AN ABSTRACT OF THE THESIS OF

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MCBs and SCBs are short DNA elements found near the start site of yeast genes that are preferentially expressed in the late G1 phase of the cell cycle. The elements bind complexes containing Swi6 and either Swi4 or Mbp1. Basal promoters dependent on the elements for UAS activity are inactive in *Δswi6* yeast. Mutagenized *Δswi6* yeast carrying MCB-dependent *HIS3* and *LacZ* reporter genes were screened for mutants in which the reporter genes were active. The mutants identified a single complementation group, which was termed *ROM1*, for repressor of MCBs. In addition to specifically activating MCB and SCB reporter genes in *Δswi6* yeast, *rom1* mutations augmented reporter gene expression in *SWI6* yeast. The mutations did not affect asynchronous cell levels of endogenous MCB gene mRNAs, but advanced the time of peak accumulation of these mRNAs in synchronized cells.

A *rom1* mutant was used to screen libraries for plasmids that returned the *LacZ* reporter gene to an off state. All three plasmids isolated contained permutations of the same locus on chromosome IV. Subcloning established that the *TRR1* gene encoding thioredoxin reductase was

responsible for *rom1*-complementing activity. Linkage analysis confirmed *TRR1* and *rom1* were allelic. Cells in which *TRR1* was deleted were viable, but grew slowly, and recapitulated the stimulatory effect of *rom1* mutations on MCB reporter gene expression, as did deletion of thioredoxin genes *TRX1* and *TRX2*. The results suggest that thioredoxin oxidation directly or indirectly activates MCB elements and suggest a link between thioredoxin-oxidizing processes such as ribonucleotide reduction and cell cycle specific gene transcription at G1/S.

Thioredoxin Reductase-Dependent Repression of MCB Cell Cycle Box Elements in *Saccharomyces cerevisiae*

by

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CHAPTER 1

Introduction

The cell cycle

The eukaryotic cell cycle is broken into four temporally distinct phases: S, G2, M and G1. After undergoing chromosomal replication during S phase, cells continue to accumulate mass during G2 phase, undergo mitosis and cell division (cytokinesis) during M phase, and then enter G1 phase. During the G1 phase, cells monitor intracellular and extracellular signals and if conditions are appropriate for cell-cycle progression, an irreversible decision is made and cells commit to a new round of replication. Alternatively, if conditions are inappropriate, G1 cells withdraw from the cell cycle (reviewed by Norbury and Nurse, 1992). For all eukaryotes, both multicellular and unicellular, the ability to correctly decide to enter or exit the cell cycle is vital for its survival.

In yeast, commitment to enter a new cell cycle is called START (reviewed by Johnston and Lowndes, 1992; Merrill *et al.*, 1992; Nasmyth, 1993: McIntosh, 1993; Küntzel *et al.*, 1994). Low nutrient levels or the presence of mating pheromones will cause withdrawal from the cell cycle. Once START has been traversed, nutrient deprivation or mating pheromones do not prevent another round of DNA synthesis and division. In vertebrate cells a similar decision to commit to a new cell cycle occurs

in late G1 and is referred to as the restriction point (Pardee *et al.*, 1978). In vertebrate cells in culture, extracellular conditions that prevent restriction point traversal include serum deprivation or removal of purified growth factors. Similar to yeast, once the restriction point has been traversed, serum or growth factor removal does not prevent another round of replication.

After committing to another cell cycle, and before entering S phase, eukaryotic cells prepare for efficient and accurate genome replication during an interval termed late G1 or G1/S phase. In yeast, once START has been traversed, three parallel pathways are initiated, leading to the replication of chromosomes, the duplication of the spindle pole body (SPB), and the formation of a new bud (Pringle and Hartwell, 1981). One aspect of this preparation is the enhanced transcription of genes encoding proteins needed for DNA synthesis. The research described herein is focused on induction of DNA synthesis gene expression in late G1, in the yeast *Saccharomyces cerevisiae*.

Saccharomyces cerevisiae, a model eukaryote

The budding yeast, *Saccharomyces cerevisiae*, is widely recognized as a model microorganism for studying eukaryotic cells. It has many parallels with higher organisms, and is the only eukaryote to date to have its total genome sequenced (Williams, 1996). It provides both ease and power in biochemical and genetic manipulations. Some of the properties that make *S. cerevisiae* particularly suitable for genetic studies include the existence of both stable haploids and diploids, rapid growth, clonability, ease of replica plating, mutant isolation, and the ability to obtain segregated

spores through ascus dissection. More recently, the development of DNA transformation has made yeast particularly accessible to gene cloning and genetic engineering techniques. Plasmid can be introduced into yeast cells either as replicating molecules or by integration into the genome. In contrast to most other organisms, integrative recombination of transforming DNA in yeast generally occurs via homologous recombination, allowing for easy gene disruption, tagging or replacement. Most of the advantages associated with working with *S. cerevisiae* were exploited in the research described herein.

DNA synthesis genes

In *S. cerevisiae* many genes involved in DNA synthesis have been identified. Some are genes directly involved in DNA synthesis. They are genes that participate in DNA precursor formation such as *TMP* (thymidylate synthase) and *RNR1* (ribonucleotide reductase subunit 1) and participate in DNA replication such as *POL1* (DNA polymerase I) and *CDC9* (DNA ligase). Others are genes which act in late G1 and are involved in the regulation of DNA synthesis initiation. These regulatory genes have been reviewed (Johnston and Lowndes, 1992; Nasmyth, 1993; Küntzel *et al.*, 1994), and are going to be discussed briefly. As earlier discussed, after START, yeast cells initiate DNA replication, duplicate their SPB, and form buds. Activation of the cyclin-dependent kinase (CDK) Cdc28 by the G1 cyclins Cln1, Cln2, and Cln3 is needed for timely execution of START (reviewed by Reed, 1992). Subsequent to START, the B-type cyclins Clb5 and Clb6 associated with Cdc28 are directly required for entry into S phase (Epstein and Cross, 1992; Schwob and

Nasmyth, 1993). Accumulation of Cln1,2/Cdc28 kinases is necessary for activating *CLB5,6* gene transcription and triggering proteolysis of Sic1, an inhibitor of Clb5,6/Cdc28 kinases (Schwob *et al.*, 1994). Transcription of *CLN1,2* and *CLB5,6* peaks around START (Cross, 1990; Epstein and Cross, 1992; Richardson *et al.*, 1992; Schwob and Nasmyth, 1993), whereas *CLN3* mRNA and protein remain constant during the cell cycle (Tyers *et al.*, 1993). The G1 cyclins Pcl1 and Pcl2 associate with CDK Pho85 and might also have a role at START (Espinoza *et al.* 1994; Measday *et al.*, 1994). Sic1 proteolysis depends on three genes: Cdc34, Cdc4, and Cdc53 (Schwob *et al.*, 1994). *CDC34* encodes an E2-type ubiquitin-conjugating enzyme. The functions of the Cdc4 and Cdc53 proteins are currently not understood. *DBF4* encodes an activator of Cdc7 protein kinase which is required for the initiation of DNA replication (Kitada *et al.*, 1992). Cdc46 controls the initiation of replication together with three other proteins (Cdc45, Cdc47, and Cdc54), by interacting with autonomously replicating sequence (ARS) regions of the genome (Hennessy *et al.*, 1990; Hennessy *et al.*, 1991; Chen *et al.* 1992). Cdc46 protein shows sequence similarity to Mcm2 and Mcm3, two other proteins important for ARS activity. The *CDC6* gene product is another protein involved in the control of initiation of replication (Pringle and Hartwell, 1981).

MCB elements and cell cycle-dependent transcription

In *S. cerevisiae* most genes encoding DNA replication enzymes are transcribed shortly after START at the G1/S boundary of the cell cycle. This pattern of transcription is dependent on an upstream activation sequence (UAS) element, usually found within a few hundred base pairs of

the translation start codon, that is common to these genes (McIntosh *et al.* 1991; Johnston and Lowndes, 1992). The conserved core of this element is a hexameric sequence (5'-ACGCGT-3') that, coincidentally, corresponds to the recognition site of the restriction endonuclease *Mlu1*. Thus, this consensus is referred to as a *Mlu1* Cell-cycle Box, or MCB element. A list of *S. cerevisiae* genes containing putative MCB sequences in their promoter region is shown in Table 1. DNA fragments from three DNA synthesis genes, *TMP1*, *POL1* and *CDC9*, which contain MCB elements, confer late G1 expression on heterologous reporter genes (Lowndes *et al., 1991*; McIntosh *et al.*, 1991; Gordon and Campbell, 1991). Deletions and point mutations within the MCB elements of *TMP1*, *POL1* and *CDC9* promoters abolishes periodic expression of these genes (Lowndes *et al., 1991*; McIntosh *et al.*, 1991; Verma *et al.*, 1991).

An alignment of 32 MCB like sequences from 18 periodically expressed late G1 genes showed the core MCB element to be (5'-ACGCGT _ R-3') (where empty space is any nucleotide and R is a purine, usually A) (Johnston and Lowndes, 1992). An even longer consensus (5'-GTGACGCGT _ A _ _ T-3') has also been suggested (McIntosh, 1993). It is important to note that not all MCB like elements used in the alignment have been shown to be *bona fide* MCB elements in the context of their native promoter. For example, the *TMP1* promoter has two potential MCB elements, but only the distal one (from the start codon) proved to confer cell cycle regulation (McIntosh *et al.*, 1991). Only one MCB element is needed in the promoter of *TMP1* and *POL1* for these genes to achieve near normal levels of transcription *in vivo* (McIntosh *et al.*, 1991; Gordon and Campbell, 1991). Insertion of the MCB consensus into a heterologous promoter has shown that the consensus alone is sufficient for

Table 1. *Saccharomyces cerevisiae* genes expressed in late G1 that have MCB and SCB elements in their promoter. Most of the genes are involved in DNA synthesis and its regulation. They were grouped according to their function in DNA synthesis. References to the DNA sequences of these genes are given in McIntosh (1993) and Johnston and Lowndes (1992).

Gene	Gene function	UAS
dNTP synthesis		
TMP1	Thymidylate synthase	MCB
CDC8	Thymidylate kinase	MCB
RNR1	Ribonucleotide reductase subunit 1	MCB
TRR1	Thioredoxin reductase	MCB
DNA polymerization		
POL1	DNA polymerase I	MCB
POL2	DNA polymerase II	MCB
DPB2	DNA polymerase II subunit B	MCB
DPB3	DNA polymerase II subunit C	MCB
POL3	DNA polymerase III	MCB
POL30	PCNA (replication factor)	MCB
PRI1	DNA primase I	MCB
PRI2	DNA primase II	MCB
Other DNA replication genes		
RFA1	Origin-binding protein	MCB
RFA2	Origin-binding protein	MCB
RFA3	Origin-binding protein	MCB
CDC9	DNA ligase	MCB
TOP2	Topoisomerase II	MCB
CDC6	Initiation of replication	
DBF4	Cofactor of Cdc7 kinase	MCB
SWI4	Activator of *HO* and G1 cyclins	MCB
Cyclins		
CLN1	G1 cyclin	MCB, SCB
CLN2	G1 cyclin	MCB, SCB
PCL1	G1 cyclin	SCB
PCL2	G1 cyclin	SCB
CLB5	B-type cyclin	MCB
CLB6	B-type cyclin	MCB
Other genes		
HO	Endonuclease	SCB

G1/S regulation. A single copy of the sequence 5'-ACGCGTTAA-3' confers G1/S regulation to the constitutive *CYC1* minimal promoter (McIntosh *et al.*, 1991), and whereas one ACGCGT hexamer has little activity, three tandemly arrayed ACGCGT hexamers have strong UAS activity and cause reporter gene mRNA levels to increase in late G1 (Lowndes et al., 1991). The expression of reporter gene constructs tends to increase in proportion to the number of MCB elements placed upstream of the gene (Lowndes *et al.*, 1991; Pizzagalli *et al.*, 1992; Verma *et al., 1992*). The fact that most of the genes listed in Table 1 contain one or more MCB elements may indicate that the number of these elements dictates the relative level of expression of the adjacent gene. Therefore the number of MCB elements, their proximity to each other, their homology to the consensus, and their distance from the start codon will all influence the level of transcription. Thus, a genes like *TMP1*, which contains only two MCB elements, might be expressed at a lower level in vivo than other genes, such as *RNR1*, which contains several MCB elements (Elledge et al., 1992).

Apart from the MCB element, only one other UAS element has been identified to confer G1/S-specific transcription in *S. cerevisiae*. The SCB (Swi4,6-dependent Cell-cycle Box) element (consensus 5'-CACGAAAA-3') was originally identified in the promoter of the *HO* endonuclease gene, which is periodically expressed in late G1. *HO* gene expression is dependent on two genes, *SWI4* and *SWI6*, which encode transcription factors that bind to the SCB element (Breeden and Nasmyth, 1987; Andrew and Herskowitz, 1989). The SCB element has since been found in the promoter region of the G1 cyclin genes *CLN1*, *CLN2* , *PCL1, and PCL2* (Breeden and Nasmyth, 1987; Ogas *et al.* 1991; Espinoza *et al.* 1994).

Table 1 lists genes that contain putative SCB sequences. Deletion and site-directed mutagenesis has shown that SCB elements in the upstream regions of *CLN2* and *HO* are required for efficient gene expression (Stuart and Wittenberg, 1994; Cross *et al.*, 1994; Breeden and Nasmyth, 1987). Attachment of SCBs to basal promoters fused upstream from reporter genes shows that these elements possess G1/S-specific UAS activity (Breeden and Nasmyth, 1987).

Transcription factors that bind to MCB and SCB elements

Band shift assays using wildtype and mutant yeast extracts show that SCB elements bind the SCB binding factor (SBF) composed of the transcription factors Swi6 and Swi4 (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989; Nasmyth and Dirick, 1991; Taba *et al.* 1991) and MCB elements bind the MCB binding factor (MBF) composed of the transcription factors Swi6 and Mbp1 (Lowndes *et al.*, 1992; Dirick *et al.*, 1992; Koch *et al.*, 1993). MBF is also known as DSC1 (Lowndes *et al.*, 1991). Band shift assays using purified proteins or *in vitro* translation products indicate that Swi4 and Mbp1 provide the primary DNA recognition function and that Swi6 enhances the affinity of the complex for its target (Primig *et al.*, 1992; Sidorova and Breeden, 1993; Koch *et al.*, 1993). Although it is often implied that Mbp1 binds MCBs and Swi4 binds SCBs, it may be more accurate to view these proteins as having affinities for both consensi. A hybrid consensus sequence (CRCG – – A) has been proposed (Koch *et al.*, 1993). Although the bulk of the MCB binding activity in yeast extracts is dependent on an intact *MBP1* gene and G1/S gene periodicity is disturbed in Δ*mbp1* yeast (Koch *et al.*, 1993), binding

of Swi4 to SCBs is competed by MCB oligonucleotides (Dirick *et al.*, 1991; Primig *et al.*, 1992; Sidorova and Breeden, 1993). Although Swi4 and Mbp1 are structurally very similar and Δ*swi4* Δ*mbp1* double mutations give synthetic lethality, Swi4 appears to have functions lacking in Mbp1. Overexpression of *SWI4* in Δ*swi6* yeast activates MCB reporter genes (Morgan *et al.*, 1995), whereas overexpression of *MBP1* does not (Merrill, unpublished). Also, Δ*swi4* deletion mutants grow more slowly than wildtype cells and have an abnormal morphology (Breeden and Nasmyth, 1987), whereas Δ*mbp1* deletion mutants do not. Finally, Δ*swi6* Δ*swi4* double mutations give synthetic lethality (Breeden and Nasmyth, 1987), whereas Δ*swi6* Δ*mbp1* double mutations do not (Koch *et al.*, 1993).

Connection of the MCB activation system to START

The mechanism linking START and MCB gene induction at G1/S is unresolved. START has been proposed to consist of a positive feedback loop involving G1 cyclin/Cdc28-dependent activation of Swi4/Swi6 as transcription factors, and Swi4/Swi6-dependent stimulation of G1 cyclin gene transcription (Cross and Tinkelenberg, 1991; Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). In considering how Swi6 activity might be regulated by Cdc28, it has been noted that Swi6 contains several potential Cdc28 phosphorylation sites, is a phosphoprotein *in vivo*, and can be phosphorylated *in vitro* by Cdc28 (Lowndes *et al.*, 1992). However, Sidorova and Breeden (1994) showed that mutation of all of the Cdc28 potential phosphorylation sites in Swi6 does not eliminate G1/S gene periodicity. *SWI4* is maximally expressed at G1/S and contains functional MCB elements in its upstream region (Foster *et al.*, 1993), suggesting that

Swi4 autostimulation may be a key process in inducing MCB gene transcription at G1/S. However, Breeden and Mikesell (1994) showed that constitutive expression of *SWI4* from a heterologous promoter does not eliminate G1/S gene periodicity. MCB binding activity measured in band shift assays is mildly periodic, with peak levels at G1/S (Lowndes *et al.*, 1991). However, Lowndes *et al.* (1991) showed that MCB binding activity is abundant in α-factor arrested cells, even though such cells show repressed MCB gene expression. Thus, MCB binding activity, at least as measured *in vitro*, is not sufficient for MCB gene induction. Deletion of *SWI6* disrupts the normal periodicity of *TMP1*, *RNR1*, *CLN1*, *CLN2* and *CDC9* mRNA (Lowndes *et al.*, 1992; Dirick and Nasmyth, 1992). However, Breeden and Mikesell (1994) showed that *SWI6* deletion only ameliorates and does not completely eliminate G1/S periodicity of *CLN1* and *CLN2* mRNA. Thus, although important *cis*- and *trans*-acting elements have been identified, the actual biochemical link 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 Δ*swi6* yeast. Analysis of the mutations revealed that thioredoxin reductase represses MCB gene expression, and suggests a model whereby thioredoxin oxidation at the onset of DNA replication may stimulate G1/S gene transcription.

CHAPTER 2

Materials and Methods

Plasmids

The 2μ-based plasmids containing the MCB/*LacZ* and mutMCB/*LacZ* reporter genes were described previously (Lowndes *et al.*, 1991), where they were referred to as pLGΔ178.3M and pLGΔ178.3mut, respectively. MCB/*LacZ* contains three *Mlu*1 sites, separated and flanked by *Xho*1 sites, and cloned into the *Xho*1 site at *CYC1* base -178 (with respect to the start codon) in plasmid pLGΔ178 (Guarante and Mason, 1983). In mutMCB/*LacZ*, the MCB consensus ACGCGT was mutated to ACtaGT, which destroys UAS activity *in vivo* and band shifting activity *in vitro* (Lowndes *et al.*, 1991). In Δ312/*LacZ* (plasmid pLGΔ312, Guarente and Mason, 1983) the 5' flanking sequence includes the native *CYC1* UAS. In SCB/*LacZ* (plasmid pLB178-43, Breeden and Nasmyth, 1987) three SCB elements from the *HO* 5' region are fused upstream of the Δ178*CYC1* promoter. The plasmid pBd177, obtained from L. Breeden (Fred Hutchinson Cancer Research Center), consists of a 3-kb HindIII/BglII *SWI6* fragment cloned into pZUC. The plasmid p3-24 consists of a 5-kb *Sau*3a partial fragment containing *SWI4* isolated from yeast library YL3 during a screen for high copy activators of MCB reporter genes in Δ*swi6* yeast (unpublished). The plasmid pGAD2F was the parental vector used in constructing the YL1, YL2 and YL3 Gal4 fusion protein libraries (Chien *et al.*, 1992).

The plasmid pMCB/*HIS3* (Figure 1) was constructed in two steps. First, a 317-bp fragment containing three tandemly linked MCB elements

Figure 1. Diagram of integrative MCB-dependent *HIS3* reporter gene plasmid pMCB/*HIS3*. Salient features of the plasmid are the presence of three MCB elements (sequence ACGCGT) upstream from the Δ178*CYC1* basal promoter, the presence of an intact *HIS3* protein coding region downstream from the *CYC1* promoter, the absence of a potential "translation-inhibiting" upstream ATG present in many other *HIS3* reporter genes, and the unique *Hpa*1 site in the *LEU2* gene used for plasmid linearization and efficient integration at the chromosomal *leu2* locus. Superscript numerals refer to distance in basepairs with respect to native ATG start codon of each gene.

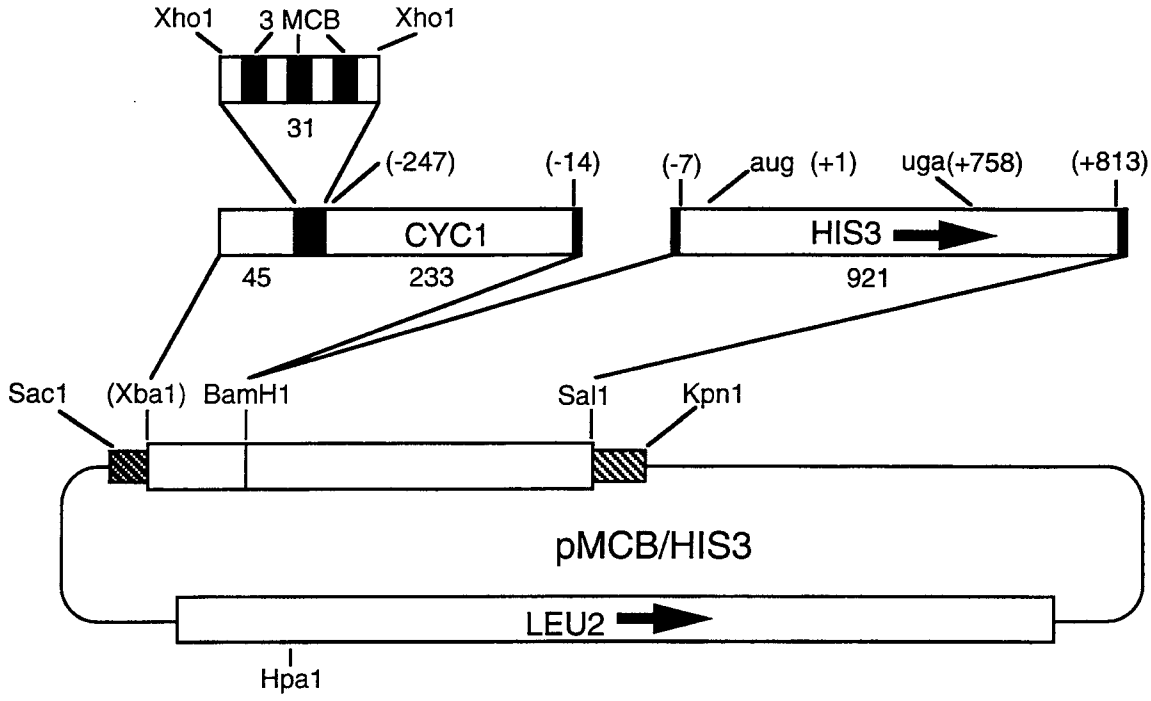
Xho1
3 MCB
Xho1
31
(-247)
(-14)
(-7)
aug
(+1)
uga(+758)
(+813)
CYC1
HIS3
45
233
921
Sac1
(Xba1)
BamH1
Sal1
Kpn1
pMCB/HIS3
LEU2
Hpa1

Figure 1.

and the *CYC1* basal promoter was generated by PCR using *Xba*1-linearized MCB/*LacZ* plasmid as template and the oligonucleotides 5'-CTAAACTCA CAAATTAGAG and 5'-CGGGATCCTGTGTATTTGTGTTTGG as primers. The latter primer introduced a terminal *BamH*1 site 3' to *CYC1* base -14 (with respect to the start codon). The PCR product was cut with *BamH*1 and inserted into pRS305 vector (Sikorski and Hieter, 1989) that had been cut with *Xba*1, filled-in with T4 DNA polymerase and cut with *BamH*1, thus creating an interim plasmid containing the MCB/*CYC1* promoter. A second PCR product, extending from -7 to +813 with respect to the *HIS3* start codon was generated using *EcoR*1-linearized pRS303 (Sikorski and Hieter, 1989) as template and the oligonucleotides 5'-CGGG ATCCGGCAAAGATGACAGAGC and 5'-GCCGTCGACGCGCGCCTCG TTCAGAATG as primers. The first primer introduced a terminal *BamH*1 fragment 5' to *HIS3* base -7 and the second primer introduced a *Sal*1 site 3' to *HIS3* base +813. The second PCR product was cut with *BamH*1 and *Sal*1 and inserted into a vector prepared by cutting the interim plasmid with *BamH*1 and *Sal*1. Functionality of the MCB/*CYC1* promoter and *HIS3* coding region in the resulting plasmid, pMCB/*HIS3*, was confirmed by transforming *Hpa*1-linearized pMCB/*HIS3* into W303-1*a* using *LEU2* selection and confirming that transformants grew in the absence of histidine and the presence of 50 mM 3-amino-1,2,4-triazole (ATZ). ATZ inhibits His3 protein (imidazoleglycerol-P dehydratase) by competing with its normal substrate (imidazoleglycerol-P). Thus the level of *HIS3* gene expressed is directly proportional to the yeast survival rate in increasing ATZ concentrations.

The *S. cerevisiae* genomic DNA libraries used in this study were the high copy, 2μ-based, Gal4 fusion protein libraries YL1, YL2, and YL3

(Chien *et al.*, 1992), and the single copy, *ars/cen*-based library YpH1 (obtained from P. Heiter).

Yeast strains

Strains are listed in Table 2. To obtain a Δ*swi6:TRP1* strain that lacked an integrated *ho-LacZ* gene and lacked uncharacterized *ade* and *met* mutations present in BY600, BY600 was mated to W303-1*a*. With the knowledge that the integrated *ho-LacZ* gene is not active in *swi6*$^-$ yeast but is active in *SWI6*$^+$ yeast, diploids were sporulated, and dissected tetrads were assayed for ß-galactosidase and nutritional auxotrophies. Tetrads yielding two *TRP*$^+$ ß-galactosidase-negative colonies and two *trp*$^-$ ß-galactosidase-positive colonies were used to identify Δ*swi6:TRP1* yeast that had lost the *ho-LacZ* gene. From these nonparental ditype tetrads, strains MY1001 and MY1003 were selected based on the additional criteria of methionine prototrophy and red color. (In *ade2* yeast a red colored intermediate accumulates in the adenine anabolic pathway and the yeast colony has a dark red color, if other *ade* mutations acting upstream from *ade2* are present, the red intermediate does not accumulate and the yeast colony has a wildtype white color. Thus red color indicates an *ade2* mutation unaccompanied by the other *ade* mutation.)

