-hand panels show cells exposed to H2O2. Significantly, in Δswi6 cells, a ring of high β-galactosidase activity was observed in the region where cells were presumably exposed to the highest non-lethal concentration of H2O2. Induction of the MCB/LacZ reporter gene by H2O2 is consistent with the idea that oxidation of thioredoxin results in MCB element activation.

DISCUSSION

Although trr1 mutations activated the MCB/HIS3 reporter and MCB/LacZ reporter genes, they did not noticeably affect

Fig. 4. Effect of hydrogen peroxide on viability and MCB/LacZ gene activity in Δswi6 strains with or without Δtrr1 mutations. Ten minutes after spreading 10⁶ exponentially growing MY2 or MY213 cells on YEPD medium, 5 µl of 30% H2O2 was deposited in the center of each plate. After allowing growth at 30 °C for 24 h, cells were replica-plated to filters and assayed for β-galactosidase activity.
endogenous MCB gene mRNA levels. The lack of effect of \textit{trr1} mutations on \textit{TMP1}, \textit{RNR1}, and \textit{SWI4} mRNA levels is reminiscent of the disparate effect of deleting \textit{SWI6} on reporter gene and endogenous gene expression. Deletion of \textit{SWI6} strongly represses \textit{A178CYC1LacZ} reporter genes that are dependent on either synthetic MCB element clusters (8) or on natural MCB elements as they are found in the context of a 55-base pair fragment of the \textit{TMP1} upstream region (9). In contrast, deletion of \textit{SWI6} has little effect on the levels of several mRNAs encoded by endogenous MCB-containing genes such as \textit{TMP1}, \textit{CDC9}, \textit{POL1}, \textit{RNR1}, \textit{SWI4}, \textit{CLN1} and \textit{CLN2} (8, 9). Foster et al. (7) showed a small but significant effect of deleting \textit{SWI6} on \textit{SWI4} mRNA levels. Despite the lack of a strong effect of deleting the \textit{trans}-acting factor \textit{Swi6}, in cases where it has been examined, mutation of the \textit{cis}-acting MCB sites have a strong negative effect on endogenous gene mRNA levels (3, 7). To summarize, \textit{cis}-acting mutations that destroy MCB sites have strong effects on both endogenous and reporter gene expression, whereas \textit{trans}-acting mutations such as deletion of \textit{SWI6} or mutation of \textit{TRR1} have little effect on endogenous gene expression but strong effects on reporter gene expression.

One plausible model to explain the disparity is that in the absence of \textit{Swi6} protein, MCB elements are targets for both inhibitory and stimulatory mechanisms affecting transcription. In the context of most native promoters, MCB elements may be targeted primarily by the positive-acting mechanism, which results in a moderate level of constitutive transcription. In the context of the heterologous \textit{CYC1} promoter, MCB elements may be targeted primarily by the negative-acting mechanism, which results in a low level of transcription. If the negative-acting mechanism requires thioredoxin reductase for its activity, it would explain why \textit{trr1} mutations activate MCB reporter genes in \textit{Δswi6} yeast.

The above explanation raises the question of whether thioredoxin reductase normally has any role in the regulation of endogenous MCB gene expression. At present, we do not know the answer. Neither deletion nor overexpression of \textit{TRR1} affected the level of expression of any of the endogenous MCB genes monitored. However, Muller (31) has shown that deletion of both yeast thioredoxin genes results in increased \textit{RNR1} and \textit{RNR2} mRNA levels. We were not able to duplicate this result using identical strains and similar conditions, raising the possibility that some aspect of the way in which we grow yeast or analyze mRNA may obscure an effect of thioredoxin or thioredoxin reductase gene mutations on MCB gene mRNA levels. Furthermore, other endogenous MCB genes, outside the subset analyzed in our study, may behave more like the MCB reporter genes in terms of \textit{Swi6}-dependence and \textit{Trr1}-sensitivity.

How might \textit{trr1} mutations activate MCB elements? Thioredoxin reductase regenerates reduced thioredoxin from oxidized thioredoxin using NADPH as electron donor. Diminished levels of reduced thioredoxin in \textit{trr1} mutants could lead to oxidation of regulatory thiols in proteins that either directly or indirectly control MCB element activity. Oxidation may either activate a positive-acting control protein, or inactivate a negative-acting control protein. Redox control of transcription factor activity has been suggested for NFκB (32), Fos/Jun (33), glucocorticoid receptor (34), and the MyoD-interacting protein E2A (35). In these vertebrate examples, protein oxidation is correlated with loss of DNA binding or transcriptional activity. However, in bacteria, oxidation of the OxyR regulatory protein is associated with enhanced transcriptional activity (36). A direct role for thioredoxin in redox control of transcription has been suggested for NFκB (32). An indirect role for thioredoxin has been suggested for Fos/Jun regulation, where the proximal redox effector protein is thought to be Ref1 (33). Recently, in S. pombe, a mutation in a gene encoding thioredoxin reductase was shown to circumvent the cell cycle arrest induced by human p53 (37). Outside the realm of transcription factors \textit{per se}, thioredoxin has been implicated in the folding or conformational regulation of several eucaryotic and prokaryotic proteins (reviewed by Buchanan et al. (38)).