Strains with *lys2* mutations were selected by growth on appropriately supplemented ammonium sulfate-free YNB agar containing 0.2% DL-α-aminoadipate (α-AA), and confirmed by showing that lysine prototrophy was restored by transforming cells with the *LYS2*-containing plasmid p8LYS2 (S. Sedgewick, National Institute for Medical Research). Strains carrying mutations in the *LYS2* gene are able to grow on α-aminoadipate

Table 2. Yeast Strains

Strain	Genotype[a]	Source/Comments
CG378	*MATa ade5 ura3-52 leu2-3 trp1-289*	Lowndes *et al.* (1991)
W303-1*a*	*MATa ade2-1 ura3 leu2-3 trp1-1 his3-11,-15 can1-100*	R. Rothstein
W303-1α	*MATα ade2-1 ura3 leu2-3 trp1-1 his3-11,-15 can1-100*	R. Rothstein
BY600	*MATa Δswi6:TRP1 ade2 adeX ura3 leu2-3,-112 trp1-1 his3 metS can1-100 ho:LacZ-46*	Breeden & Nasmyth (1987)
BY604	*MATα Δswi4:LEU2 ade2 adeX ura3 leu2-3,-112 trp1-1 his3 metS can1-100 ho:LacZ-46*	Breeden & Nasmyth (1987)
MY1000	same as BY600 except *LEU2*:MCB/*HIS3*	MCB/*HIS3* integrated at *leu2*
MY1001	*MATα Δswi6:TRP1 ade2 ura3 leu2 trp1-1 his3*	spore from BY600 x W303-1α NPD tetrad
MY1002	same as MY1004 except *lys2*	selected from MY1004 on aminoadipate
MY1003	same as MY1001 except *MATa*	spore from BY600 x W303-1α NPD tetrad
MY1004	same as MY1001 except *ho:lacZ-46*	spore from BY600 x W303-1α NPD tetrad
YM2061	*MATa ade2-101 ura3-52 LEU2:GAL1/LacZ his3Δ200 metT lys2-801*	M. Johnson
MY2090	*MATa rom1-21 Δswi6:TRP1 ade2 adeX ura3-52 LEU2*:MCB/*HIS trp1-1 his3 metS can1-100 ho:LacZ-46*	selected from mutagenized MY1000 by MCB reporter gene activation

Table 2. (Continued)

MY2043	*MATα rom1-21 Δswi6:TRP1 ade2 adeX ura3-52 leu2-3 trp1-1 his3 lys2 ho:LacZ-46*	random spore from MY2090 x MY1002 diploid
MY2046	*MATa rom1-21 ade2 ura3-52 leu2-3 trp1-1 his3 ho:LacZ-46*	spore from MY2043 x W303-1*a* NPD tetrad
MY2162	*MATα Δswi6:TRP1 ade2 adeX leu2 ura3 trp1-1 ±ho:LacZ-46*	spore from MY2043 x W303-1*a* NPD tetrad
MY2171	*MATα leu2 ura3 trp1 metS ho:LacZ-46*	random spore from CG378 x MY2043 diploid
MY2177	*MATα rom1-21 Δswi6:TRP1 ade2 adex ura3 leu2 trp1 his3 metS*	random spore from W303-1a x MY2043 diploid
MY2179	*MATα ade2 ura3 leu2 trp1 his3Δ200 metT ho:LacZ-46*	random spore from YM2061 x MY2171 diploid
MY2182	*MATa/α rom1-21/rom1-21 SWI6/Δswi6:TRP1 ade2/ade2 ura3-52/ura3-52 leu2-3/leu2-3 trp1-1/trp1-1 his3/his3 metS/metS ho:LacZ-46/ HO*	MY2046 x MY2177 diploid
MY2191	*MATa/α rom1-21/ROM1 SWI6/Δswi6:TRP1 ade2/ade2 ura3-52/ura3-52 leu2-3/leu2-3 trp1-1/trp1-1 his3/his3 metS/METS ho-LacZ-46/±ho:LacZ-46*	MY2046 x MY2162 diploid
MY2183	*MATa/α TRR1/TRR1 ade2-101/ade2 ura3-52/ura3-52 LEU2:GAL1/LacZ/leu2 TRP1/trp1 his3Δ200/his3Δ200 metT/metT lys2-801/lys2-801* HO/*ho:LacZ-46*	YM2061 x MY2179 diploid
MY2196	same as MY2183 except *TRR1* allele disrupted by *HIS3*	this study, transformant #11

Table 2. (Continued)

MY2197	same as MY2183 except *TRR1* allele disrupted by *HIS3*	this study, transformant #6
MY2199	*MATa Δtrr1:HIS3 ade2 ura3-52 LEU2:GAL1-Z trp1 his3 Δ200 met lys2-801*	from MY2196 tetrad
MY2202	*MATa Δtrr1:HIS3 ade2 adeXura3-52 leu trp1 his3 Δ200 metT lys2-801 ho:lacZ-46*	form MY2196 tetrad, this study segregant A2
MY2203	*MATa/α Δtrr1:HIS3/TRR1 ade2/ade2 ura3-52/ura3-52 LEU2:GAL1-Z/leu2 his3Δ200/his3 trp1/trp1 SWI6/Δswi6:TRP1 met/MET lys2-801/LYS2*	MY1001 x MY2199 diploid
MY2209	*MATα Δtrr1:HIS3 Δswi6:TRP1 ade2 ura3 leu2 trp1 his3 met*	random spore from MY2203
MY2221	*MATα Δtrr1:HIS3 ade2 ura3 leu2 trp1 his3*	random spore from MY2203
SSC18	*MATa ade2 ura3-1 leu2-3,112 trp1-1 his3-11 cdc15-1^{ts}*	Price (1991)
MY2226	*MATα ade2 ura3 leu2 trp1 his3 mets cdc15-1^{ts}*	random spore from MY2209 x SSC18 diploid
MY2157	same as BY600 except *TRR1:LEU2*	*LEU2* integrated at *TRR1* locus
MY2233	*MATα ade2 ura3 leu2 trp1 his3 met^{s} Δswi6:TRP1 TRR1:LEU2 ho:LacZ-46*	random spore from MY2043 x MY2157 diploid
MY2281	*MATα ade2 ura3 leu2 trp1 his3 TRR1:LEU2 cdc15-1^{ts} ±ho:LacZ-46*	random spore from MY2233 x SSC18 diploid

Table 2. (Continued)

MY2282	*MATα ade2 adex ura3 leu2 trp1 his3 trr1-21 cdc15-1^{ts} ±ho:LacZ-46*	random spore from MY2281 x MY2046 diploid
MY2283	*MATα ade2 adex ura3 leu2 trp1 his3 TRR1:LEU2 cdc15-1^{ts} ±ho:LacZ-46*	random spore from MY2281 x MY2046 diploid
EMY60	*MATa ade2 ade3 ura3-1 leu2-3,112 trp1-1 his3-11 lys2 can1-1*	Muller (1994)
EMY63	*MATa Δtrx1:TRP1 Δtrx2:LEU2 ade2 ade3 ura3-1 leu2-3,112 trp1-1 his3-11 lys2 met can1-1*	Muller (1994)
EMY56-5D	*MATα Δtrx1:LYS2 Δtrx2:LEU2 ade2 ade3 ura3-1 leu2-3,112 trp1-1 lys2 met can1-1*	Muller
MY2257	*MATa Δtrx1:LYS2 Δtrx2:LEU2 Δswi6:TRP1 ade2 ade3 ura3-1 leu2-3,112 trp1-1 lys2 met can1-1 ho:LacZ-46*	random spore from BY600 x EMY56-5D diploid
MY2276	same as MY1003 except *lys2*	selected from MY1003 on aminoadipate

[a] Two different genes responsible for Met auxotrophies in the strains are designated *metS* (from BY600) and *metT* (from YM2061). The presence or absence of *ho:LacZ-46* in all of the strains derived from *BY600* was confirmed experimentally, except for some strains in which *ho:LacZ-46* status remained unknown and they were designated as ±*ho:LacZ-46*. The status of *can1-1* in MY1000 and all its derivatives was not determined. NPD = non parental ditype. In text, strains labeled with suffix Z implies strain was transformed with MCB/*LacZ* plasmid.

as sole nitrogen source, whereas *LYS*+ strains are not. This difference in growth provides the basis for positive selection for *lys2* mutants (Chatoo *et al.*, 1979).

Strain MY1000 was derived by transforming BY600 with *Hpa1*-linearized pMCB/*HIS3* (Figure 1) and selecting for leucine prototrophy. Integration at *leu2* was confirmed genetically by mating MY1000 to W303-1*a* and showing by random spore analysis that all *leu*- spores were also *his*- and all *LEU*+ spores were also *HIS*+.

Tetrads were dissected on YEPD plates using a Lawrence micromanipulator-equipped Zeiss scope. Asci were pretreated with 5% glusulase in water for 5 min at 25°C.

Random spores were prepared by incubation at 30°C in 5% glusulase in water for 2 hrs on a rotating wheel, with addition of 500 µg glass beads at 1 hr. For *lys2* heterozygotes, spores were plated on aminoadipate plates (see above), to select against residual diploid cells. For cells carrying *trr1*:*HIS3* alleles, it was necessary to plate spores on YEPD, and use other criteria to identify contaminating diploid cells.

Mutagenesis and isolation of *rom1* mutants

A pilot experiment was done to determine optimal mutagenesis conditions. Aliquots of 10^8 MY1000Z cells (MY1000 cells transformed with the MCB/*LacZ* reporter gene) were collected by microcentrifugation, washed twice with and then resuspended in 1 ml 100 mM $NaPO_4$ pH 7, and shook 1 hr at 30°C with 0, 30, 60 or 100 µg/ml ethylmethylsulfonate (EMS), a mutagenic compound. Cells were then collected by microcentrifugation, resuspended in water, transferred to fresh tubes,

washed twice with 5% sodium thiosulfate, and spread on YEPD or aminoadipate plates (see above). Treatment with 30 µg/ml EMS gave optimal results, reducing viability by 50% and increasing the frequency of α-aminoadipate-resistant clones 25-fold above the control level of 10^{-5} per viable cell.

To isolate *rom* mutants, 10^8 MY1000Z cells were either treated with 30 µg/ml EMS or mock-treated, and 2 x 10^6 cells were spread on five 10-cm plates containing YNB agar supplemented with Ade, Met and 10 mM 3-amino-1,2,4-triazole (ATZ). By 2.5 days after plating, EMS-treated and mock-treated cells gave 50 and 8 ATZ-resistant colonies per plate, respectively. When corrected for the number of viable cells plated in each group (determined by spreading aliquots on His-supplemented plates), the frequency of ATZ-resistant cells was 125 x 10^{-5} in the EMS-treated population and 6 x 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 ß-galactosidase assay. Sixty-four gave blue color in the assay, and of these, thirty were selected for further analysis.

RNA isolation from yeast by hot phenol method

RNA was isolated from yeast using glass beads and hot phenol (Aves *et al.*, 1985). Yeast were harvested by spinning for 15 seconds in a microcentrifuge and the pellets washed twice with 500 µl cold saline. At this point yeast pellets could be frozen and stored at -80°C to be processed later. Yeast were resuspended in 25 µl cold SETH (0.32 M sucrose, 10 mM EDTA, 20 mM Tris pH 7.5, 0.5 mg/ml heparin). Acid-washed glass

beads were then added to the meniscus and samples were vortexed for 5 min. To the sample 250 µl cold TNESH (50 mM Tris pH 7.5, 100 mM NaCl, 15 mM EDTA, 1% SDS, 0.5 mg/ml heparin) and 750 µl 65°C QDW-saturated phenol (hot phenol) were added. Samples were incubated 5 min at 65°C, and vigorously shaken every minute for 20 sec. Samples were microcentrifuged 3 min and the aqueous phase and interface were transferred to fresh tubes. The aqueous phase and interface were extracted twice more with hot phenol and then once with 750 µl 1:1 (V/V) phenol:chloroform. To the aqueous phase 1 ml of 100% EtOH was added and RNA was precipitated 1 hr at -80°C or overnight at -20°C. Samples were microcentrifuged 15 min and pellets were washed with cold 70% EtOH and resuspended in 100 µl DEP-QDW (milli-Q filtered distilled H_2O treated with 1% diethyl pyrocarbonate and autoclaved). RNA concentration was determined by A_{260}, assuming 1 OD was equal to 40 µg/ml.

Isolation of genomic DNA for Southern blot analysis

Yeast were grown in 10 ml YEPD culture to saturation and cells were collected by centrifugation in a table top centrifuge. Pellets were resuspended in 500 µl of water, transferred to 1.5 ml microcentrifuge tubes and microcentrifuged 5 sec. To the pellets 200 µl of TSNTE (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl pH 8, 1 mM Na_2 EDTA), 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3 g of acid-washed glass beads were added. Tubes were vortexed 2 min, microcentrifuged 5 min and the aqueous phase transfered to a fresh tube. After addition of 1 ml of 100% ethanol, samples were mixed, microcentrifuged and the pellets were resuspended in 400 µl TE plus 3 µl

10 mg/ml RNase A. After incubation at 37°C for 5 min, 10 µl of 4 M ammonium acetate plus 1 ml 100% ethanol was added. The samples were mixed by inversion, microcentrifuged 2 min, and the pellets air dried. After resuspending pellets in 50 µl of TE, 10µl (approximately 2-4 µg of DNA) from each sample were analyzed by the Southern blot method.

Northern and Southern blot analysis

RNA (10 µg) was denatured and fractionated by electrophoresis through 1% agarose, 2.2 M formaldehyde gels as described by Lehrach *et al.*, (1977), except that 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 4 mM sodium acetate, 0.5 mM EDTA was used as buffer. Gels were rinsed 5 min with water, stained 15 min with 1 µg/ml EtBr, rinsed 90 min with water, and blotted overnight to buffer-equilibrated GeneScreen using 10X SSC (1X SSC = 60 mM NaCl, 15 mM sodium citrate pH 7) as transfer buffer. After UV-crosslinking using a Stratalinker at the default setting of 1200 joules and baking 2 hr at 80°C under vacuum, blots were rinsed in 2X SSC, prehybridized 3 hrs in 10 ml Starks buffer (50% formamide, 5X SSC, 25 mM sodium phosphate pH 6.5, 0.02% each of bovine serum albumin, Ficoll 400 and polyvinylpyrrolidone, and 250 µg/ml salmon sperm DNA), and hybridized in 10 ml 4:1 Starks buffer:dextran sulfate containing 2 x 10^6 cpm radiolabeled probe.

Probes were labeled with [α^{32}P]dCTP (3000 Ci/mM, New England Nuclear) using a Random Priming DNA Labeling System kit (GIBCO) as described by the manufacturer, except that incorporated radioactivity was isolated by chromatography through a 5-ml Sephadex G50 column equilibrated with 1X TES buffer (10 mM Tris pH 7.6, 10 mM EDTA, 1%

SDS). Hybridization probes used were: *RNR1*, 2.6 kb *EcoR1* fragment from pSE738 (S. Elledge, Baylor College of Medicine); *SWI4*, 3.2 kb *HindIII* fragment from p3-24; *SWI6*, 1.9 kb *HindIII/EcoR1* fragment from pBd177; *TMP1*, 1.3 kb *HindIII/EcoR1* fragment from pEM54 (McIntosh *et al.*, 1991); *LEU2*, 2.1 kb *EcoR1* fragment from pEM54; *TRR1*, 1.9 kb *EcoR1/Xho1* fragment from pGAD29; *LIGASE*, 2.7 kb *Sst1* fragment from pR12Sclig2 and *H2A/PROTEIN1 (P1)*, 2.3 kb *Sst1* from pTRT1 (L. H. Johnston NIMR, London). Blots were washed twice for 5 minutes at room temperature with 2X SSC, 0.1% SDS, and three times for 15 min at 50°C with 0.1X SSC, 0.1% SDS. Washed blots were exposed to X-ray film and to a Phosphorimager intensifying screen. Intensifying screens were analyzed using Phosphorimager (Molecular Dyanamics) and ImageQuant software (Microsoft). Adobe Photoshop was used to process the images.

Southern blots were done essentially as described for northern blots except that agarose gels containing 1X TAE (100 mM Tris pH 7.5, 100 mM acetate/acetic acid, 10 mM EDTA) were used and BA85 nitrocellulose (Schleicher and Schuell) was used instead of GeneScreen. To confirm Δ*trr1*:*HIS3* disruption, transformant DNA digested with *EcoR*1 was blotted and probed with a radiolabeled 1.9 kb *EcoR1/Xho1 TRR1* fragment. To confirm nondisruptional tagging of the *TRR1* locus, DNA digested with *HindIII* and *HindIII/Xho1*, was blotted and probed with a radiolabeled 2.1 kb *BglII/Xho1 ELP1* fragment.

Yeast transformation

Yeast were grown to 10^7 cells/ml in YEPD (for most strains 1 OD at 600 nm corresponds to 5 x 10^7 cells/ml, for Δ*swi6* strains 1 OD

corresponds to 2 x 10^7 cell/ml) and were harvested by spinning 5 min at 2500 rpm in a table top centrifuge. Yeast pellets were washed twice with TE (10 mM Tris pH 7, 1 mM EDTA), resuspended in LITE (100 mM LiAc pH 7, 10 mM Tris pH 7.4, 1 mM EDTA) to a final concentration of 10^9 cells/ml, and 200 µl of yeast suspension was immediately added to 1.5 ml microcentrifuge tubes containing a mixture of 1 µg of the plasmid to be transformed and 50 µg of denatured salmon sperm DNA (for library transformations, 5µg of plasmid DNA was used). Immediately, 1.2 ml PEG/LITE (40% polyethylene glycol, mw 3300, in LITE) was added and the mixture was shaken horizontally for 30 min at 30°C. After a 15 min heatshock at 42°C, 100 µl of the mixture was spread on selection plates.

Sequencing

The insert sequence shared by all *rom1*-complementing plasmids (Figure 8) was determined by the dye-on-terminator dideoxysequencing method, using an Applied Biosystems DNA Sequencer operated by the Oregon State University Center for Gene Research and Biotechnology Central Service Facility. Oligonucleotides were made using an Applied Biosystems Oligonucletide Synthesizer also operated by the Central Service Facility.

Primer extension analysis of *LacZ* mRNA

The oligonucleotide primer 5'-CGGGGAGAGGCGGTTTGCGTA TTGG, which was complementary to *Lac* sequences 93 bases downtream

from the MCB/*LacZ* reporter gene start codon, was end-labeled to a specific activity of 3 x 10^6 cpm/pmol using a 50-µl reaction containing 10 pmol primer, 50 mM Tris pH 8, 10 mM $MgCl_2$, 1.5 mM spermidine, 20 pmol [γ^{32}P]-ATP (3000 Ci/mmol, New England Nuclear) and 3U T4 polynucleotide kinase (ICN). After 30 min at 37°C, the reaction was terminated by incubation for 5 min at 65°C. Radiolabeled primer was purified by chromatography through a 5-ml column of Biogel P6 (BioRad) equilibrated with 1 mM Tris pH 7, 1 mM EDTA buffer, precipitated and washed with ethanol, and resuspended in 25 µl DEP-treated water. Primer (0.8 pmol) and 20 µg yeast RNA were mixed, adjusted to 250 mM NaCl, precipitated with 2.5 volumes ethanol, washed with 70% ethanol, air dried 30 min, and resuspended in 30 µl hybridization buffer (80% deionized formamide, 400 mM NaCl, 40 mM piperazine-N,N'-bis [2-ethanesulfonic acid] [PIPES] pH 6.4, 1 mM EDTA). Samples were incubated 10 min at 94°C, 1 hr at 55°C and then overnight at 25°C. Samples were precipitated with 2.5 volumes ethanol, washed with 70% ethanol, resuspended in 20 µl reverse transcriptase buffer (60 mM KCl, 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 1 mM dNTPs, 1mM dithiothreitol, 1 U/µl RNasin [Promega] and 50 µg/ml actinomycin D [Calbiochem]), and incubated 2 hr at 37°C with 5 units AMV reverse transcriptase (a mixture of U.S. Biochemical and Life Sciences enzymes). EDTA was added to 25 mM, and samples were incubated 30 min at 37°C with 5 ng RNase A. Samples were adjusted to 200 µl with 100 mM NaCl, 10 mM Tris pH 7.6, 1 mM EDTA; extracted with 200 µl phenol:chloroform; precipitated with 2.5 volumes ethanol; washed with 70% ethanol; air dried; dissolved in 4 µl DEP-treated water; mixed with 6 µl loading buffer (80% formamide, 10 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol); denatured 5 min at 94°C; rapidly

cooled on ice slush; loaded on a 40 cm x 30 cm x 0.4 mm, 8% acrylamide/0.4% bis-acrylamide, 8M urea, 0.5X TBE sequencing gel; and run at 30 watts until the bromphenol blue reached the bottom. Radioactive bands on dried gels were detected using X-ray film and a Phosphorimager. To determine the length of primer extension products, a dideoxysequencing ladder generated using the above oligonucleotide as primer and the MCB/*LacZ* reporter gene as template was run in parallel lanes.

Gene disruption

The *TRR1* gene was disrupted using the approach developed by Baudin *et al.* (1993). Diploid yeast strain MY2183, which was homozygous for the *his3*Δ200 deletion mutation, was transformed with a PCR fragment containing the intact *HIS3* gene sandwiched between the first 38 and last 38 nucleotides of the *TRR1* protein coding region (Figure 15). The two PCR primers were 5'-ATGGTTCACAACAAAGTTACTATCAT TGGTTCAGGTCCTGATGCGGTATTTTC and 5'-TTCTAGGGAAGTTA AGTATTTCTCAGCATCCAAAGCTGTCTGTAAGCGGATGC. DNA from *HIS*$^+$ transformants was analyzed by the Southern blot method to determine whether *TRR1* was disrupted. Of seven transformants analyzed, only two (MY2196 [#11] and MY2197 [#6]) had the *EcoR1* restriction map expected for a Δ*trr1*:*HIS3* disruption mutation (Figure 16).

The *ELP1* gene was disrupted by the same method using a *HIS3*-containing PCR fragment generated with the primers 5'-ATGTCGTGCTC AAACGGCATCTGGCCTACTGTATCTCCTGATGCGGTATTTTC and 5'-GTTTGCGTTTCACCAGACAACAGTGATGTACGTTCTTGTCTGTA AGCGGATGC.

Gene tagging

A 2.1 kb *BglII/XhoI ELP1* gene fragment from pGAD29 was cloned into *BamHI/XhoI* digested pRS305 (an integration plasmid carrying *LEU2*). The resulting plasmid was partially digested with *EcoRV* and transformed into BY600. *LEU2*$^+$ transformants were isolated, genomic DNA was extracted and digested with *HindIII* and *HindIII/XhoI*, separated by agarose gel electrophoresis, blotted to nitrocellulose and probed with a radiolabeled 2.1 kb *BglII/XhoI ELP1* fragment.

ß-galactosidase assays

For liquid ß-galactosidase assays, exponentially growing yeast were harvested by centrifugation, resuspended in 250 µl breakage buffer (100 mM Tris pH 8, 1 mM DTT, 20% glycerol) and transferred to 1.5 ml microcentrifuge tubes on ice. After adding 500 µ glass beads (SIGMA) to the meniscus and 12 µl 40 mM PMSF (phenylmethy-sulfonyl fluoride), yeast were disrupted by intermittent vortexing and chilling six times for 15 sec. Cells were checked microscopically for greater than 50% disruption, and vortexed longer if necessary. Lysate was siphoned to fresh tubes, clarified by microcentrifugation for 15 min, and aliquots of the supernatant were diluted with breakage buffer to 90 µl and mixed with 800 µl Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, adjusted to pH 7), 2 µl 12 M ß-mercaptoethanol and 180 µl 13 µM ONPG (o-nitrophenyl-ß-D-galactoside). After 30 min at 30°C, reactions were stopped by addition of 450 µl 1 M Na_2CO_3 and absorbance at 420 nm was determined to measure the extent of ONP production, assuming 1 OD =

222 nmol/ml. The protein concentration of clarified lysates were determined by the Bradford method (1976), using bovine serum albumin as standard.

For filter ß-galactosidase assays, yeast colonies were replicaplated to Whatman filters, frozen in liquid N_2, thawed, laid on a duplicate filter presoaked with 2 ml Z buffer, 2 µl 12 M ß-mercaptoethanol and 50 µl 40 mg/ml XGAL (5-bromo-4-chloro-3-indolyl-ß-galactopyranoside) in DMSO, and incubated at 30°C until blue color developed (1 to 2 hrs).

Preparing yeast for flow cytometry

The procedure was adapted from Dien *et al.* (1994). Cells were harvested, fixed in 70% EtOH (to stop them from growing and to permeabilize them) and microcentrifuged 15 sec. The pellets were resuspended in 500 µl saline (140 mM NaCl), vortexed, microcentrifuged 15 sec and resuspended in 50 µl saline. To each sample 2.5 µl 10 mg/ml RNase A was added. After incubating 2 hrs at 37°C, cells were microcentrifuged and pellets resuspended in 200 µl 300 µg/ml propidium iodide in saline. Samples were sonicated twice for 6 sec at maximum power and diluted with saline to about 10^6/ml for flow cytometry.

Extracting plasmid from yeast

Yeast were grown to 10^7/ml in 25 ml selective medium and harvested by spinning 5 min in a table top centrifuge. Pellets were washed with saline (140 mM NaCl), resuspended in 200 µl TNE (50 mM Tris pH 7.6, 100 mM NaCl, 10 mM EDTA) and transferred to 1.5 ml microcentrifuge

tubes. Glass beads (0.5 mm; acid washed; baked) were added to just below the meniscus and vortexed violently 4 times for 30 sec (storing on ice between vortexing). After adding 100 µl TNE containing 3% sarkosyl, samples were vortexed and incubated 15 min at 65°C. Samples were extracted once with 600 µl TE-saturated phenol, once with 300 µl phenol:chloroform 1:1, and once with 300 µl chloroform. The aqueous phase of each extraction was transferred to a fresh 1.5 ml tubes. Plasmid DNA precipitated with EtOH (30 µl 3M NaOAc, 750 µl 100% EtOH at -20°C for 1 hr and microcentrifuged 15 min). Pellets were washed with 500 µl 70% EtOH and resuspended in 50 µl TE.

Transforming bacteria

Competent bacterial cell stocks, prepared by the Chung method (1988), were stored at -70°C as 100 µl aliquots. Tubes containing 100 µl competent cells were thawed on ice and the DNA to be transformed (5 µl of yeast-extracted plasmid or 0.1 µg of bacteria-extracted plasmid) was added immediately. Tubes were incubated 15 min on ice, heatshocked 2 min 42°C, and returned briefly to ice. At room temperature, 0.9 ml of LB was added, and the tubes were shaken horizontally 37°C. After 60 min, 250 µl was plated to LB selective plates (tetracycline or ampicillin/methicillin).

Yeast synchronization

Two different methods of cell synchrony were used. First cells were synchronized using centrifugal elutriation (Lowndes et al., 1991). Yeast

were grown overnight in 500 ml of YNB media at 30°C and harvested by centrifugation at mid-log phase. Cells were washed twice with cold saline (140 mM NaCl) and concentrated to a final volume of 5 ml. Yeast were sonicated for 20 sec and injected into a saline equilibrated spinning rotor (Beckman J6M/E centrifuge, JE 5.0 rotor). By carefully adjusting the saline flow entry and the rotor speed, small daughter cells (early G1 yeast) were elutriated. Fractions were collected until budded cells began to elute. Fractions containing small, unbudded cells were centrifuged, resuspended in 50ml YEPD medium, and incubated at 30°C with shaking. Cells were collected every 15 min and either used for A_{600} determination, budding index determination, or were washed with saline and frozen at -80°C for later RNA extraction or FACS analysis.

The second method of synchronization employed a temperature sensitive (*ts*) allele of the *CDC15* gene. Conditional mutations in *CDC15* cause cell cycle arrest in late anaphase (Cullotti and Hartwell, 1971). Exponentially growing cells were collected in late anaphase by 3 hr incubation at the nonpermissive temperature of 37°C. Cells were then shifted back to the permissive temperature of 25°C for a further 270 min incubation. Samples were removed at 15 min intervals, washed with saline and frozen at -80°C for later RNA extraction.

The *cdc15* block was also used to synchronize cells for release into medium with or without 200 mM hydroxyurea (HU), an inhibitor of ribonucleotide reductase (RNR).

CHAPTER 3

rom1 Mutations Activate MCB Reporter Genes

Identification of *rom1* by mutations that activate MCB reporter genes

To identify mutations that activated MCB reporter genes in *Δswi6* yeast, strain MY1000Z, which carries an integrated MCB-dependent *HIS3* reporter gene (MCB/*HIS3*) and a plasmid-borne MCB-dependent *LacZ* reporter gene (MCB/*LacZ*) was mutagenized with ethylmethylsulfonate (EMS) and plated on medium lacking histidine and containing 3-amino-1,2,4-triazole (ATZ). Cells that formed colonies on ATZ were assayed for ß-galactosidase using a filter assay. Sixty-four out of 250 ATZ-resistant colonies gave blue color. Thirty ATZ-resistant, ß-galactosidase-positive mutants were randomly selected for further characterization. The mutants were grown in liquid culture and lysates were assayed quantitatively for ß-galactosidase activity. Table 3 shows ß-galactosidase levels in the mutants, arranged in descending order of *LacZ* activation. As evident from the second column in Table 3, ß-galactosidase levels varied from 7-fold to 70-fold over the background levels of *Δswi6* parental strain MY1000Z.

To assess whether the mutations were recessive, each mutant was mated to *Δswi6* yeast strain MY1001, and the resulting diploids were assayed for ß-galactosidase activity. As shown in the third column of Table 3, ß-galactosidase activity was extinguished in all cases when mutants were mated to nonmutant cells, indicating that all thirty MCB-activating mutations were recessive. When the diploid cells were sporulated, and inheritance of the MCB-activating phenotype was determined by random

Table 3. MCB reporter gene activity of *rom* mutants and assignment to complementation groups. Mutants carrying the MCB/*LacZ* reporter gene were assayed for ß-galactosidase activity either directly, or after mating to nonmutant Δ*swi6* strain MY1001 or to a MAT-α *lys2* derivative of primary mutant MY2074*a*. The data shows all mutations were recessive and allelic, defining a complementation group which we refer to as *rom1*. Strain genotypes are identical to that listed for MY2090 in Table 2, except that MY2066, 2082 and 2100 had converted to Met prototrophy during isolation.

Table 3.

Strain[a] Name	ß-galactosidase activity (nmol ONP/min-mg protein)			Allele
	Primary isolate	After mating to MY1001(Δ*swi6*)	After mating to MY2074α(Δ*swi6 rom*)	
MY2082	1218	11	1073	*rom1-1*
MY2072	789	11	204	*rom1-2*
MY2100	723	28	458	*rom1-3*
MY2066	699	16	272	*rom1-4*
MY2080	655	17	369	*rom1-5*
MY2073	561	10	274	*rom1-6*
MY2087	551	11	660	*rom1-7*
MY2101	502	11	600	*rom1-8*
MY2050	493	25	312	*rom1-9*
MY2091	478	12	239	*rom1-10*
MY2068	473	10	282	*rom1-11*
MY2071	469	11	254	*rom1-12*
MY2079	464	13	825	*rom1-13*
MY2095	409	9	256	*rom1-14*
MY2065	361	15	296	*rom1-15*
MY2081	357	10	306	*rom1-16*
MY2092	336	13	507	*rom1-17*
MY2051	326	14	311	*rom1-18*
MY2070	292	14	512	*rom1-19*
MY2054	288	24	292	*rom1-20*
MY2090	281	8	541	*rom1-21*
MY2078	245	14	204	*rom1-22*
MY2059	234	14	235	*rom1-23*
MY2093	216	11	253	*rom1-24*
MY2060	216	22	887	*rom1-25*
MY2074	163	10	136	*rom1-26*
MY2057	138	13	410	*rom1-27*
MY2061	132	16	407	*rom1-28*
MY2062	128	13	332	*rom1-29*
MY2053	122	16	216	*rom1-30*
MY1000	18	12	7	*ROM1*
MY1000 +pBd177(*SWI6*)	1140	nd	nd	*ROM1*

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 MAT-α strain carrying mutation 310, obtained by sporulating a MY2074 x MY1001 diploid, was mated to all thirty of the original MAT-*a* mutants, and the resulting diploids were assayed for ß-galactosidase. As shown in the fourth column of Table 3, all of the resulting diploids showed high ß-galactosidase activity. The failure of any diploids to show the low ß-galactosidase activity characteristic of nonmutant cells indicated that all thirty mutations were in the same complementation group. As the wildtype allele of the gene identified by the mutations presumably functions as a repressor of MCB reporter genes, we tentatively named the gene *ROM1*. In the fifth column of Table 3, each *rom1* allele is identified by a suffix indicating its relative placement in the array of phenotypes. The various *rom1* alleles gave varying degrees of MCB reporter gene activation, suggesting that at least the more poorly activating *rom1* alleles were not null alleles. Strains with more strongly activating *rom1* mutations grew significantly slower than parental cells. The strain carrying the *rom1-21* mutation grew with a generation time nearly equivalent to parental cells and was selected for further characterization.

One feature concerning the behavior of *rom1* mutants deserves mention. When diploids formed between a *rom1* mutant and a *ROM1* wildtype cell were selected for MCB reporter gene activation (by growth in the absence of histidine and presence of ATZ), the cells showed an extremely high conversion rate to the *rom1* phenotype. We estimate that nearly 1% of heterozygous *ROM1/rom1* diploid cells were able to convert to a *rom1* phenotype when incubated in ATZ. In contrast, homozygous

ROM1 diploids never converted to the *rom1* phenotype when incubated in ATZ. We suspect that the *rom1* locus is very recombinogenic, a suspicion borne out by the ease with which plasmids integrate at the *rom1* locus (described later).

Although they restored MCB reporter gene expression, *rom1* mutations did not suppress other aspects of the *swi6* phenotype. Yeast that are Δ*swi6* are larger than wildtype cells and have irregularly shaped buds (Breeden and Nasmyth, 1987). None of the *rom1* mutations listed in Table 3 corrected the abnormal morphology of Δ*swi6* cells, and at least the *rom1-21* mutation (the only one tested) did not suppress the synthetic lethality of *swi6 swi4* double mutations.

Specificity of *rom1* effect on MCB and SCB activation

Using genetic screens similar to ours, a number of global suppressors of transcription have been isolated in yeast (Lycan *et al.*, 1994). 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 Δ178*CYC1* promoter. To determine whether *rom1* mutations enhanced transcription from a *CYC1* promoter lacking a functional UAS, the effect of the *rom1-21* mutation on expression of a mutant MCB/*LacZ* gene in which the upstream MCB elements were mutated to ACtaGT was determined. The effect of the *rom1-21* mutation on the expression of a Δ*312CYC1/LacZ* reporter gene carrying the native *CYC1* UAS was also determined. In addition, the effect of the *rom1-21* mutation on the expression of a SCB/*LacZ* reporter gene carrying three upstream SCB elements derived from the *HO* gene, consensus

CACGCAAAA, was determined. As earlier discussed, SCBs are elements structurally related to MCBs and are implicated in G1/S gene induction.

As shown in Table 4, the *rom1-21* mutation elevated ß-galactosidase activity 25-fold in cells carrying either the MCB/*LacZ* or SCB/*LacZ* reporter gene. In contrast, the *rom1-21* mutation had no effect on the high levels of ß-galactosidase activity in cells carrying the Δ312*CYC1*/*LacZ* reporter gene, and relatively little effect on the low level of ß-galactosidase activity in cells carrying the mutMCB/*LacZ* reporter gene. Control experiments on cells that were not transformed with any plasmid, indicated that the small, two-fold effect of *rom1-21* in mutMCB/*LacZ* transformants was due to an integrated *ho-LacZ* gene present in all the strains tested. On the basis of the data in Table 4, we concluded that *rom1* mutations specifically activated MCB- or SCB-containing reporter genes, and that the Rom1 polypeptide therefore was not a nonspecific global repressor of transcription.

Thus far, all the effects of *rom1* had been studied in a Δ*swi6* background, where MCB reporter genes are repressed (Lowndes *et al.*, 1991). To determine whether *rom1* affected MCB reporter gene expression in Swi6-containing cells, the effect of introducing the *SWI6*-containing plasmid pBd177 was determined. Also, as earlier unpublished work in our laboratories had determined that highcopy expression of *SWI4* allowed efficient MCB reporter gene expression in Δ*swi6* yeast, we investigated the effect of *rom1* on reporter gene expression in yeast transformed with the *SWI4*-containing plasmid p3-24. Both the *SWI6* and *SWI4* plasmids were 2μ-based and *LEU2*-marked. Therefore, control cells were transformed with the 2μ-based *LEU2*-marked plasmid pGAD2F (Chien *et al.*, 1991). As shown in Table 5, in cells transformed with the

Table 4. Specificity of *rom1* effect on MCB and SCB reporter genes[a]

Strain	Relevant Genotype	Reporter Gene			
		MCB/*LacZ*	mutMCB/*LacZ*	Δ312/*LacZ*	SCB/*LacZ*
		ß-galactosidase activity (nmol ONP/min-mg protein)			
BY600	*Δswi6*	9±1	11±2	1770±25	14±2
MY2043	*Δswi6 rom1-21*	231±26	20±2	1492±198	357±64

[a] Lysates from exponentially growing cells transformed with the indicated reporter genes were assayed for ß-galactosidase activity. In mutMCB/*LacZ* the central "GC" residues of the ACGCGT MCB consensus were mutated to "TA" (Lowndes *et al.*, 1991). In Δ312/*LacZ* (plasmid pLGΔ312, Guarente and Mason, 1983) the 5' flanking sequence includes the native *CYC1* UAS. In SCB/*LacZ* (plasmid pLB178-43, Breeden and Nasmyth, 1987) three SCB elements from the *HO* 5' region are fused upstream of the Δ178*CYC1* promoter. The mean ± standard error of the mean for four independent transformant clones is shown.

MCB/*LacZ* reporter gene and either the *SWI6* or *SWI4* plasmid, the *rom1* mutation significantly augmented the already high levels of ß-galactosidase activity. In cells transformed with the mutMCB/*LacZ* reporter gene and the *SWI6* plasmid, the *rom1* mutation also augmented ß-galactosidase activity; but subsequent analyses showed that ß-galactosidase in these *SWI6*+ cells was generated from an integrated *ho-LacZ* gene, rather than the mutMCB/*LacZ* reporter gene.

Nondistortion of cell cycle compartment duration in *rom1* mutants

MCB-controlled genes are activated shortly after START and inactivated in mid-S phase (McIntosh *et al.*, 1991; Lowndes *et al.*, 1991). Thus, MCB reporter gene activation in *rom1* mutants could be a consequence of disproportional expansion of a cell cycle compartment during which MCB elements are active. For example, if *rom1* mutations prolonged S and delayed cytokinesis, 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 *rom1* mutations disproportionately affected the duration of cell cycle compartments, exponentially growing *ROM1* wildtype and *rom1-21* mutant cells were stained with propidium iodide and analyzed by flow cytometry. As shown in Figure 2A, in both a *SWI6* background or Δ*swi6* background, the DNA profiles of *ROM1* wildtype and *rom1* mutant cells were indistinguishable, indicating that the *rom1-21* mutation did not disproportionately expand the S/G2 phases of the cell cycle. In contrast to the lack of effect of *rom1* on the frequency of G1 and

Table 5. MCB reporter gene augmentation in *rom1-21* cells expressing Swi6 and Swi4[a]

Strain	Relevant Genotype	Activating Plasmid	Reporter Gene	
			MCB/*LacZ*	mutMCB/*LacZ*
			ß-galactosidase activity (nmol ONP/min-mg protein)	
BY600	*Δswi6*	pGAD2F	7±2	5±1
		pBd177(*SWI6*)	232±8	45±3
		p3-24(*SWI4*)	521±15	nd.
MY2043	*Δswi6 rom1-21*	pGAD2F	141±9	6±1
		pBd177(*SWI6*)	625±81	132±8
		p3-24(*SWI4*)	1006±24	nd.

[a] Lysates from exponentially growing cells transformed with the indicated activating plasmid and reporter gene plasmid (see Table 4) were assayed for ß-galactosidase. The plasmids pBd177 and p3-24 are high copy plasmids containing full length *SWI6* and *SWI4* genes, respectively. The mean ± standard error of the mean for three independent transformant clones is shown.

Figure 2. Flow cytometric analysis showing *rom1* does not affect cell cycle distribution or cell size. Growing yeast were stained with propidium iodide and analyzed for DNA content by fluorescence (F), or for cross-sectional area by forward angle light scatter (FALS). A) Fluorescence profile showing distribution of cells with 1N, 2N and intermediate DNA contents. B) FALS profile of total population, showing modal size of all cells FALS. C) Fluorescence plotted versus FALS showing boxed data points used to obtain FALS profile of 2N cells. D) FALS profile of 2N cells showing threshold setting required to reduce event frequency by 10% (an estimate of the smallest G2 cells).

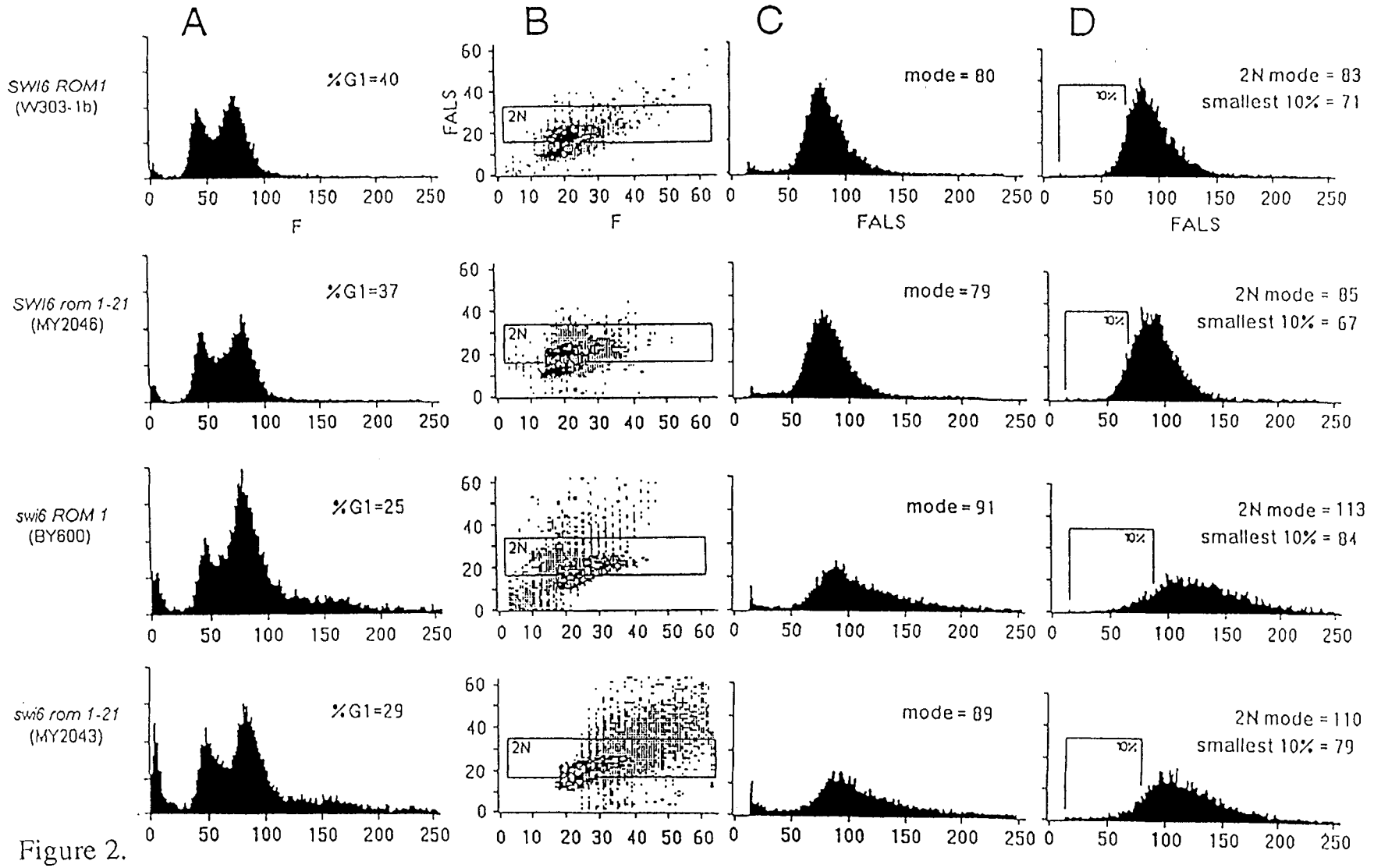
A
B
C
D
SWI6 ROM1
(W303-1b)
%G1=40
mode = 80
2N mode = 83
smallest 10% = 71
SWI6 rom 1-21
(MY2046)
%G1=37
mode = 79
2N mode = 85
smallest 10% = 67
swi6 ROM 1
(BY600)
%G1=25
mode = 91
2N mode = 113
smallest 10% = 84
swi6 rom 1-21
(MY2043)
%G1=29
mode = 89
2N mode = 110
smallest 10% = 79
2N
10%
F
FALS

Figure 2.

S/G2 cells, in both *ROM1* and *rom1-21* cells, deletion of *SWI6* had a marked effect, increasing the percentage of G2/S cells at the expense of G1 cells (compare top two panels with bottom two panels of Figure 2A).

If *rom1* mutations delayed cytokinesis without affecting mass accumulation, *rom1* cells should be larger than *ROM1* wildtype 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 *rom1* on cell size. As shown in Figure 2B, the *rom1-21* mutation did not affect the modal FALS value of either *SWI6* or *Δswi6* cells, indicating that *rom1* did not increase average cell size. As expected, deletion of *SWI6* did increase the modal FALS, consistent with the microscopic observation that *Δswi6* cells are larger than *SWI6* cells (Breeden and Nasmyth, 1987). To specifically investigate an effect of *rom1* on G2 cell size, we used the approach of Lew *et al.* (1992), in which only cells with a G2 DNA content (Figure 2C, boxed data points) were used to derive a profile of FALS values. The results, shown in Figure 2D, showed that *rom1-21* did not affect the modal size of G2 cells. Furthermore, even when the smallest 10% of the G2 populations were compared, *rom1-21* did not cause an observable increase in cell size.

Based on DNA content and FALS data, the *rom1-21* allele did not alter the relative distribution of cells within the cell cycle and did not increase cell size. The *rom1-21* mutant was initially selected for characterization because it had a doubling time that was only slightly longer than *ROM1* parental cells (2.55 hrs for MY2090 versus 2.20 hrs for MY1000). Other *rom1* alleles, that more strongly activated MCB reporter gene expression (see Table 3), generally had significantly longer doubling

times. However, flow cytometric analysis of eight of the strains listed in Table 3, covering the whole range of activation phenotypes, showed that even the most strongly activating *rom1* alleles caused no shift in the relative proportion of G1 and S/G2 cells (Table 6). We conclude that even in the more slowly growing *rom1* mutants, that showed high levels of MCB reporter gene activation, all phases of the cell cycle were expanded equally.

Characterization of MCB gene mRNA levels in *rom1* yeast

The *rom1* mutants were isolated on the basis of ATZ resistance and elevated ß-galactosidase activity. To confirm that the *rom1* mutations elevated reporter gene mRNA levels, a primer extension assay was used to determine *LacZ* mRNA levels in *ROM1* and *rom1* cells in the presence or absence of Swi6. Initial attempts to measure *LacZ* mRNA by northern analysis were noninformative due to probe hybridization to abundant Swi6-independent transcripts initiating downstream from the translation start codon. Similar to earlier findings using the intact *CYC1* promoter (McNeil and Smith, 1985), primer extension assays revealed that the *CYC1* promoter of the MCB/*LacZ* gene gave rise to heterogeneous transcription initiation events in all genetic backgrounds assayed (Figure 3A). Because of upstream AUG triplets, transcripts initiating more than 126 bases upstream from the translation start codon would not likely be translated. Thus, only prominent transcripts initiating within 126 nucleotides of the start codon were quantitated (see arrows in Figure 3A and histograms in Figure 3B). Three initiation events, designated a, b and c in Figure 3, matched previously identified *CYC1* transcription initiation sites (McNeil and Smith, 1985). The other three sites were not precisely assignable, but

Table 6. Flow cytometric analysis of different *roml* mutants

Strain[a]	Relevant Genotype	%G1	Cell Size		
			All	G2	Smallest 10% G2
MY2082	Δ*swi6 roml-01*	31	89	125	84
MY2087	Δ*swi6 roml-07*	31	94	130	86
MY2068	Δ*swi6 roml-11*	32	91	130	83
MY2095	Δ*swi6 roml-14*	30	95	120	84
MY2051	Δ*swi6 roml-18*	31	96	125	88
MY2090	Δ*swi6 roml-21*	28	89	130	82
MY2061	Δ*swi6 roml-27*	32	91	122	85
MY2053	Δ*swi6 roml-30*	28	86	120	79

[a] Exponentially growing yeast were analyzed by flow cytometry. They were stained with propidium iodide and analyzed for DNA content by fluorescence or cell size (in arbitrary units) by forward angle light scatter (FALS). The %G1 was obtained from fluorescence profile; cell size of the indicated populations was determined by FALS. Values for eight different *roml* mutants are shown. Data show *roml* mutation does not affect cell cycle distribution or cell size.

Figure 3. Primer extension analysis of *rom1* 1-21 effect on *LacZ* mRNA levels. A) Autoradiogram of primer extension products obtained using 20 μg of RNA from exponentially growing yeast carrying the MCB/*LacZ* reporter gene. The strains analyzed were MY2043 and BY600 that were transformed with either the *SWI6*-encoding plasmid pBd177 (*rom1 SWI6* and *ROM1 SWI6* strains, respectively) or the control plasmid pGAD2F (*rom1 Δswi6* and *ROM1 Δswi6* strains, respectively). RNA from *SWI6 ROM1* yeast lacking the MCB/*LacZ* reporter gene was analyzed in lane 5 as a negative control. Signals corresponding to mRNAs starting in the transcription initiation region of the *CYC1* gene are designated by lower case letters and quantitated below. A sequencing ladder run in parallel lanes did not have the resolution to identify the exact location of the d-h transcription initiation events. B) Histogram showing phosphoimager quantitation of primer extension signals designated in autoradiogram. Each signal was corrected by subtracting the background fluorescence in the corresponding region of control lane 5. Signals were normalized to the strongest signal in lane 4, arbitrarily set at 100.