In addition to its activity as a protein disulfide reductase, thioredoxin is the proximal donor of electrons during reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates by ribonucleotide reductase, during reduction of sulfate to sulfite by adenosine 3′-phosphate 5′-phosphosulfate reductase, and during reduction of \textit{H}_2\textit{O}_2 to \textit{H}_2\textit{O} by thioredoxin-dependent peroxidase (27).

In light of the activities of thioredoxin, one intriguing model for thioredoxin involvement in \textit{G}_1/S transcriptional regulation involves ribonucleotide reductase. According to the model, MCB genes may be subject to negative control by a thioredoxin-dependent regulatory protein. In non-\textit{G}_1/S cells the regulatory protein would be maintained in a reduced state by an adequate supply of reduced thioredoxin. However, after replication origins are triggered at \textit{G}_1/S, the cell deoxynucleoside triphosphate (dNTP) pools would be quickly consumed through incorporation into DNA. Muller (31) estimated the dNTP pools in budding yeast to be only 5% of the 6 × 10^7 bases minimally needed to replicate the genome. Freed from dNTP feedback inhibition, ribonucleotide reductase would begin to rapidly convert ribonucleotides to deoxyribonucleotides, quickly depleting the pool of reduced thioredoxin. Proteins with thioredoxin-dependent thiols would become oxidized, triggering conformational changes that either directly or indirectly activate transcription of MCB/SCB-dependent genes. As proteins involved in DNA precursor synthesis accumulate and the dNTP demand becomes satisfied, reduced thioredoxin would begin to reaccumulate, and the thioredoxin-sensitive transcription system would be returned to an off state. In considering how accumulation of DNA precursor synthesizing enzymes could satisfy the dNTP demand, it is noteworthy that the \textit{TRR1} gene itself contains an upstream MCB element and is maximally expressed at \textit{G}_1/S.

As an initial test of the idea that an episode of RNR-mediated thioredoxin oxidation at \textit{G}_1/S may contribute to MCB gene induction, \textit{cdc15}-synchronized cells were incubated in the presence or absence of the RNR inhibitor hydroxyurea (HU), and endogenous MCB gene mRNA levels were measured by Northern blot analysis. Both the nontreated and HU-treated populations began to bud about 45 min after release from the non-permissive temperature, indicating that HU did not block \textit{START} or replication-independent processes downstream from \textit{START}. Coincident with budding, nontreated cells showed a severalfold increase in \textit{RNR1}, \textit{SWI4} and \textit{TRR1} mRNA. The increase in MCB gene mRNA was followed by an increase in \textit{H2A} message, which indicated that the nontreated cells had crossed the \textit{G}_1/S border by 60 min and were actively replicating DNA. In contrast, most of the increase in \textit{RNR1} mRNA and all of the increase in \textit{SWI4} and \textit{TRR1} mRNA did not occur when \textit{cdc15}-synchronized cells were incubated in HU. The increase in \textit{H2A} mRNA also did not occur in the HU-treated population, consistent with the idea that DNA replication was blocked in the absence of DNA precursor biosynthesis. Similar effects of HU on MCB gene induction were observed in α-factor-synchronized cells, as long as the drug was added several minutes before release from the pheromone block. The experiments with HU did not distinguish whether suppression was due to inhibition of ribonucleotide reductase \textit{per se} or was due to inhibition of replication. It is difficult to distinguish experimentally between an effect on RNR activity and an effect on replication.
Drugs or mutations that block replication would also be expected to inhibit RNR activity due to dNTP feedback inhibition, and drugs that inhibit RNR would also be expected to block replication due to exhaustion of dNTP pools. In either case, our results showed that MCB gene induction, unlike other post-START events such as bud emergence, was inhibited by hydroxyurea.

Critical testing of the model that RNR-mediated oxidation of thioredoxin contributes to MCB gene induction at G1/S will require the development of an assay capable of measuring the REDOX state of thioredoxin during the cell cycle. One attractive feature of the model is that it suggests a biochemical mechanism for linking the onset of DNA replication to induc- tion of specific gene transcription at G1/S.

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