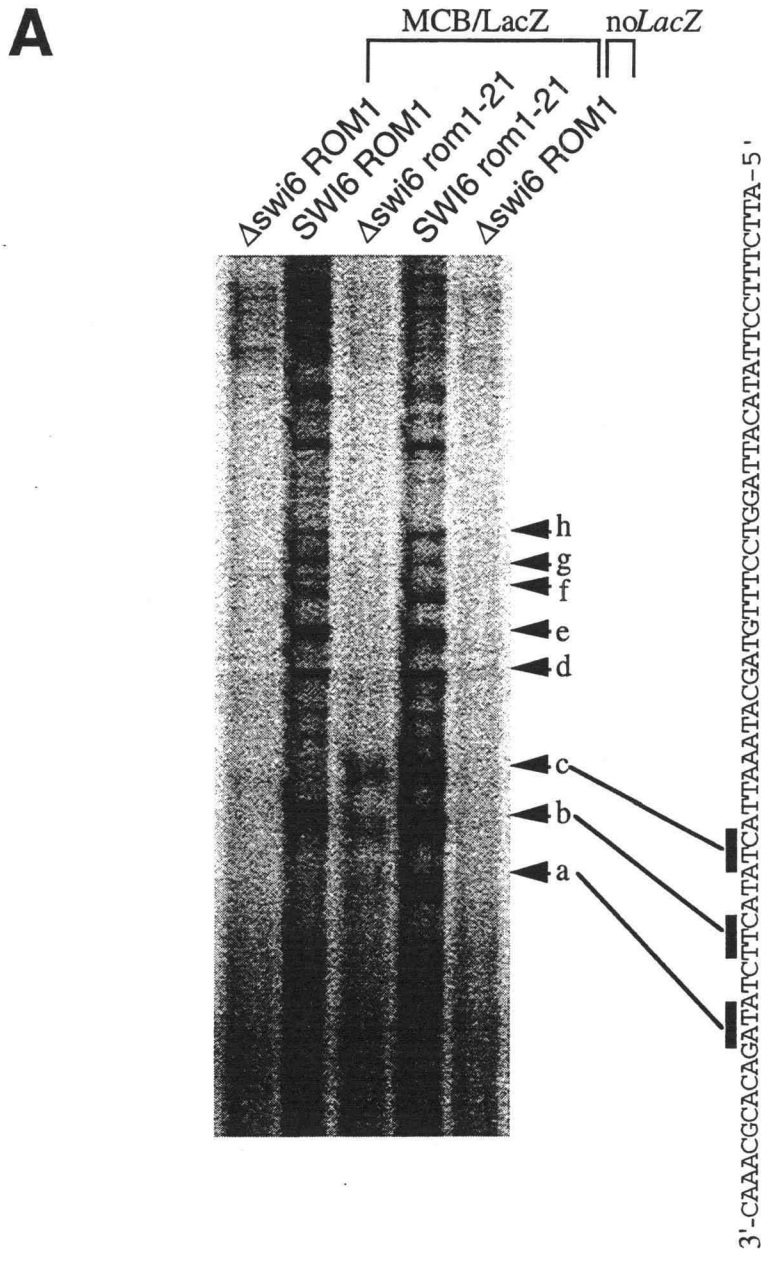
A
MCB/LacZ
noLacZ
Δswi6 ROM1
SWI6 ROM1
Δswi6 rom1-21
SWI6 rom1-21
Δswi6 ROM1
h
g
f
e
d
c
b
a
3'-CAAACGCACAGATATCTTCATATCATTAAATACGATGTTTCCTGGATTACATATTCCTTTCTTA-5'

Figure 3A.

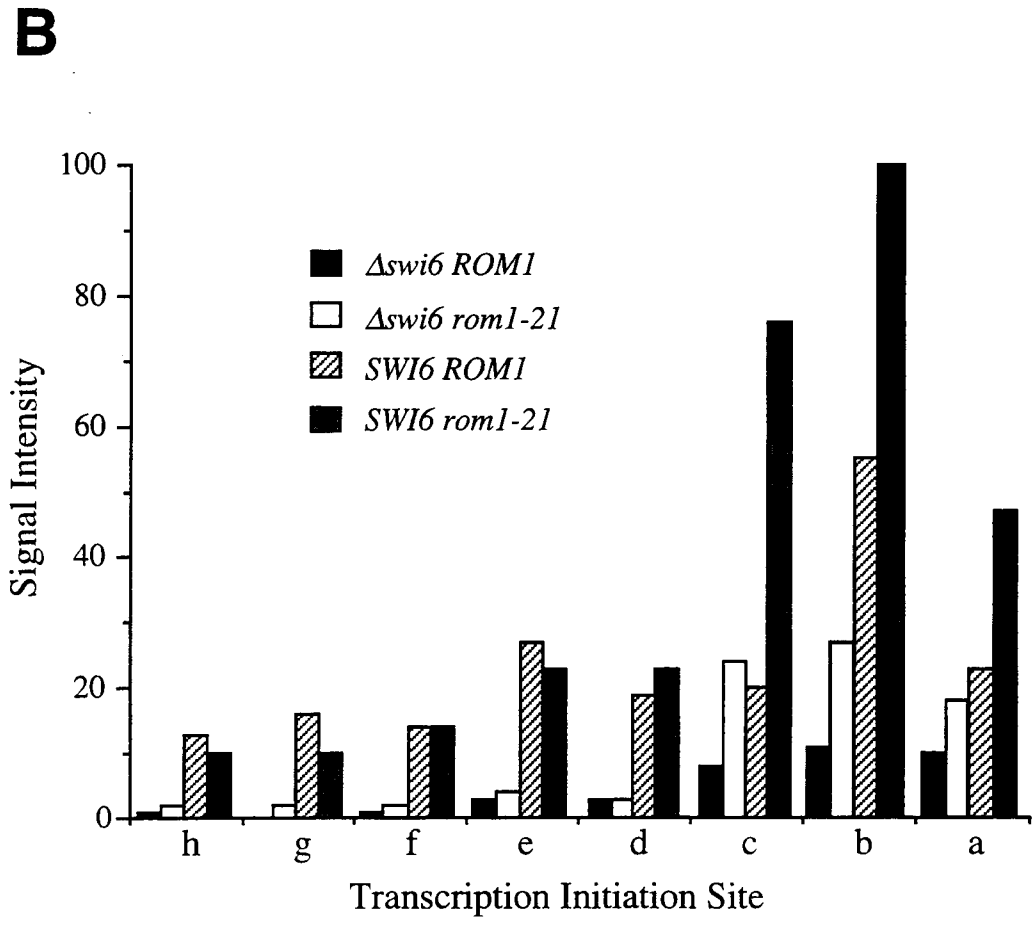
B
100
80
60
40
20
0
Signal Intensity
Δswi6 ROM1
Δswi6 rom1-21
SWI6 ROM1
SWI6 rom1-21
h
g
f
e
d
c
b
a
Transcription Initiation Site

Figure 3B.

mapped to a region within 126 bases of the start codon. As shown in Figure 3A, all six initiation events were several-fold stronger in *SWI6* cells (lane 2) than Δ*swi6* cells (lane 1), thus confirming earlier conclusions based on ß-galactosidase measurements that deletion of *SWI6* greatly reduced MCB/*LacZ* reporter gene transcription (Lowndes *et al.*, 1992; Dirick *et al.*, 1992). Significantly, in *Δswi6* carrying the *rom1-21* allele (lane 3), *LacZ* mRNA levels were partially restored. Finally, again consistent with ß-galactosidase activity measurements, in *SWI6* carrying the *rom1-21* allele (lane 4), *LacZ* mRNA levels were augmented beyond *SWI6 ROM1* wildtype levels (compare lanes 4 and 2). Figure 3B quantitates the primer extension signals and shows that the *rom1-21* mutation elevated transcription from the a, b and c start sites about 2-fold in *Δswi6* cells and 3-fold in *SWI6* cells. Thus, in addition to increasing ß-galactosidase enzyme expression from the MCB/*LacZ* reporter gene, *rom1* mutations elevated reporter gene mRNA levels. We note however that, in Δ*swi6* cells, the effect of *rom1* on *LacZ* mRNA was not as great as the effect on ß-galactosidase activity.

Having established that *rom1* mutations elevated MCB reporter gene mRNA levels, we next investigated whether the *rom1-21* mutation affected expression of the *CDC9* gene encoding DNA ligase, the *RNR1* gene encoding the large subunit of ribonucleotide reductase, or the *SWI4* gene, all of which contain MCB elements and have been shown to be maximally expressed at G1/S (Lowndes *et al.*, 1991; Foster *et al.*, 1993). Figure 4 shows a northern blot of RNA from exponentially growing cells with the indicated genotypes that was sequentially probed with radiolabeled *CDC9*, *RNR1* and *SWI4* DNA fragments. Although band intensities varied between lanes in a particular experiment, when data from additional

Figure 4. Northern analysis of *rom1-21* effect on endogenous MCB gene expression in asynchronous cells. RNA from exponentially growing cells (5 μg/lane) was sequentially hybridized with radiolabeled *CDC9*, *RNR1*, and *SWI4* DNA probes (the strains analyzed were identical to those assayed in Figure 3). Relative *CDC9*, *RNR1*, and *SWI4* mRNA levels were calculated by normalizing hybridization signals to rRNA levels.

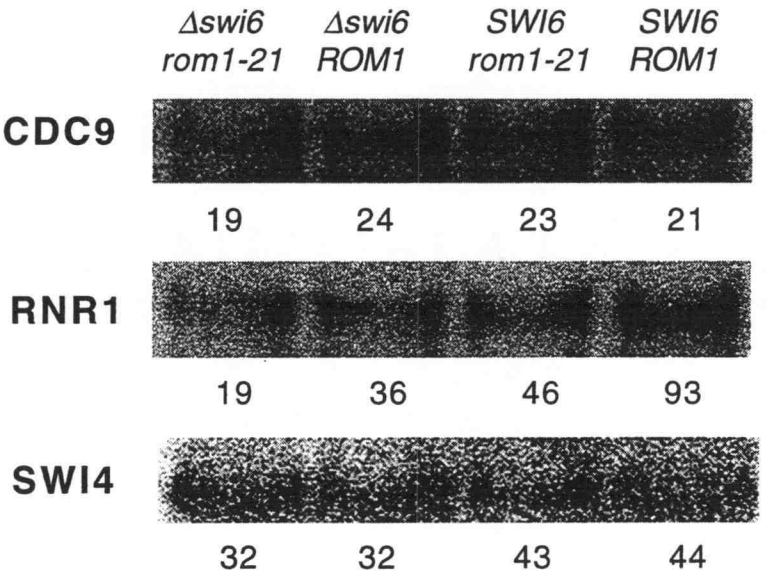
Δswi6 rom1-21
Δswi6 ROM1
SWI6 rom1-21
SWI6 ROM1
CDC9
19
24
23
21
RNR1
19
36
46
93
SWI4
32
32
43
44

Figure 4.

replicate blots was considered, *CDC9*, *RNR1* and *SWI4* mRNA levels were essentially equal in *Δswi6 rom1*, *Δswi6 ROM1*, *SWI6 rom1* and *SWI6 ROM1* cells. Thus, in contrast to the elevation of *LacZ* reporter gene mRNA levels, the *rom1-21* mutation did not elevate endogenous MCB gene mRNA levels. Two caveats should be noted. First, we only investigated effects of the *rom1-21* allele. Other, more strongly activating, *rom1* mutations (see Table 3) may affect endogenous MCB gene expression. We did not focus on these mutations because they affected cell growth rates, and effects would therefore be more difficult to interpret. Second, we only determined *CDC9*, *RNR1* and *SWI4* mRNA levels by northern blot assay. Although the mRNAs observed were of the expected size, northern blots did not have the resolution to reveal whether the mRNAs began and ended at their *bona fide* termini and were therefore translatable.

Although the *rom1-21* mutation did not noticeably affect endogenous MCB gene mRNA levels, neither did deletion of *SWI6*, a gene known to be an important MCB element activator. Previous studies have also reported little effect of *SWI6* deletion on asynchronous cell *CDC9*, *RNR1* and *TMP1* mRNA levels (Lowndes *et al.*, 1992; Dirick *et al.*, 1992). However, in these previous studies, deletion of *SWI6* did disrupt the normal periodicity of these transcripts. We therefore sought to determine the effect of the *rom1-21* mutation on endogenous MCB gene periodicity.

As *Δswi6* cells were difficult to synchronize and were already known to show altered DNA synthesis gene periodicity (Lowndes *et al.*, 1992; Dirick *et al.*, 1992), we investigated the effect of *rom1* on DNA synthesis gene periodicity in *SWI6* cells. Cells were synchronized using centrifugal elutriation (Lowndes *et al.*, 1991). To improve the size homogeneity of the population, diploid cells were used. Cells that were either *rom1-21/rom1-*

21 homozygotes or *ROM1/rom1-21* heterozygotes were analyzed. Both cell types carried the *TMP1*-encoding plasmid pEM54. Synchrony was monitored by determining the budding index. As shown in Figure 5A, bud emergence in both populations began about 60 minutes after elutriation. In contrast to the simultaneous onset of budding, peak *TMP1* mRNA levels in *rom1-21* cells occurred about 15 minutes earlier than in *ROM1* cells (Figure 5B). Figure 5C shows *TMP1* mRNA levels, normalized to *LEU2* mRNA levels. *LEU2* is carried on the pEM54 plasmid but is not a G1/S-specific gene. RNA for the sixty minute timepoint in *ROM1* cells was artificially low due to RNA degradation. Although *rom1* advanced the peak of *TMP1* mRNA levels, it did not prolong the peak.

RNA from the synchronized cells was also analyzed for *RNR1* and *SWI4* mRNA (Figure 6A). *RNR1* and *SWI4* mRNA levels, normalized to *LEU2* mRNA levels is shown in Figure 6B. As was previously observed for the plasmid-encoded *TMP1* gene product, peak expression of the chromosomal *RNR1* and *SWI4* gene products was also advanced in *rom1* cells. As was observed for *TMP1* mRNA, *rom1* did not prolong the peak of *RNR1* or *SWI4* mRNA, perhaps explaining why *rom1* did not elevate asynchronous cell *RNR1* and *SWI4* mRNA levels.

In order to rule out the possibility that advancement of MCB gene expression in *rom1* cells was an artifact of the method of synchrony, a second method was employed. A *ts* allele of the *CDC15* gene (Price *et al.*, 1991) was used to generate *rom1-21 cdc15-1* and *ROM1 cdc15-1* haploid yeast. At the nonpermissive temperature *cdc15* cells arrest at M/G1. Cells were synchronized by releasing *cdc15* cells arrested at the nonpermissive to the permissive temperature. Synchrony was monitored by determining the

Figure 5. Northern analysis of *rom1-21* effect on high copy *TMP1* expression in elutrially synchronized cells. All cells harbored the *TMP1*-encoding 2μ plasmid pEM54 (McIntosh *et al.*, 1993) and the MCB/*LacZ* plasmid. Diploid strains MY2182 (*rom1-21/rom1-21* homozygote) or MY2191 (*ROM1/rom1-21* heterozygote) were fractionated by elutriation, and small early G1 cells were inoculated into YEPD and incubated at 30°C. At indicated times, budding index was determined by scoring 200 cells (A), or RNA was prepared for northern blot analysis. B) Northern blot of RNA (5 μg/lane) hybridized with radiolabeled *TMP1* or *LEU2* DNA probes. *LEU2*, the selectable marker on pEM54, was analyzed as a control for plasmid copy number and RNA loading. C) *TMP1* mRNA levels, normalized to *LEU2* mRNA levels, were quantitated and plotted as a function of time after inoculation.

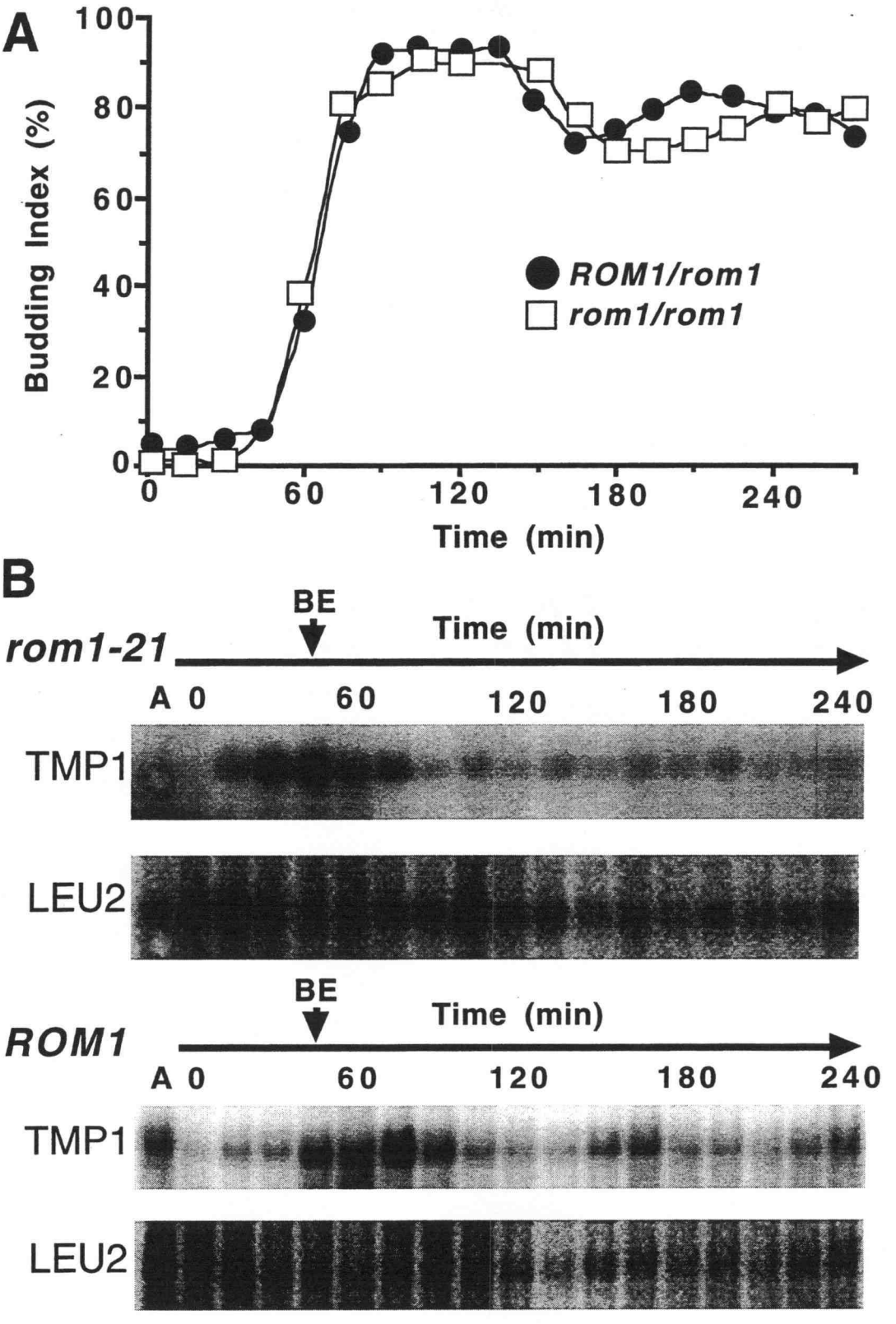
A
100
80
60
40
20
0
Budding Index (%)
0
60
120
180
240
Time (min)
ROM1/rom1
rom1/rom1
B
rom1-21
BE
Time (min)
A 0
60
120
180
240
TMP1
LEU2
ROM1
BE
Time (min)
A 0
60
120
180
240
TMP1
LEU2

Figure 5A, 5B.

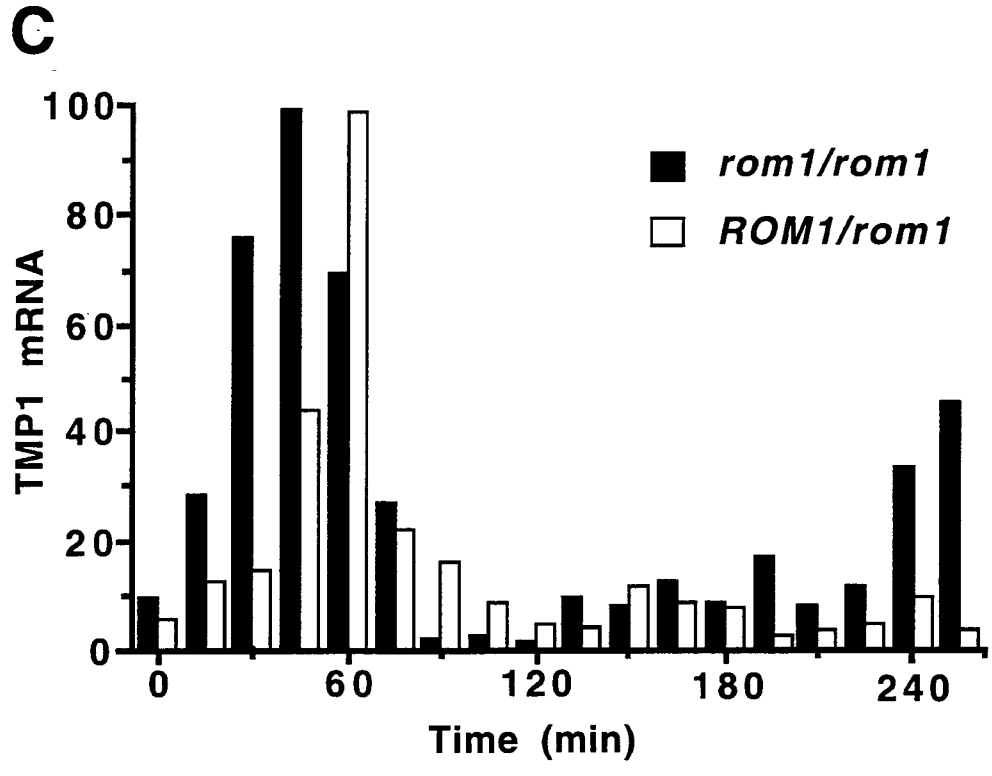
C
100
80
60
40
20
0
TMP1 mRNA
rom1/rom1
ROM1/rom1
0
60
120
180
240
Time (min)

Figure 5C.

Figure 6. Northern analysis of *rom1-21* effect on endogenous *RNR1* and *SWI4* gene expression in elutrially synchronized cells. A) The same RNA that was analysed for *TMP1* mRNA in Figure 5 was hybridized with radiolabeled *RNR1* or *SWI4* probes. B) *RNR1* and *SWI4* mRNA levels, normalized to *LEU2* mRNA levels, were quantitated and plotted as a function of time after inoculation.

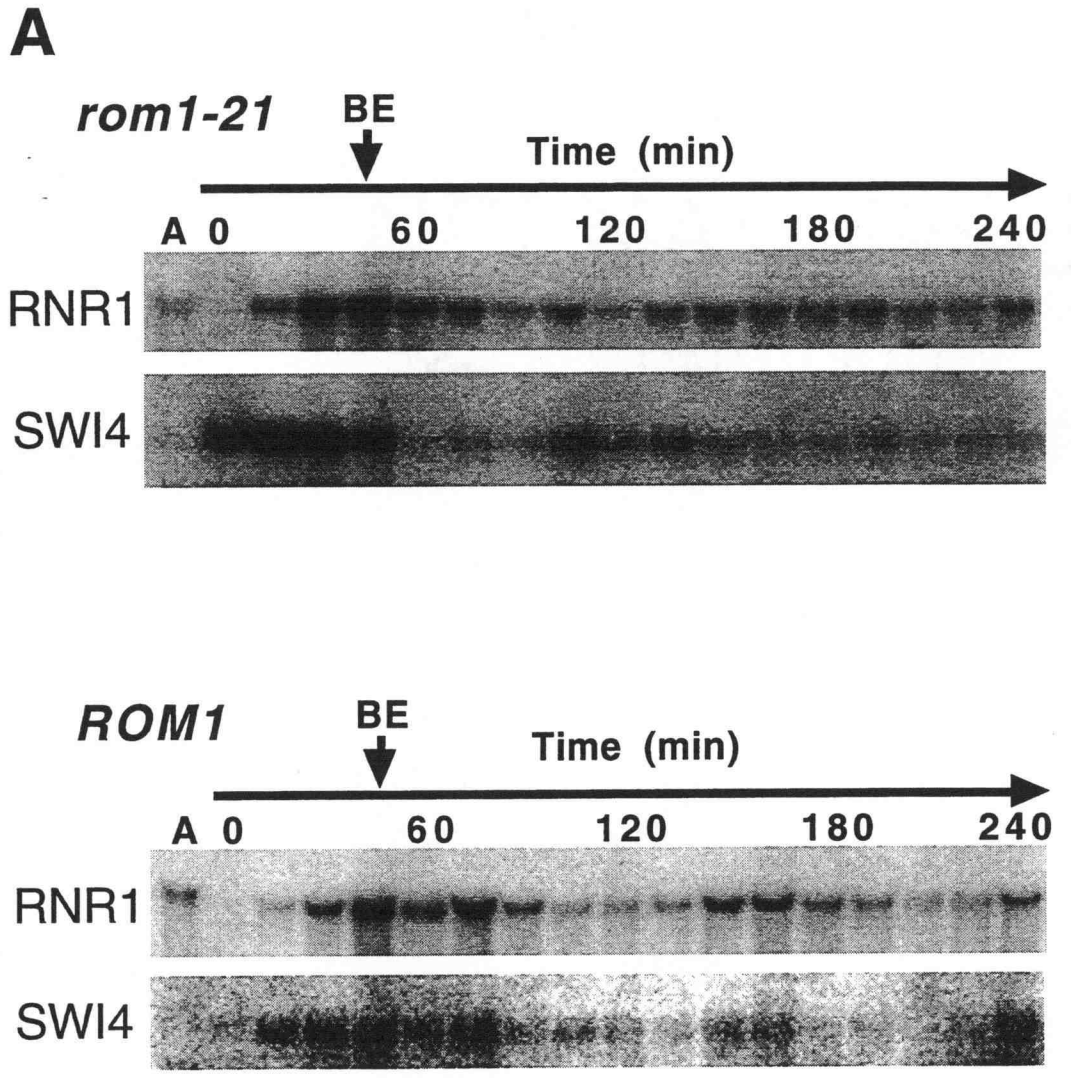
A
rom1-21
BE
Time (min)
A 0
60
120
180
240
RNR1
SWI4
ROM1
BE
Time (min)
A 0
60
120
180
240
RNR1
SWI4

Figure 6A.

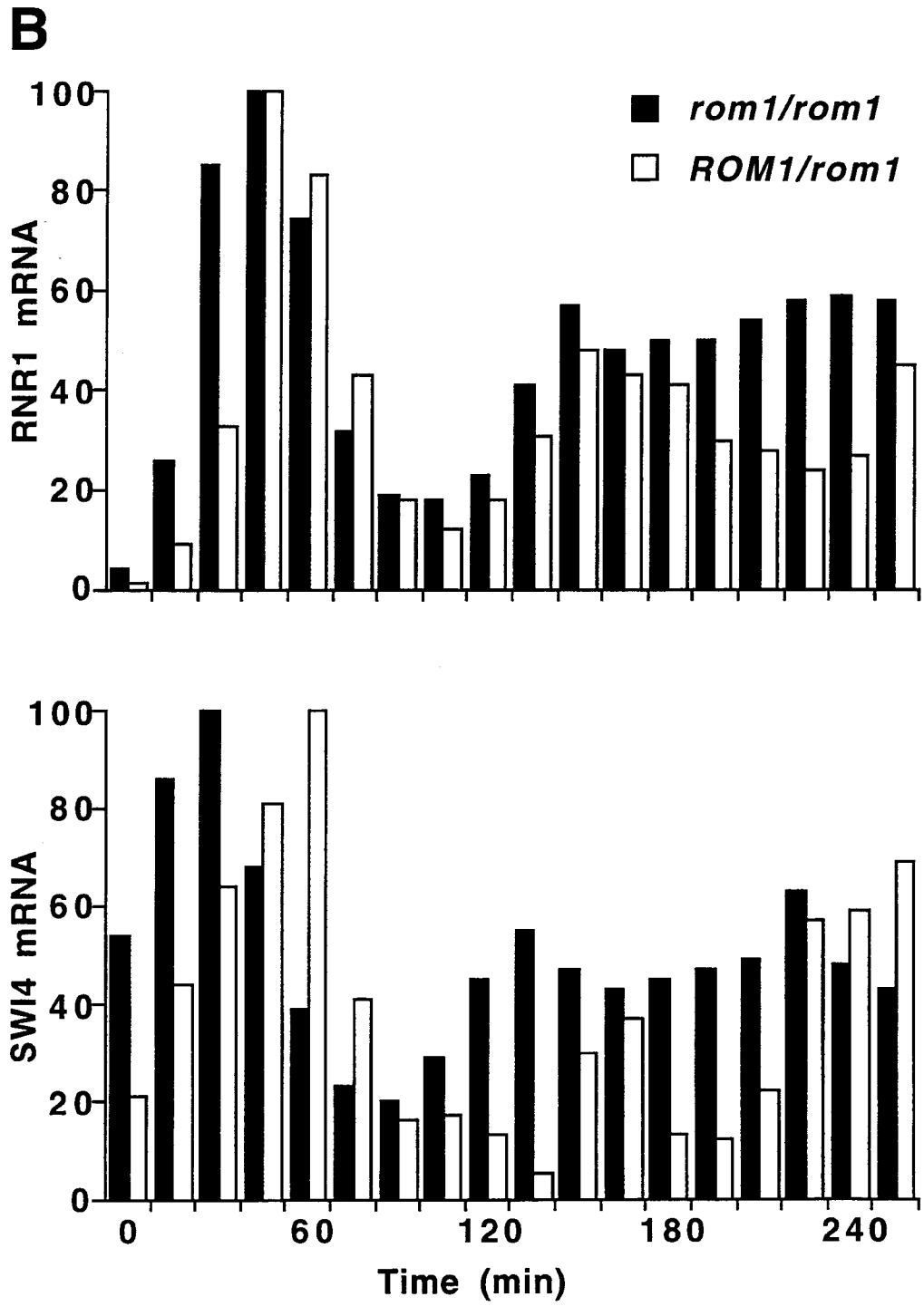
B
rom1/rom1
ROM1/rom1
RNR1 mRNA
SWI4 mRNA
100
80
60
40
20
0
0
60
120
180
240
Time (min)

Figure 6B.

budding index. The kinetics of budding were identical in both populations; buds began to emerge at 48 minutes after release. *RNR1* mRNA levels, normalized to the constitutively expressed *P1* (*Protein-1*) message, are shown in Figure 7A. As was previously observed in elutrially-synchronized cells, the *rom1* mutation advanced the peak in *RNR1* mRNA in *cdc15* synchronized cells (Figure 7B). Also, as was previously observed, *rom1* did not prolong the peak in *RNR1* mRNA level.

The data in Figures 5, 6, 7 and replicate analyses, indicated that the *rom1-21* mutation advanced the onset of MCB gene activation, without advancing budding. A later transcriptional event in the cell cycle, histone *H2A* gene activation in mid S phase, also was advanced by about 15 minutes in *rom1* cells. Possibly, MCB gene activation was immediately triggered after START in *rom1* mutants, whereas MCB gene activation was delayed until a product synthesized by Rom1 protein was depleted in *ROM1* wildtype cells. The fact that *rom1* did not increase asynchronous cell levels of MCB gene mRNA suggested that early induction of MCB genes in *rom1* mutants may trigger downstream cell cycle regulatory events that result in early silencing of MCB genes.

Figure 7. Northern analysis of *rom1-21* effect on endogenous *RNR1* gene expression in *cdc15* synchronized cells. Strains MY2283 (*cdc15 ROM1:LEU2*) or MY2282 (*cdc15 rom1-21*) were synchronized by release from a *cdc15* block into the permissive temperature. At indicated times RNA was prepared for northern blot analysis (A). Northern blot of RNA (5 μg/lane) were hybridized with radiolabeled *RNR1*. Budding kinetics were identical in both populations, with bud emergence (BE) beginning at 48 min. B) *RNR1* mRNA levels, normalized to *P1* mRNA levels, were quantitated and plotted as a function of time after release to the permissive temperature.

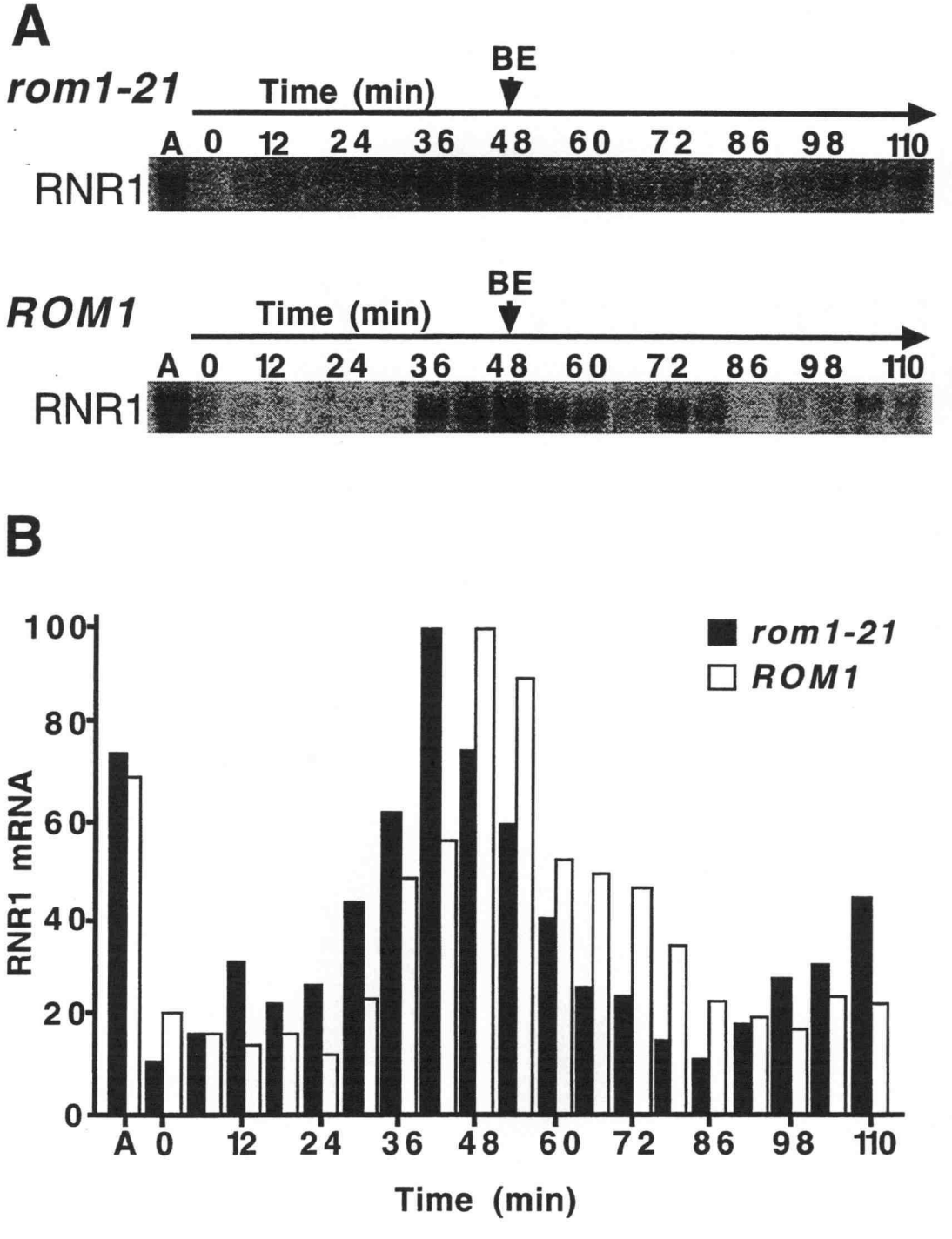
A
rom1-21
Time (min)
BE
A 0 12 24 36 48 60 72 86 98 110
RNR1
ROM1
Time (min)
BE
A 0 12 24 36 48 60 72 86 98 110
RNR1
B
rom1-21
ROM1
RNR1 mRNA
100
80
60
40
20
0
A 0 12 24 36 48 60 72 86 98 110
Time (min)

Figure 7A, 7B.

CHAPTER 4

ROM1 Encodes Thioredoxin Reductase

Cloning wildtype *ROM1* gene

Having genetically identified a gene that encodes a protein that specifically and strongly represses MCB reporter genes and prevents premature activation of endogenous MCB genes, cloning the wildtype *ROM1* gene was a necessary next step in determining its mode of action. To isolate the *ROM1* gene, strain MY2043Z, which carried the MCB/*LacZ* reporter gene, was transformed with the pGAD-based yeast DNA libraries YL1, 2 and 3 developed by Chien and colleagues (1992). Transformants were selected using the *LEU2* marker on the library plasmid and were then screened for white color in filter ß-galactosidase assays. Of 70,000 transformants screened, 200 were white. Of these, only twelve were white when replated and reassayed by filter ß-galactosidase assay. When the twelve were assayed by liquid ß-galactosidase assay only three showed complete suppression of reporter gene activity (Table 7). Plasmids from the three transformants were isolated and reshuttled to MY2043Z cells to confirm that they complemented the *rom1* mutation. All three restored ß-galactosidase activity to the low level expected for a *Δswi6* cell.

Restriction mapping and partial sequencing of the *rom1*-complementing plasmids established that all three plasmids contained permutations of the same genetic locus, upstream from *TRP4* on yeast chromosome IV. A restriction map of the three plasmids is shown in Figure 8. Only two complete open reading frames (designated orf 1 and 2) were common to all three plasmids and were thus candidates for encoding

Table 7. MCB reporter gene activity in yeast transformed with 2μ-based *rom1*-complementing plasmid[a]

Host Cell Genotype	2μ Transformant Clone	MCB/*LacZ* Reporter Gene Activity (nmol ONP/min-mg protein)
Δswi6 rom1-21	31	12
"	38	12
"	29	15
"	25	20
"	8	36
"	22	49
"	23	57
"	6	67
"	7	100
"	39	128
"	37	182
"	32	190
"	pGAD2F	184
Δswi6 ROM1	pGAD2F	12

[a] MY2043Z (*Δswi6 rom1-21*) 2μ-based library transformants selected on the basis of loss of blue color in filter ß-galactosidase assay, were assayed for ß-galactosidase. Three transformants, 31, 38, and 29, showed complete suppression of ß-galactosidase activity. The *Δswi6 ROM1* strain used as a control was MY1001Z.

Figure 8. Restriction map of three highcopy plasmids that restored MCB reporter genes to an "off" state in *Δswi6 rom1-21* yeast. The three plasmids were permutations of the same genetic locus, near *TRP4* on chromosome IV. Two open reading frames (open rectangles), designated orf 1 and orf 2, were common to all three plasmids and were therefore candidates for being the gene encoding the *rom1*-complementing activity. Partial open reading frames are designated by rectangles with one dotted side. Subscript numerals refer to the distance in basepairs from the unique *Kpn1* site upstream from orf1. Restriction site abbreviations are: R1, *EcoR1*; Bg, *BglII*; R5, *EcoRV*; K, *Kpn1*; X, *Xho1*. Polarity of the insert is evident from the vector *EcoR*1 site, which is proximal to the ADH promoter in the library vector. The nucleotide sequence from position -539 to position 3494 was determined.

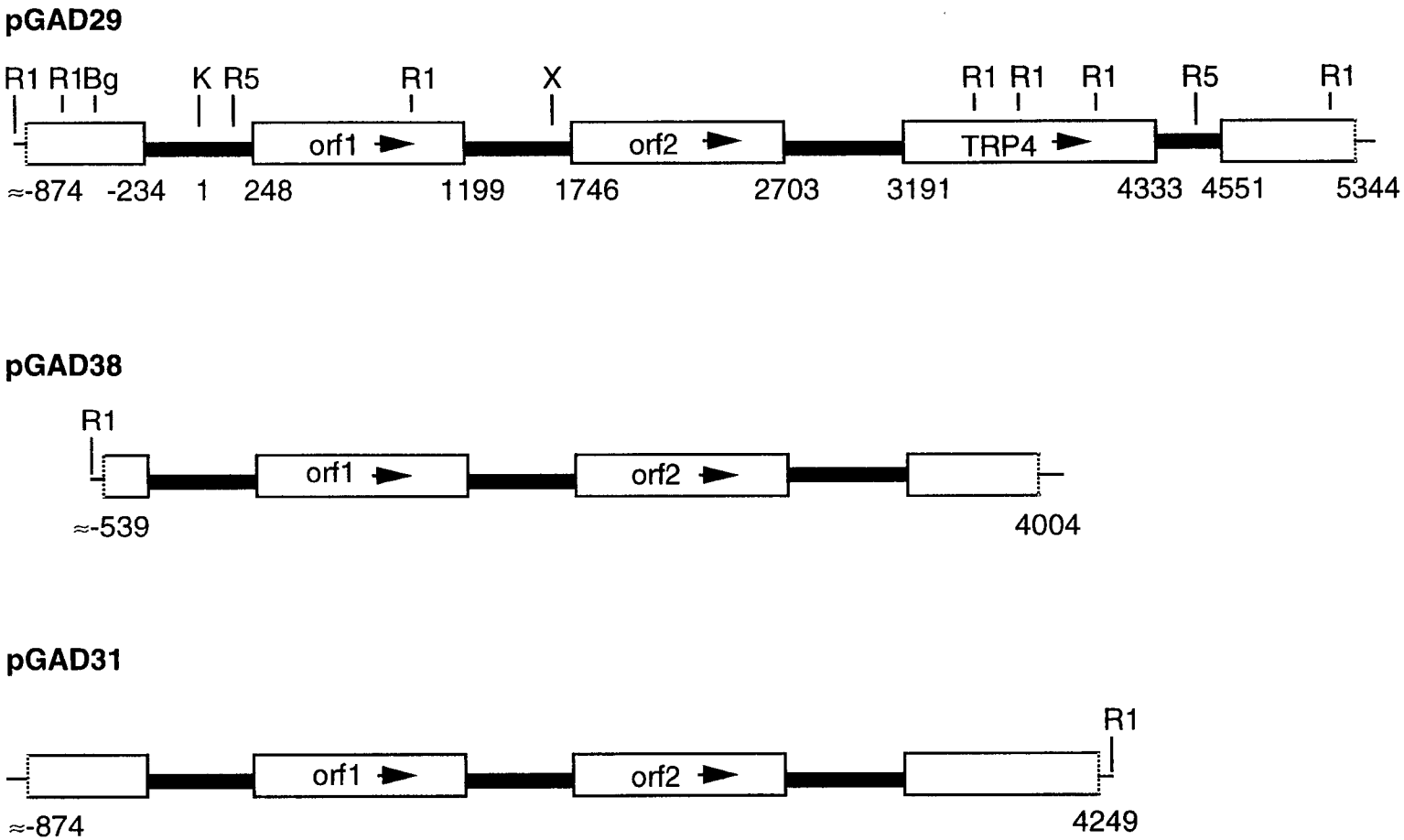
pGAD29
R1 R1Bg K R5 R1 X R1 R1 R1 R5 R1
orf1
orf2
TRP4
≈-874 -234 1 248 1199 1746 2703 3191 4333 4551 5344
pGAD38
R1
orf1
orf2
≈-539 4004
pGAD31
R1
orf1
orf2
≈-874 4249

Figure 8.

the *rom1*-complementing activity. To look for additional *rom1*-complementing genes, additional libraries were screened. One additional plasmid showing complete suppression of reporter gene activity was isolated (Table 8). Restriction mapping established it too was a permutation of chromosome IV DNA containing orfs 1 and 2. In contrast to the first three plasmids obtained, which were isolated from a high copy 2μ-based library, the final plasmid was isolated from a single copy *ars/cen*-based library, YpH1 (obtained from P. Heiter).

The nucleotide sequence common to all of the *rom1*-complementing plasmids was determined and the two open reading frames conceptually translated. The first open reading frame (orf1 in Figure 8) encoded a protein with the seven hydrophobic domains characteristic of G protein-coupled receptors and serpentine membrane proteins. The orf1 protein showed 31% identity and 55% similarity to open reading frame YBR147w on chromosome II (emb|Z36016|) and 29% identity and 55% similarity to open reading frame YOL092w on chromosome XV (emb|X83121|). All three hypothetical proteins showed similarity to the yeast protein Ers1, and therefore the chromosome IV, II and XV Ers1-like proteins were tentatively named Elp1, Elp2 and Elp3, respectively. Expressed sequence tag (EST) clones with sequences identical to *ELP1*, *ELP2* and *ELP3* have recently been reported, suggesting that all three genes are expressed. *ERS1* was originally identified as a high copy suppressor of the slow growth phenotype of yeast *erd1* null mutants. The protein encoded by yeast *ERD1* is located in the endoplasmic reticulum, where it functions to retain proteins that would otherwise be transported to other destinations (Hardwick *et al.*, 1990). Genes similar to *ERS1* and *ERD1* have been identified in *Caenorhabditis elegans*. An intriguing structural feature of

Table 8. MCB reporter gene activity in yeast transformed with *ars/cen*-based *rom1*-complementing plasmid[a]

Host Cell Genotype	*ars/cen* Transformant Clone	MCB/*LacZ* Reporter Gene Activity (nmol ONP/min-mg protein)
Δswi6 rom1-21	33	9
"	15	30
"	16	40
"	5	52
"	14	58
"	18	150
"	13	191
"	YCP50	188
Δswi6 ROM1	YCP50	9

[a] MY2043Z (*Δswi6 rom1-21*) *ars/cen*-based library transformants selected on the basis of loss of blue color in filter ß-galactosidase assay, were assayed for ß-galactosidase. One transformant, 33, showed complete suppression of ß-galactosidase activity. The *Δswi6 ROM1* strain used as a control was MY1001Z.

the Ers1-like family of proteins was the presence a conserved direct repeat in regions predicted to comprise the first and third cytosolic loops (assuming the proteins are oriented across the plasma or ER membrane in the same way as in most seven transmembrane proteins). The repeats have conserved proline (P), glutamine (Q) and serine (S) residues and thus were named PQS repeats. The Elp1 and Elp2 proteins are aligned in Figure 9A, which shows the conserved placement of the PQS repeats with respect to the putative transmembrane domains. The PQS repeats of Elp1, Elp2, Elp3 and Ers1 were aligned and a consensus determined (Figure 9B). A search of the databases using an algorithm that looked for additional proteins with two copies of the consensus (where z was polar, j was nonpolar, ø was aromatic and (-) was acidic residues) failed to find any matches.

Seven transmembrane proteins frequently function as receptors for specific ligands and interact specifically with intracellular signal transduction proteins. Elp1 thus was a member of a class of proteins capable of specific regulatory activities. To determine if Elp1 carried the *rom1*-complementing activity, a 2.1 kb *Bgl2/Xho1* fragment of pGAD29, containing the entire *ELP1* open reading frame plus flanking sequences, was subcloned into YEp181 and transformed into MY2043Z. Transformants continued to show high levels of ß-galactosidase activity (Table 9), suggesting Elp1 did not have *rom1*-complementing activity. Also, in a *ROM1* Δ*swi6* strain, when the chromosomal *ELP1* gene was disrupted by insertion of a *HIS3* PCR product, MCB reporter gene was not activated. Thus, *ELP1* did not complement *rom1* mutations. Furthermore, disruption of *ELP1* did not affect the growth rate or morphology of the yeast in any obvious way. Thus *ELP1* was not essential for *S. cerevisiae*

Figure 9. The first open reading frame (orf1) of the *rom1*-complementing plasmids predicts a seven transmembrane-type serpentine protein and reveals a repeated cytosolic loop region conserved in other serpentine proteins. A) The amino acid sequence of the protein predicted by orf1 (U28372.4), which we have termed Ers1-like protein, or Elp1 and that of a similar protein (Elp2) predicted by open reading frame YBR147w on chromosome 2 were aligned using the GCG GAP program at default settings. The seven hydrophobic domains characteristic of serpentine-type membrane proteins are boxed. The conserved proline/glutamine/serine-containing motif in predicted cytosolic loops 1 and 3 of both proteins are labeled PQS repeat. B) The first and second PQS repeat of four PQS-repeat-containing serpentine proteins were aligned to show conserved features of the motif. Residues identical in most of the repeats are boxed and indicated in the consensus by capital letters. Other residues in the consensus are coded j for nonpolar, z for polar, ø for aromatic, (-) for acidic and x for any residue.

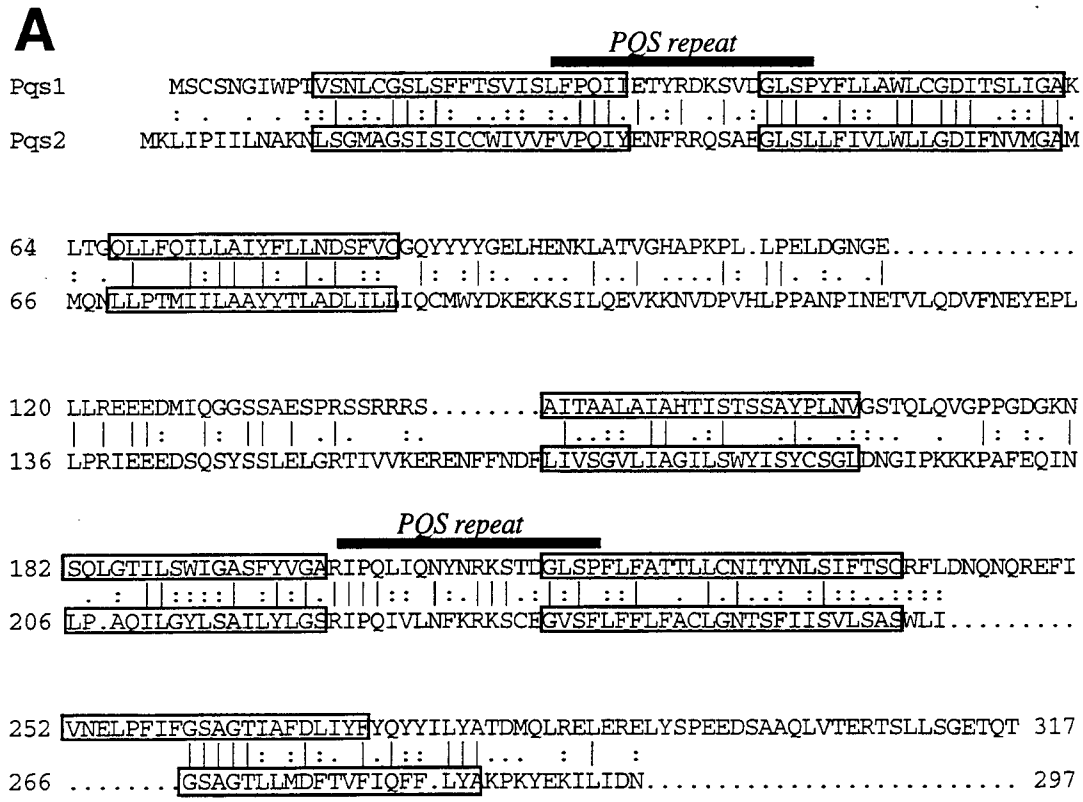
A
PQS repeat
Pqs1
Pqs2
PQS repeat
B
Elp1 (29)
Elp1 (202)
Elp2 (31)
Elp2 (221)
Elp3 (31)
Elp3 (229)
Ers1 (22)
Ers1 (168)
consensus
P Q i j x N ø z r K S x - g j S

Figure 9.

Table 9. Effect of orf1 and orf2 on MCB reporter gene expression[a]

Host Cell Genotype	Plasmid	Orf's Present	MCB/*LacZ* Reporter Gene Activity
			(nmol ONP/min-mg protein)
Δ*swi6 rom1-21*	pGAD2F	none	221±39
"	pGAD29	orf1 + orf2	11±4
"	pGAD31	orf1 + orf2	12±3
"	pGAD38	orf1 + orf2	13±4
Δ*swi6 rom1-21*	YEP181	none	226±35
"	YEP-ELP1	orf1	194±32
"	YEP-TRR1	orf2	10±3
Δ*swi6 ROM1*	YEP181	none	11±3

[a] Lysates from exponentially growing MY2043Z (Δ*swi6 rom1-21*) transformed with the indicated plasmid were assayed for ß-galactosidase activity. All strains carried the MCB/*LacZ* reporter gene. Open reading frames orf1 and orf2 are described in Figure 8. The mean ± standard deviation of the mean for three replicates is shown in the right column. Data show that orf2 and not orf1 complements *rom1-21*. MY1001Z (*Δswi6 ROM1) strain was used as a control.*

growth under laboratory conditions. (While this thesis was in preparation, the sequence of *ELP1* was determined as part of the yeast genome sequencing project; it appears as residues 9743-10697, open reading frame U28372.4, of cosmid clone YSCL9476, gb|U28372|; our sequence exactly matches the deposited sequence in the *ELP1* coding region.)

The second open reading frame (orf2 in Figure 8) common to all of the *rom1*-complementing plasmids encoded a 317-amino protein that was 62% identical to *E. coli* thioredoxin reductase. We therefore named the gene *TRR1*. While we were carrying out our experiments, Chae *et al.* (1994) reported the cloning and sequencing of the orf2 thioredoxin reductase gene (emb|U10274|) *TRR1*. Our sequence varies from the deposited sequence at two residues, which predict Thr for Ala amino acid substitutions at residues 119 and 133. A protein 82% identical to Trr1 is predicted by open reading frame YHR106w on chromosome VIII (gp|Z23109|), which we tentatively named Trr2. There are no other *TRR* gene homologs in *S. cerevisiae*. Both Trr1 and Trr2 contain virtually every conserved domain found in all six NADPH thioredoxin reductases thus far sequenced (Figure 10). The existence of two closely related thioredoxin reductase genes in yeast suggested that *trr1* mutations were unlikely to be the basis for the *rom1* phenotype, because recessive mutations in redundant genes would not give a phenotype. However, it was possible that small structural differences gave the two thioredoxin-reducing proteins different biological activities. For example, Trr2 might be targeted to a different intracellular compartment than Trr1, and thus be unable to complement Trr1 deficiencies. In this regard it is worth noting that Trr2 has a segment of N-terminal amino acids not found in other

Figure 10. The second open reading frame (orf2) of the *roml*-complementing plasmids encodes a thioredoxin reductase. The sequence of the orf2 thioredoxin reductase (Trr1) and a homolog predicted by orf YHR106w on yeast chromosome VIII (Trr2), as well as the nine other known NADPH-type thioredoxin reductases from other species were aligned using the GCG PILEUP program, and the indicated consensus was derived using a plurality of 7. The asterices denote two absolutely conserved cysteines that probably represent the REDOX active thiols involved in shuttling electrons from NADPH to thioredoxin. For the *A. thaliana* protein only part of sequence was available. For the *S. typhimurium* F52a protein (Acc. no. J05478) only the part homologous to thioredoxin reductase is shown. For all other proteins, the complete sequence is shown.

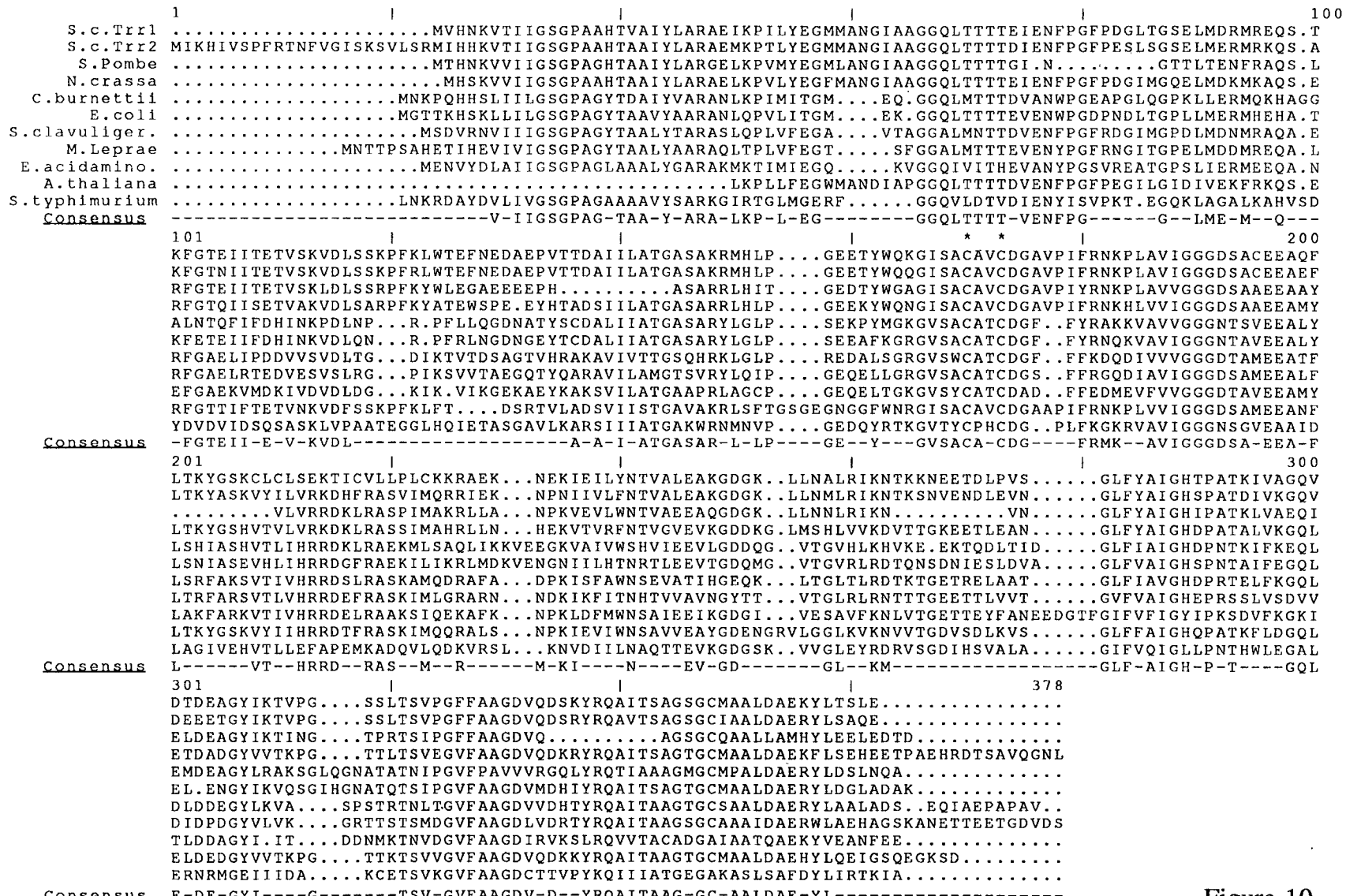
1
100
S.c.Trr1 .....MVHNKVTIIGSGPAAHTVAIYLARAEIKPILYEGMMANGIAAGGQLTTTTEIENFPGFPDGLTGSELMDRMREQS.T
S.c.Trr2 MIKHIVSPFRTNFVGISKSVLSRMIHHKVTIIGSGPAAHTAAIYLARAEMKPTLYEGMMANGIAAGGQLTTTTDIENFPGFPESLSGSELMERMRKQS.A
S.Pombe .....MTHNKVVIIGSGPAGHTAAIYLARGELKPVMYEGMLANGIAAGGQLTTTTGI.N.....GTTLTENFRAQS.L
N.crassa .....MHSKVVIIGSGPAAHTAAIYLARAELKPVLYEGFMANGIAAGGQLTTTTEIENFPGFPDGIMGQELMDKMKAQS.E
C.burnettii .....MNKPQHHSLIILGSGPAGYTDAIYVARANLKPIMITGM....EQ.GGQLMTTTDVANWPGEAPGLQGPKLLERMQKHAGG
E.coli .....MGTTKHSKLLILGSGPAGYTAAVYAARANLQPVLITGM....EK.GGQLTTTTEVENWPGDPNDLTGPLLMERMHEHA.T
S.clavuliger. .....MSDVRNVIIIGSGPAGYTAALYTARASLQPLVFEGA....VTAGGALMNTTDVENFPGFRDGIMGPDLMDNMRAQA.E
M.Leprae .....MNTTPSAHETIHEVIVIGSGPAGYTAALYAARAQLTPLVFEGT.....SFGGALMTTTEVENYPGFRNGITGPELMDDMREQA.L
E.acidamino. .....MENVYDLAIIGSGPAGLAAALYGARAKMKTIMIEGQ.....KVGGQIVITHEVANYPGSVREATGPSLIERMEEQA.N
A.thaliana .....LKPLLFEGWMANDIAPGGQLTTTTDVENFPGFPEGILGIDIVEKFRKQS.E
S.typhimurium .....LNKRDAYDVLIVGSGPAGAAAAVYSARKGIRTGLMGERF.....GGQVLDTVDIENYISVPKT.EGQKLAGALKAHVSD
Consensus ----------V-IIGSGPAG-TAA-Y-ARA-LKP-L-EG--------GGQLTTTT-VENFPG------G--LME-M--Q---
101
200
KFGTEIITETVSKVDLSSKPFKLWTEFNEDAEPVTTDAIILATGASAKRMHLP....GEETYWQKGISACAVCDGAVPIFRNKPLAVIGGGDSACEEAQF
KFGTNIITETVSKVDLSSKPFRLWTEFNEDAEPVTTDAIILATGASAKRMHLP....GEETYWQQGISACAVCDGAVPIFRNKPLAVIGGGDSACEEAEF
RFGTEIITETVSKLDLSSRPFKYWLEGAEEEEPH.....ASARRLHIT....GEDTYWGAGISACAVCDGAVPIYRNKPLAVVGGGDSAAEEAAY
RFGTQIISETVAKVDLSARPFKYATEWSPE.EYHTADSIILATGASARRLHLP....GEEKYWQNGISACAVCDGAVPIFRNKHLVVIGGGDSAAEEAMY
ALNTQFIFDHINKPDLNP...R.PFLLQGDNATYSCDALIIATGASARYLGLP....SEKPYMGKGVSACATCDGF..FYRAKKVAVVGGGNTSVEEALY
KFETEIIFDHINKVDLQN...R.PFRLNGDNGEYTCDALIIATGASARYLGLP....SEEAFKGRGVSACATCDGF..FYRNQKVAVIGGGNTAVEEALY
RFGAELIPDDVVSVDLTG...DIKTVTDSAGTVHRAKAVIVTTGSQHRKLGLP....REDALSGRGVSWCATCDGF..FFKDQDIVVVGGGDTAMEEATF
RFGAELRTEDVESVSLRG...PIKSVVTAEGQTYQARAVILAMGTSVRYLQIP....GEQELLGRGVSACATCDGS..FFRGQDIAVIGGGDSAMEEALF
EFGAEKVMDKIVDVDLDG...KIK.VIKGEKAEYKAKSVILATGAAPRLAGCP....GEQELTGKGVSYCATCDAD..FFEDMEVFVVGGGDTAVEEAMY
RFGTTIFTETVNKVDFSSKPFKLFT....DSRTVLADSVIISTGAVAKRLSFTGSGEGNGGFWNRGISACAVCDGAAPIFRNKPLVVIGGGDSAMEEANF
YDVDVIDSQSASKLVPAATEGGLHQIETASGAVLKARSIIIATGAKWRNMNVP....GEDQYRTKGVTYCPHCDG..PLFKGKRVAVIGGGNSGVEAAID
Consensus -FGTEII-E-V-KVDL----------A-A-I-ATGASAR-L-LP----GE--Y---GVSACA-CDG----FRMK--AVIGGGDSA-EEA-F
201
300
LTKYGSKCLCLSEKTICVLLPLCKKRAEK...NEKIEILYNTVALEAKGDGK..LLNALRIKNTKKNEETDLPVS......GLFYAIGHTPATKIVAGQV
LTKYASKVYILVRKDHFRASVIMQRRIEK...NPNIIVLFNTVALEAKGDGK..LLNMLRIKNTKSNVENDLEVN......GLFYAIGHSPATDIVKGQV
.........VLVRRDKLRASPIMAKRLLA...NPKVEVLWNTVAEEAQGDGK..LLNNLRIKN..........VN......GLFYAIGHIPATKLVAEQI
LTKYGSHVTVLVRKDKLRASSIMAHRLLN...HEKVTVRFNTVGVEVKGDDKG.LMSHLVVKDVTTGKEETLEAN......GLFYAIGHDPATALVKGQL
LSHIASHVTLIHRRDKLRAEKMLSAQLIKKVEEGKVAIVWSHVIEEVLGDDQG..VTGVHLKHVKE.EKTQDLTID.....GLFIAIGHDPNTKIFKEQL
LSNIASEVHLIHRRDGFRAEKILIKRLMDKVENGNIILHTNRTLEEVTGDQMG..VTGVRLRDTQNSDNIESLDVA.....GLFVAIGHSPNTAIFEGQL
LSRFAKSVTIVHRRDSLRASKAMQDRAFA...DPKISFAWNSEVATIHGEQK...LTGLTLRDTKTGETRELAAT......GLFIAVGHDPRTELFKGQL
LTRFARSVTLVHRRDEFRASKIMLGRARN...NDKIKFITNHTVVAVNGYTT...VTGLRLRNTTTGEETTLVVT......GVFVAIGHEPRSSLVSDVV
LAKFARKVTIVHRRDELRAAKSIQEKAFK...NPKLDFMWNSAIEEIKGDGI...VESAVFKNLVTGETTEYFANEEDGTFGIFVFIGYIPKSDVFKGKI
LTKYGSKVYIIHRRDTFRASKIMQQRALS...NPKIEVIWNSAVVEAYGDENGRVLGGLKVKNVVTGDVSDLKVS......GLFFAIGHQPATKFLDGQL
LAGIVEHVTLLEFAPEMKADQVLQDKVRSL...KNVDIILNAQTTEVKGDGSK..VVGLEYRDRVSGDIHSVALA......GIFVQIGLLPNTHWLEGAL
Consensus L------VT--HRRD--RAS--M--R------M-KI----N----EV-GD-------GL--KM----------GLF-AIGH-P-T----GQL
301
378
DTDEAGYIKTVPG....SSLTSVPGFFAAGDVQDSKYRQAITSAGSGCMAALDAEKYLTSLE.....
DEEETGYIKTVPG....SSLTSVPGFFAAGDVQDSRYRQAVTSAGSGCIAALDAERYLSAQE.....
ELDEAGYIKTING....TPRTSIPGFFAAGDVQ.....AGSGCQAALLAMHYLEELEDTD.....
ETDADGYVVTKPG....TTLTSVEGVFAAGDVQDKRYRQAITSAGTGCMAALDAEKFLSEHEETPAEHRDTSAVQGNL
EMDEAGYLRAKSGLQGNATATNIPGVFPAVVVRGQLYRQTIAAAGMGCMPALDAERYLDSLNQA.....
EL.ENGYIKVQSGIHGNATQTSIPGVFAAGDVMDHIYRQAITSAGTGCMAALDAERYLDGLADAK.....
DLDDEGYLKVA....SPSTRTNLTGVFAAGDVVDHTYRQAITAAGTGCSAALDAERYLAALADS..EQIAEPAPAV..
DIDPDGYVLVK....GRTTSTSMDGVFAAGDLVDRTYRQAITAAGSGCAAAIDAERWLAEHAGSKANETTEETGDVDS
TLDDAGYI.IT....DDNMKTNVDGVFAAGDIRVKSLRQVVTACADGAIAATQAEKYVEANFEE.....
ELDEDGYVVTKPG....TTKTSVVGVFAAGDVQDKKYRQAITAAGTGCMAALDAEHYLQEIGSQEGKSD.....
ERNRMGEIIIDA.....KCETSVKGVFAAGDCTTVPYKQIIIATGEGAKASLSAFDYLIRTKIA.....
Consensus E-DE-GYI----G-------TSV-GVFAAGDV-D--YRQAITAAG-GC-AALDAE-YL----------

Figure 10.

thioredoxin reductases and that the segment shares features common to mitochondrial targeting sequences (Tyler *et al.*, 1992).

To test whether Trr1 carried the *rom1*-complementing activity, a 2.6-kb *EcoR1* fragment of pGAD29 containing the *TRR1* coding region plus flanking sequences was subcloned into YEp181 and transformed into MY2043Z yeast. ß-galactosidase activity was extinguished in all transformants (Table 9), proving that Trr1 protein possessed the *rom1*-complementing activity.

The thioredoxin reductase gene *TRR1* and *rom1* are allelic

To establish that *TRR1* and *rom1* were allelic and that complementation was not due to a gene dosage phenomenon or other form of extragenic suppression, the chromosomal *TRR1* locus was non-disruptionally tagged in Δ*swi6* strain BY600 by insertion of a *LEU2*-marked plasmid (Figure 11). Integration at the *TRR1* locus was confirmed by Southern blot analysis (Figure 12). The *TRR1:LEU2* tagged strain was mated to *rom1-21* Δ*swi6:TRP1* strain MY2043 and inheritance of the *LEU*$^+$ and *rom1* phenotype was monitored. Figure 13 shows an example of a tetrad analysis. In twelve tetrads and forty-three random spores, the *LEU2* marker and the *rom1* phenotype were always inherited reciprocally. Thus establishing that *TRR1* and *rom1* were allelic.

In tagging the *TRR1* locus with the *LEU2*-marked plasmid we noticed unusually selective site-specific integration. The plasmid used to tag the locus consisted of a 2 kb *Bgl2/Xho1* restriction fragment of pGAD29 cloned into pRS305. The plasmid was linearized by partial digestion with *EcoR5* , which cut the plasmid either within the insert or

Figure 11. Strategy for *LEU2* tagging of *TRR1* locus. A 2.1 kb *BglII/Xho1 ELP1* gene fragment from *rom1* complementing plasmid pGAD29 was cloned into the *BamH1/Xho1* cut pRS305 (integration plasmid carrying *LEU2* gene). The resulting plasmid was partially digested with *EcoRV* (an *EcoRV* site in *LEU2* is not depicted) and transformed into BY600. A restriction map of the chromosome locus if the plasmid was homologously integrated is shown at the bottom. Restriction enzyme abbreviations: H3, *HindIII*; Bg, *BglII*; R5, *EcoRV*; X, *Xho*1. The solid bar shows the DNA fragment used to probe southern blots.

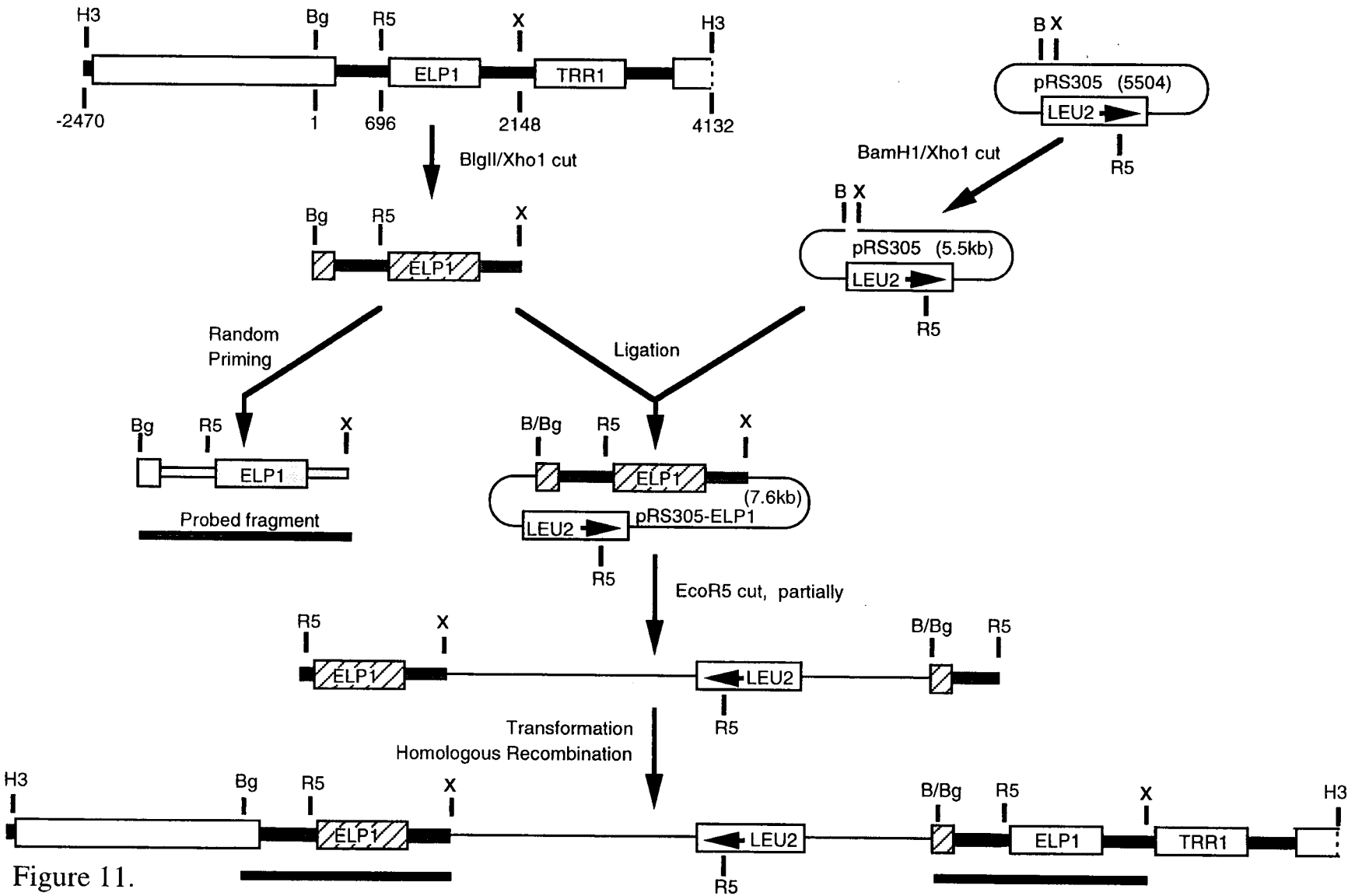
H3
Bg
R5
X
H3
ELP1
TRR1
-2470
1
696
2148
4132
BglII/Xho1 cut
B X
pRS305 (5504)
LEU2
R5
BamH1/Xho1 cut
B X
pRS305 (5.5kb)
LEU2
R5
Bg
R5
X
ELP1
Random
Priming
Ligation
Bg
R5
X
ELP1
Probed fragment
B/Bg
R5
X
ELP1
(7.6kb)
LEU2
pRS305-ELP1
R5
EcoR5 cut, partially
R5
X
ELP1
LEU2
B/Bg
R5
R5
Transformation
Homologous Recombination
H3
Bg
R5
X
ELP1
LEU2
R5
B/Bg
R5
X
H3
ELP1
TRR1

Figure 11.

Figure 12. Southern blot confirming homologous integration of *LEU2* plasmid at *TRR1* locus. *HindIII* and *HindIII/XhoI digested* DNA from *LEU2*+ pRS305-ELP1 transformants were electrophoreticaly separated and blotted. Blots were probed with a radiolabeled 2.1 kb *BglII/Xho*1 *ELP1* fragment (see Figure 11). BY600 was included in lane 1 as a control showing a non-tagged *TRR1* locus. The expected bands for the *HindIII* digest are 6.6 kb for *TRR1*, and 14.2 kb for *TRR1:LEU2*. The expected bands for the *HindIII/XhoI* digest are 4.6 kb for *TRR1*, and 7.6 kb and 4.6 kb for *TRR1:LEU2*.

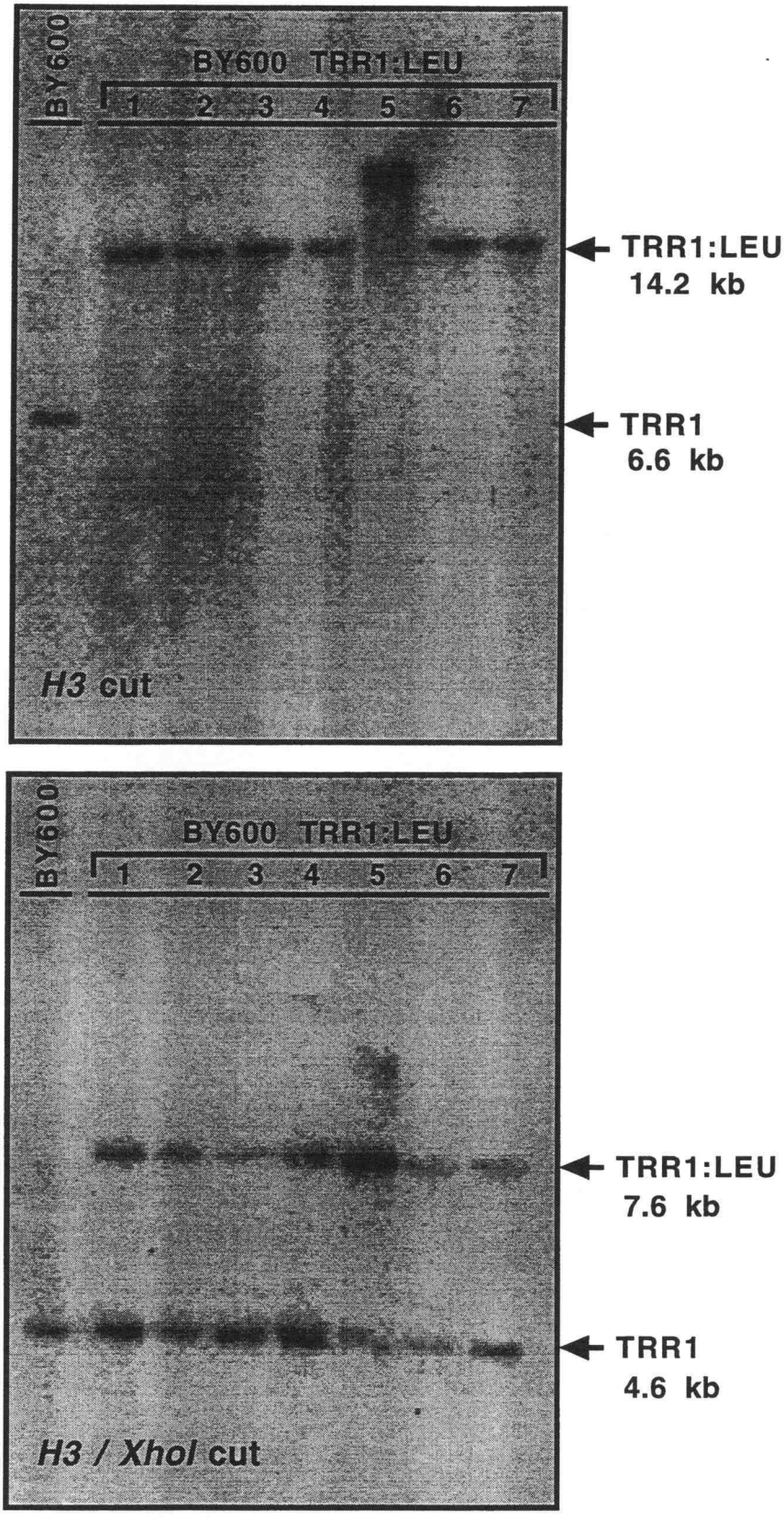
BY600
BY600 TRR1:LEU
1 2 3 4 5 6 7
TRR1:LEU
14.2 kb
TRR1
6.6 kb
H3 cut
BY600
BY600 TRR1:LEU
1 2 3 4 5 6 7
TRR1:LEU
7.6 kb
TRR1
4.6 kb
H3 / XhoI cut

Figure 12.

Figure 13. Tetrad analysis showing *TRR1* is wildtype allele of *rom1*. MY2043 (*rom1-21* Δ*swi6*) was mated to MY2157 (*TRR1:LEU2* Δ*swi6*). Three independent tetrad sets were derived from the diploid. A) Spore growth in the presence of leucine (+Leu). B) Spore growth in the absence of leucine (-Leu). Note 2:2 segregation of *LEU2* marker. C) Filter ß-gal assay of segregants. Note that *LEU2* segregants are white (indicative of *TRR1* allele), while *leu2* segregants are blue (indicative of *rom1-21* allele). Data shows that *rom1* and *TRR1* are allelic.

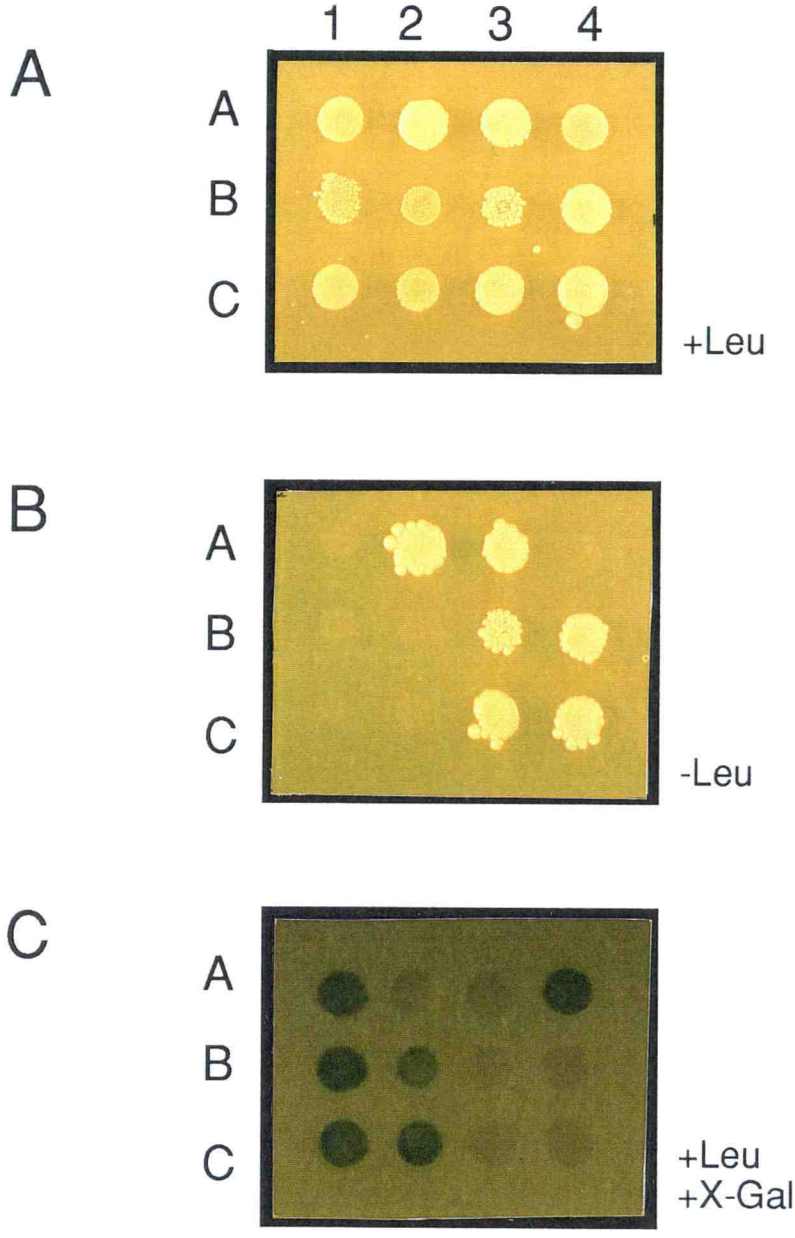

Figure 13.

within the *LEU2* open reading frame (Figure 11). Diagnostic cutting with a second enzyme (*XhoI*) confirmed that the plasmid had been linearized with roughly equal efficiency at each of the two *EcoR5* sites. However, when the linearized plasmid was gel-recovered and used to transform BY600, Southern blots showed that six out of seven *LEU*$^+$ transformants had integrated the plasmid at the *TRR1* locus (Figure 12). Preferential integration at the *TRR1* locus suggested that the region was particularly recombinogenic. High recombination activity in the *TRR1* region may explain the high rate of conversion to the *rom1* phenotype in *ROM1/rom1* heterozygotes selected for MCB/*HIS3* reporter gene activity by growth in ATZ (see earlier discussion).

Having established that *TRR1* and *rom1* were allelic, eleven different *rom1* alleles covering the whole range of reporter gene activation (Table 3), were probed with a radiolabeled *TRR1* DNA fragment. Southern blot (Figure 14) showed that ten out of eleven alleles gave a restriction fragment that was wildtype in size. Only the *rom1-13* allele generated a different pattern, which indicated the mutation grossly affected the structure of the locus.

TRR1 disruption reproduces *rom1* effects

To determine the effect of disrupting *TRR1* on cell viability and on MCB reporter gene expression, a *HIS3*-encoding disruption fragment containing *TRR1* sequences at each end was generated by PCR (Figure 15). The disruption fragment was used to transform haploid strain MY2179 and diploid strain MY2183, both of which carried mutation *his3Δ200*. Of four haploid and seven diploid *HIS*$^+$ transformants analyzed by Southern blot

Figure 14. Southern blot of *TRR1* locus in different *rom1* mutants. Genomic DNA from eleven different *rom1* mutants (see Table 3) covering the whole range of reporter gene activities, were digested with *EcoR1*, electrophoretically separated and blotted. The blot was hybridized with radiolabeled *TRR1* DNA fragment (see Figure 11). BY600 DNA was included to show the 2.6 kb band expected for the wildtype *TRR1* locus. Data show that except for *rom1-15*, all the *rom1* alleles gave a 2.6 kb *EcoR1* fragment identical in size to the wildtype *TRR1* gene. The data suggest that most of the *rom1* mutations are not due to large deletion or insertion of DNA.

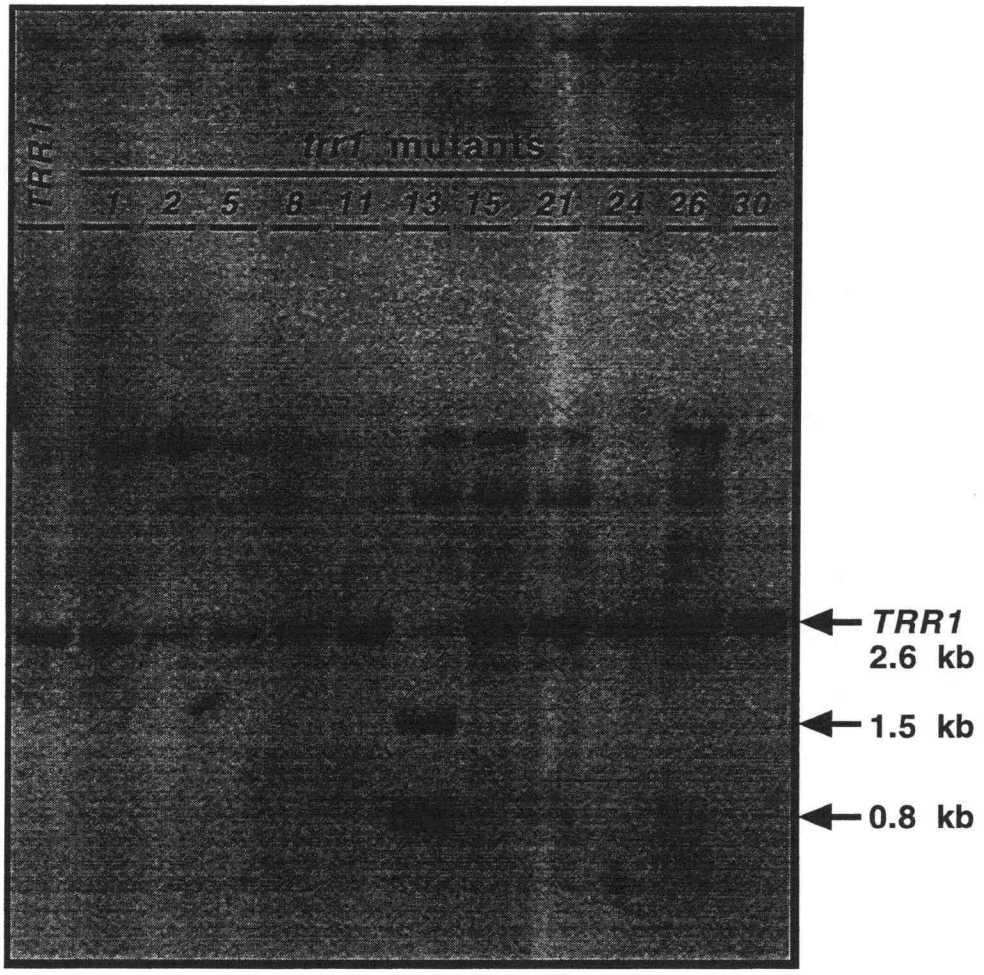

Figure 14.

Figure 15. *TRR1* disruption strategy. Two PCR oligos with homology to *HIS3* at their 3' end (filled box) and homology to *TRR1* at the 5' end (white box) were used to generate a PCR fragment containing the intact *HIS3* gene sandwiched between the first 38 and last 38 nucleotides of the *TRR1* protein coding region. Homologous integration of the *HIS3* PCR fragment at *TRR1* locus would generate a *Δtrr1:HIS3* disruptive mutation with the indicated restriction map. Restriction enzyme abbreviations: H3, *HindIII*; Bg, *BglII*; R1, *EcoR1*; X, *Xho1*.

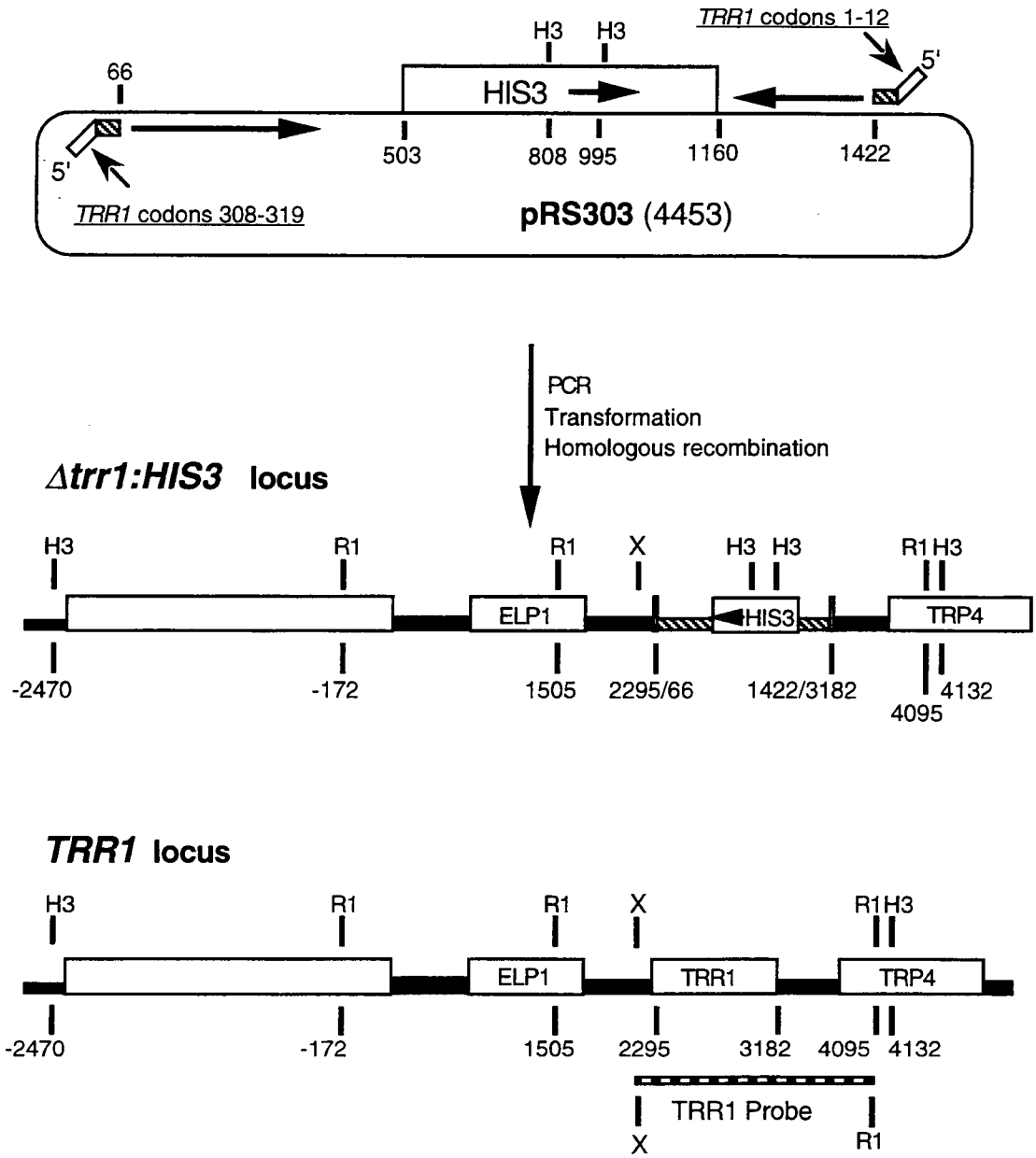
H3
H3
TRR1 codons 1-12
5'
66
HIS3
5'
503
808
995
1160
1422
TRR1 codons 308-319
pRS303 (4453)
PCR
Transformation
Homologous recombination
Δtrr1:HIS3 locus
H3
R1
R1
X
H3
H3
R1
H3
ELP1
HIS3
TRP4
-2470
-172
1505
2295/66
1422/3182
4095
4132
TRR1 locus
H3
R1
R1
X
R1
H3
ELP1
TRR1
TRP4
-2470
-172
1505
2295
3182
4095
4132
TRR1 Probe
X
R1

Figure 15.

hybridization, only two diploid transformants were found to carry a *Δtrr:HIS3* deletion mutation (Figure 16). When one of the heterozygous deletion mutant was sporulated and twenty tetrads were dissected, only one tetrad gave four colonies. Southern blot confirmed a 2:2 segregation of the *ROM1* and *Δtrr1:HIS3* alleles in the tetrad (Figure 17). The tetrad had two large *his*$^-$ colonies and two small *HIS*$^+$ colonies. Most tetrads yielded only two large colonies, both of which were *his*$^-$. In the few tetrads that gave three colonies, two were always large and *his*$^-$ and one was small and *HIS*$^+$. We concluded that disruption of *TRR1* resulted in poor viability, and that in those *Δtrr1:HIS3* disruptants that managed to form colonies, the growth rate was significantly slower than in *TRR1* cells. The fact that no *Δtrr1:HIS3* disruptants were obtained in haploid cells reinforced this assumption. The viability problem was even more evident when spores were plated on supplemented YNB plates. When glusulased random spores were directly plated on supplemented YNB plates, no *Δtrr:HIS3* spores formed colonies. When the experiment was repeated but spores were allowed to form colonies on YEPD plates and then were replicaplated to appropriately supplemented YNB plates, some small *Δtrr1:HIS3* colonies were evident, but at much lower frequency than the 50% expected. The results suggested that *Δtrr1:HIS3* spores have poor viability when germinated on YEPD plates and are not viable at all when germinated on supplemented YNB medium.

The dissected tetrad that yielded four viable spores was analyzed further to quantitate the effect of deleting *TRR1* on cell growth rate. As shown in Figure 18, the *Δtrr1:HIS3* segregants grew with an average doubling time of 3.1 hours, which was 70% longer than the 1.8-hr doubling time of *TRR1* segregants. Microscopic examination of dissected

Figure 16. Southern blot confirming *TRR1* disruption by *HIS3*. Strains MY2179 (*his3Δ200*, haploid) or MY2183 (*his3Δ200*, diploid) were transformed with the *HIS3* PCR fragment (see Figure 15). Four *HIS3*+ haploids and seven *HIS3*+ diploids transformants were obtained. DNA from the transformants or the two parental strains were digested with *EcoR1*. The intact *TRR1* gene was expected to generates a 2.6 kb fragment while a *Δtrr1:HIS3* allele would generate a 3.0 kb fragment. Most of the diploids and none of the haploids gave evidence of homologous recombination. However, two clones 6 and 11 showed a pattern consistent with homologous integration, yielding a (*TRR1/Δtrr1:HIS3*) heterozygote.

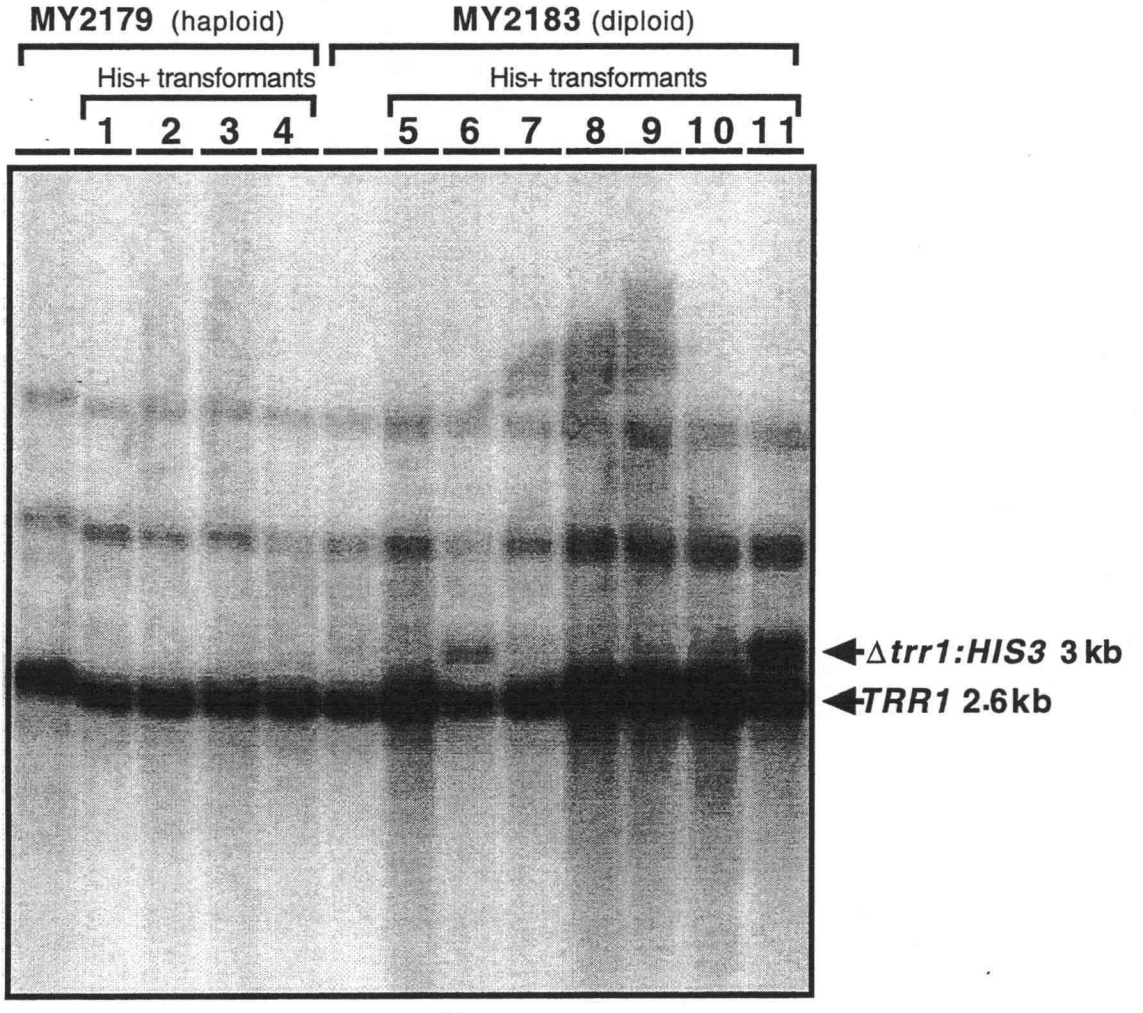
MY2179 (haploid)
MY2183 (diploid)
His+ transformants
His+ transformants
1 2 3 4
5 6 7 8 9 10 11
Δtrr1:HIS3 3 kb
TRR1 2.6kb

Figure 16.

Figure 17. Southern blot of segregants from a MY2197 (*TRR1/Δtrr1:HIS3*) tetrad. DNA from a *TRR1/TRR1* strain (MY2183), two putative *TRR1/Δtrr1:HIS3* strains (MY2196 and MY2197), and MY2197 tetrad segregants (A1, A2, A3 and A4) were digested with *HindIII* and analysed by Southern blot. The blot shows the expected 1.4 kb fragment for *Δtrr1:HIS* and the 6.6 kb fragment for *TRR1* segregating 2:2 in the tetrad segregants.

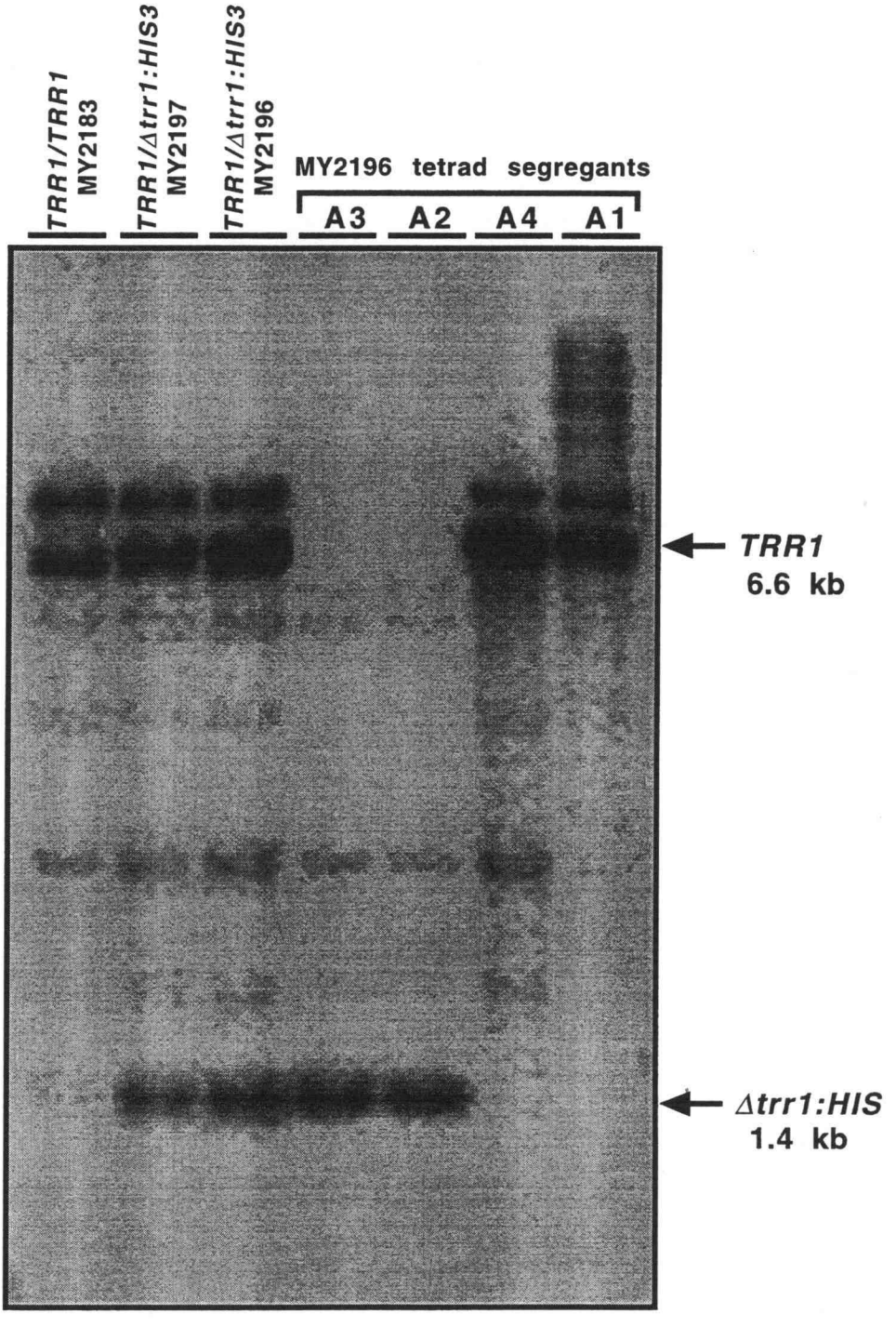
TRR1/TRR1
MY2183
TRR1/Δtrr1:HIS3
MY2197
TRR1/Δtrr1:HIS3
MY2196
MY2196 tetrad segregants
A3
A2
A4
A1
TRR1
6.6 kb
Δtrr1:HIS
1.4 kb

Figure 17.

Figure 18. Effect of deleting *TRR1* gene on growth rate. *TRR1* and *Δtrr1:HIS3* segregants from a dissected tetrad were incubated in YEPD medium and growth rate was monitored by aborbance at 600 nm.

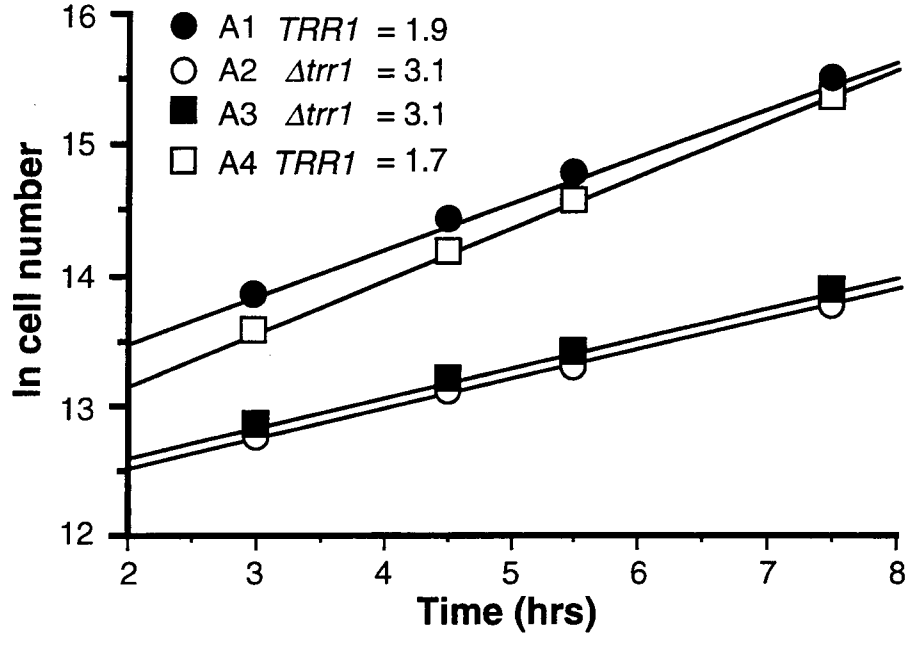
A1 TRR1 = 1.9
A2 Δtrr1 = 3.1
A3 Δtrr1 = 3.1
A4 TRR1 = 1.7
ln cell number
16
15
14
13
12
2
3
4
5
6
7
8
Time (hrs)

Figure 18.

tetrads showed that germination (the appearance of two-cell colonies) was delayed in Δ*trr1*:*HIS3* segregants, which together with the slower growth rate, caused Δ*trr1*:*HIS3* segregants to form very small colonies relative to wildtype.

The viable Δ*trr1* null mutants allowed us to test the effect of *TRR1* disruption on MCB reporter gene activity. A Δ*trr1*:*HIS* disruptant was mated to Δ*swi6*:*TRP1* strain MY1001Z, and segregants with the four expected haplotypes were isolated. As shown in Table 10, Δ*trr1*:*HIS3* Δ*swi6*:*TRP1* segregants showed 35-fold higher ß-galactosidase levels than their *TRR1* Δ*swi6* counterparts. The effect of deleting *TRR1* was also observed in *SWI6* cells, where Δ*trr1*:*HIS3* segregants showed 2.5-fold higher ß-galactosidae activity than their *TRR1* counterparts. Thus, in both a Δ*swi6* mutant and *SWI6* wildtype background, the Δ*trr1* deletion mutation had the same positive effect on MCB reporter gene expression as previously observed for the natural *trr1* alleles.

Having established that Δ*trr1* mutations elevated MCB reporter gene expression, we next investigated whether the Δ*trr1* mutation affected expression of the endogenous *RNR1* genes. Northern blot analysis of RNA from exponentially growing cells showed that Δ*trr1* deletion mutation had the same non-effect on expression of endogenous *RNR1* gene as previously observed for the natural *trr1* alleles.

TRX1 and *TRX2* disruption reproduces Δ*trr1* effects

The enzymatic function of Trr1 presumably is to reduce the active site disulfide on oxidized thioredoxin. *S. cerevisiae* contains at least two thioredoxin genes, *TRX1* and *TRX2*, both of which must be disrupted to

Table 10. Effect of deleting *TRR1* on MCB Reporter Gene Expression

Genotype[a]	Strain	MCB/*LacZ* Reporter Gene Activity	
		nmol ONP/min-mg protein	mean±SD
TRR1 SWI6	MY2220	223	235±46
	MY2223	296	
	MY2224	186	
Δtrr1:HIS3 SWI6	MY2218	479	636±171
	MY2219	555	
	MY2221	873	
TRR1 Δswi6:TRP1	MY2215	11	5±4
	MY2216	2	
	MY2217	2	
Δtrr1:HIS3 Δswi6:TRP1	MY2209	224	245±16
	MY2211	246	
	MY2212	264	

[a] Strains with the indicated haplotypes were isolated from a diploid strain heterozygous for *Δtrr1:HIS3* and *Δswi6:TRP1* (MY2203). All strains carried the MCB/*LacZ* reporter gene. Lysates from exponentially growing cells were assayed for ß-galactosidase activity. The mean ± standard deviation of the mean for the threee segregants is shown in the right column.

give a noticeable phenotype (Muller, 1991). Muller (1991) showed that Δ*trx1* Δ*trx2* double mutants grow slower than wildtype cell, have a protracted S phase and show elevated levels of *RNR1* and *RNR2* mRNA. If Δ*trr1* mutations activate MCBs by lowering reduced thioredoxin levels, disruption of *TRX1* and *TRX2* should also activate MCB reporter gene activity. To test this prediction, yeast strain EMY63, carrying Δ*trx1* Δ*trx2* double deletion mutations, and isogenic wildtype strain EMY60, were transformed with the MCB/*LacZ*, mutMCB/*LacZ* or SCB/*LacZ* reporter genes, and transformants were assayed for ß-galactosidase. The results, shown in Table 11, established that the Δ*trx1* Δ*trx2* deletion mutations recapitulated the effect of natural *rom1* mutations and Δ*trr1* deletion mutation on MCB reporter gene activity. MCB/*LacZ* and SCB/*LacZ* reporter gene activity were significantly augmented, and the mutMCB/*LacZ* reporter gene was relatively unaffected.

Although *trr1* mutations reproducibly induced MCB/*LacZ* reporter gene activity in *SWI6*$^+$ cells, the effect was much more striking in Δ*swi6* cells, where reporter gene activity is very low in the absence of *trr1* mutations (see Table 3). Having established that the Δ*trx1* Δ*trx2* deletion mutations recapitulated the two-fold effect of Δ*trr1* deletion on MCB reporter gene activity in *SWI6*$^+$ cells, we next investigated whether the Δ*trx1* Δ*trx2* deletion mutations reproduced the multifold effect of Δ*trr1* deletion on MCB reporter gene activity in Δ*swi6* cells. A Δ*trx1*:*LEU2* Δ*trx2*:*LYS2* yeast strain (EMY56) was mated to the Δ*swi6* strain MY1001, and segregants with the desired genotype were isolated and transformed with the MCB/*LacZ*, mutMCB/*LacZ* or SCB/*LacZ* reporter genes. Transformants were assayed for ß-galactosidase. The results, shown in Table 12, indicate that deletion of *TRX1* and *TRX2* recapitulates the effect

Table 11. Effect of deleting *TRX1* and *TRX2* on MCB and SCB reporter genes[a]

Strain	Relevant Genotype	Reporter Gene		
		MCB/*LacZ*	mutMCB/*LacZ*	SCB/*LacZ*
		ß-galactosidase activity (nmol ONP/min-mg protein)		
EMY60	*TRX1 TRX2*	270±44	9±5	137±39
EMY63	*Δtrx1:LEU2 Δtrx2:TRP1*	614±134	10±5	506±102

[a] Lysates from exponentially growing cells transformed with the indicated reporter genes were assayed for ß-galactosidase (see Table 4 for designation of reporter genes). The mean ± standard deviation of the mean for four independent transformant clones is shown.

Table 12. Effect of deleting both thioredoxin genes *TRX1* and *TRX2* on MCB and SCB reporter gene in Δ*swi6* yeast[a]

Strain	Relevant Genotype	Reporter Gene		
		MCB/*LacZ*	mutMCB/*LacZ*	SCB/*LacZ*
		ß-galactosidase activity (nmol ONP/min-mg protein)		
BY600	*Δswi6:TRP1 TRX1 TRX2*	4±1	5±1	8±2
MY2257	*Δswi6:TRP1 Δtrx1:LEU2 Δtrx2:LYS2*	185±53	9±2	54±2

[a] Lysates from exponentially growing cells transformed with the indicated reporter genes were assayed for ß-galactosidase (see Table 4 for designation of reporter genes). The mean ± standard deviation of the mean for four independent transformant clones is shown.

of deleting *TRR1* on MCB reporter gene activity in Δ*swi6* cells. MCB/*LacZ* and SCB/*LacZ* reporter gene activity was significantly augmented, and mutMCB/*LacZ* reporter gene was relatively unaffected.

To test if either deletion of *TRX1* or *TRX2* alone affected MCB reporter gene activity in Δ*swi6* cells, segregants from the EMY56 x MY1001 diploid in which either *TRX1* or *TRX2* were deleted were transformed with the MCB/*LacZ* reporter gene, and transformants were assayed for ß-galactosidase. The results, shown in Table 13, indicate that deletion of both *TRX1* and *TRX2* is necessary to fully recapitulate the effect of deleting *TRR1* on MCB reporter gene activity in Δ*swi6* cells. Whereas deletion of both *TRX1* and *TRX2* resulted in a 48-fold increase in ß-galactosidase level, deletion of *TRX2* alone had no effect on MCB reporter gene activity and deletion of *TRX1* alone resulted only in a 9-fold increase over the ß-galactosidase level of *TRX1 TRX2* Δ*swi6* cells.

One ancillary observation concerning the phenotype of Δ*trr1* mutations and Δ*trx1* Δ*trx2* double mutations was that the Δ*trr1* mutation had a stronger effect on the cell growth rate. Whereas Δ*trx1* Δ*trx2* double mutants grew 30% slower than wildtype, Δ*trr1* cells grew 70% slower than wildtype cells. Also, whereas the Δ*trx1* Δ*trx2* double mutations protracted only S phase (Muller, 1991), the Δ*trr1* mutation protracted all phases of the cell cycle proportionately. The stronger effect of the Δ*trr1* mutation on growth suggests that other thioredoxin genes exist and can partially fulfill the thioredoxin growth requirement in Δ*trx1* Δ*trx2* cell as long as thioredoxin reductase is present. (A recent search for *TRX1* and *TRX2* homologs in *S. cerevisiae* produced another gene (YCR083), which we tentatively named *TRX3*. *TRX1* and *TRX2* have more homology between

Table 13. Effect of singly deleting thioredoxin genes *TRX1* or *TRX2* on MCB reporter gene expression in Δ*swi6* yeast[a]

Genotype	MCB/*LacZ* Reporter Gene Activity
	(nmol ONP/min-mg protein)
Δswi6:TRP1 TRX1 TRX2	5±2
Δswi6:TRP1 Δtrx1:LEU2 Δtrx2:LYS2	242±53
Δswi6:TRP1 TRX1 Δtrx2:LYS2	3±1
Δswi6:TRP1 Δtrx1:LEU2 TRX2	45±9

[a] Haploid segregants with the indicated genotype were obtained after mating EMY56 (*Δtrx1:LEU2*, *Δtrx2:LYS2*) and MY2276 (*Δswi6:TRP1*). All strains carried the MCB/*LacZ* reporter gene. Lysates from exponentially growing cells were assayed for ß-galactosidase activity. The mean ± standard deviation of the mean for three independent transformant clones is shown in the right columm.

them, than either one does to *TRX3*.) Alternatively, thioredoxin reductase is required for functions in addition to thioredoxin reduction.

Effect of ribonucleotide reductase inhibition on MCB gene activity

In addition to its activity as a protein disulfide reductase, thioredoxin is the proximal donor of electrons during reduction of ribonucleoside diphosphates (rNDPs) to deoxyribonucleoside diphosphates (dNDPs) by ribonucleotide reductase (RNR). This activity suggested a model for thioredoxin involvement in G1/S transcriptional regulation (see Chapter 5, and Figure 20 for further elaboration). According to the model, at the start of S phase RNR1 would quickly deplete reduced Trx levels. Proteins with Trx-dependent thiols would become oxidized and activate transcription of MCB dependent genes. Such a model would explain why *trr1* mutation advanced the onset of MCB gene activity (Figure 5C and 6B). Trx levels would be more quickly exhausted in the absence of Trr1 enzyme.

A second prediction of the model is that inhibition of RNR1 should inhibit MCB gene induction at G1/S. To test this prediction *cdc15* cells (strain MY2226) blocked at M/G1 were shifted to the permissive temperature into medium with or without 200 mM hydroxyurea (HU), an inhibitor of RNR. Synchrony was monitored by determining the budding index. As shown in Figure 19A, bud emergence in both populations began about 60 minutes after release. RNA from the synchronized cells was analyzed for *RNR1*, *SWI4*, *TRR1* and *H2A* mRNA (Figure 19B). As an internal control, the blot was also hybridized with the nonperiodically

Figure 19. Northern blot analysis of hydroxyurea effect on endogenous MCB gene induction in synchronized cells. A *cdc15* strain (MY2226) was synchronized by release from the nonpermissive temperature into medium with or without the ribonucleotide reductase inhibitor hydroxyurea (HU). At indicated times, budding index was determined by scoring 200 cells (A) or RNA was prepared for northern blot analysis. B) The northern blots (5 μg RNA/lane) were hybridized with radiolabeled *RNR1, SWI4, TRR1,* or *H2A/P1* DNA probes. Lane labeled A show asynchronous cell levels of the respective mRNAs. C) *RNR1, SWI4* and *TRR1* mRNA levels, normalized to *P1* mRNA levels, were quantitated and plotted as a function of time after release from the nonpermissive temperature.

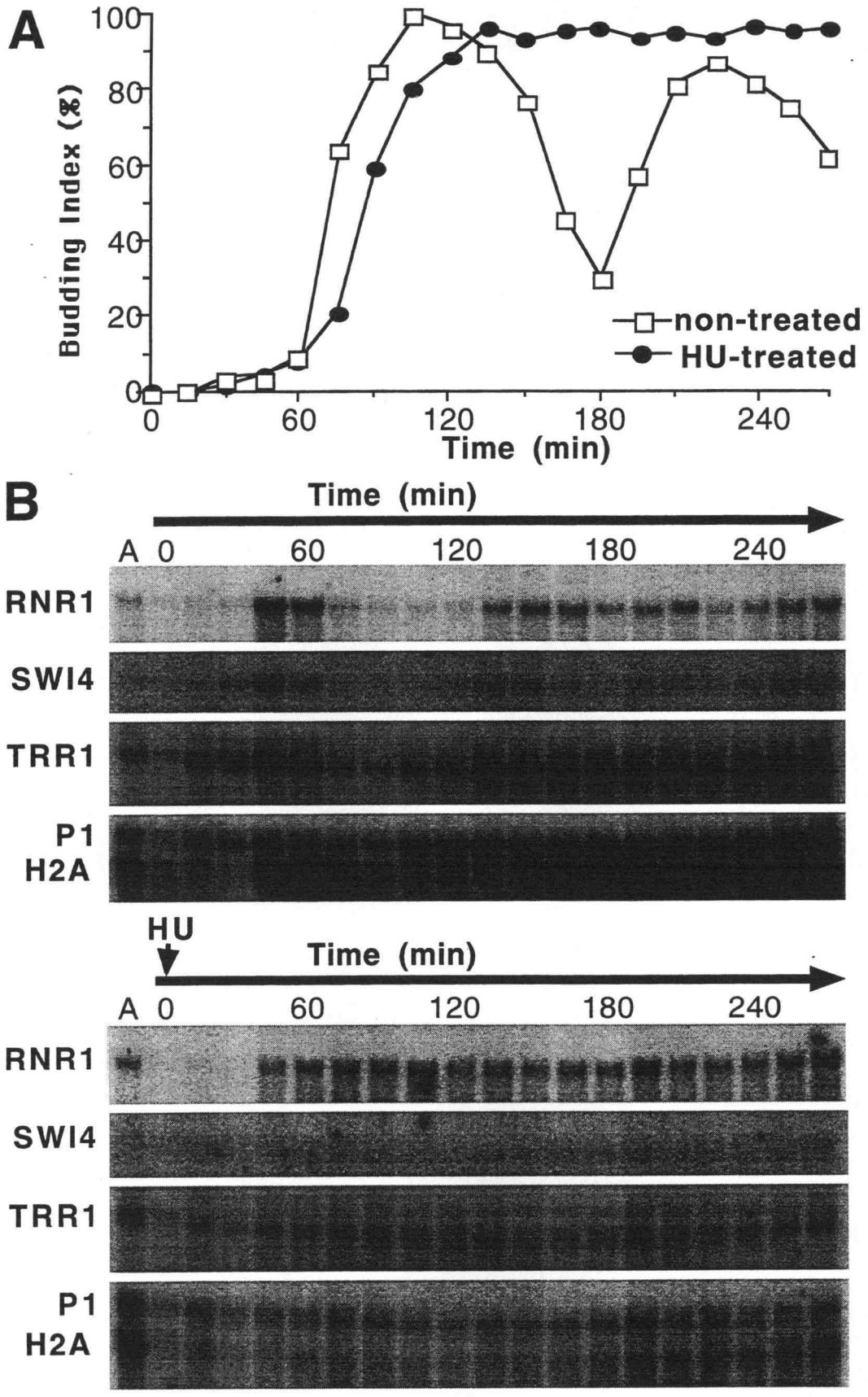

Figure 19A, 19B.

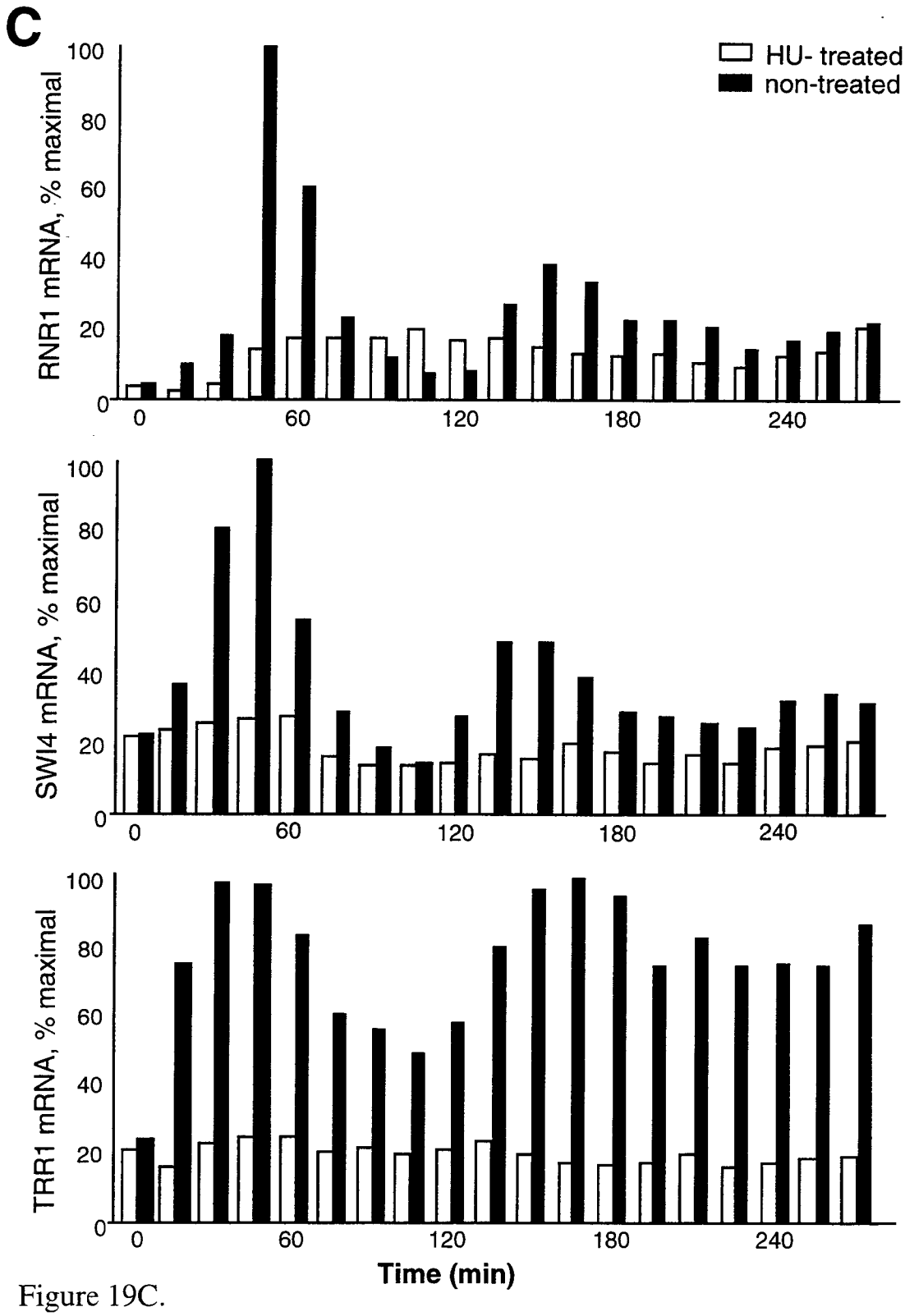
C
HU- treated
non-treated
RNR1 mRNA, % maximal
SWI4 mRNA, % maximal
TRR1 mRNA, % maximal
0
20
40
60
80
100
0
60
120
180
240
Time (min)

Figure 19C.

expressed *P1* gene. *RNR1*, *SWI4* and *TRR1* mRNA levels, normalized to *P1* mRNA levels, is shown in Figure 19C. Consistent with the prediction, the increase in MCB gene mRNA that usually occurs at G1/S was greatly suppressed in cells released into hydroxyurea. In considering how accumulation of DNA precursor-synthesizing enzymes could satisfy the dNTP demand, it is interesting to note that the *TRR1* gene itself contains an upstream MCB element and is maximally expressed at G1/S (Figure 19B).

Swi4 is not required for Trr1 repression of MCB activity

In order to establish if Trr1 repression of MCB reporter gene activity was mediated directly or indirectly by Swi4, strain BY604 (Δ*swi4*) was mated to MY2202Z (Δ*trr1*) and segregants with specific genotypes were assayed for ß-galactosidase activity. If Trr1 repression was mediated through Swi4, deleting *TRR1* in a Δ*swi4* cell should not affect MCB reporter gene activity. The results, shown in Table 14, indicated that deletion of *TRR1* in both *SWI4* and Δ*swi4* cells led to a 3- to 5-fold increase in ß-galactosidase activity. Thus, Swi4 was not necessary for Trr1 repression of MCB reporter gene activity.

Table 14. Effect of deleting *TRR1* on MCB reporter gene expression in *SWI4* and Δ*swi4* yeast[a]

Genotype	MCB/*LacZ* Reporter Gene Activity
	(nmol ONP/min-mg protein)
TRR1 SWI4	281±58
Δ*trr1:HIS3 SWI4*	973±104
TRR1 Δ*swi4:LEU2*	154±31
Δ*trr1:HIS3* Δ*swi4:LEU2*	786±84

[a] Haploid segregants with the indicated genotypes were isolated after mating MY2202Z (Δ*trr1:HIS3*) and BY604 (Δ*swi4:LEU2*). All strains carried the MCB/*LacZ* reporter gene. Lysates from exponentially growing cells were assayed for ß-galactosidase activity. The mean ± standard deviation of the mean for three independent segregants is shown in the right column.

CHAPTER 5

Discussion

Using an approach similar to ours, Lycan and colleagues (1994) isolated several suppressors of *swi4* (*ssf*) mutants by screening for efficient *ho-LacZ* expression in *swi4* yeast. The *ssf* mutations defined three complementation groups and restored efficient *HO* expression in yeast lacking either Swi4 or Swi6 protein. Cloning showed that two of the *ssf* genes were alleles of *SIN4* and *CDC68*, and therefore nonidentical to *TRR1*. The third *ssf* gene (*ssf9*) was not cloned, but the phenotype of *ssf9* mutants suggests it also is nonidentical to *TRR1*. Whereas *rom1* mutations activated only MCB- or SCB-containing promoters, *ssf9* mutations also activated UAS-less basal promoters, suggesting *SSF9* encodes a global repressor. It is surprising that Lycan *et al.* (1994) did not isolate *trr1* mutations. The report mentions, but does not elaborate on, a second class of mutants, termed *ssx* mutants, which were isolated using a similar approach, but in a *swi6* background. Perhaps, *trr1* mutations are only easily identified in a *swi6* background, and the *ssx* mutations mentioned by Lycan *et al.* (1994) will turn out to be allelic to *TRR1*.

Although *trr1* mutations activated the MCB/*HIS3* reporter and MCB/*LacZ* reporter genes, they did not noticeably affect endogenous MCB gene mRNA levels, at least as measured in asynchronous cells. The lack of a *trr1* effect on asynchronous cell levels of *CDC9*, *RNR1* and *SWI4* mRNA is reminiscent of the disparate effect of deleting *SWI6* on reporter gene and endogenous gene expression. Deletion of *SWI6* strongly represses Δ178*CYC1*/*LacZ* reporter genes that are dependent on either synthetic

MCB element clusters (Lowndes *et al.*, 1992) or on natural MCB elements as they are found in the context of a 55-bp fragment of the *TMP1* upstream region (Dirick *et al*, 1992). In contrast, deletion of *SWI6* has little effect on asynchronous cell levels of several mRNAs encoded by endogenous MCB-containing genes such as *TMP1*, *CDC9*, *POL1*, *RNR1*, *SWI4*, *CLN1* and *CLN2* (Lowndes *et al.*, 1992; Dirick *et al.*, 1992). Foster *et al.* (1993) showed a small but significant effect of deleting *SWI6* on *SWI4* mRNA levels. Despite the lack of a strong effect of deleting the *trans*-acting factor Swi6, in cases where it has been examined, mutation of the *cis*-acting MCB consensus has a strong negative effect on endogenous gene mRNA levels (McIntosh *et al.*, 1991; Foster *et al.*, 1993). To summarize, *cis*-acting mutations that destroy MCB elements have strong effects on both endogenous and reporter gene expression, whereas *trans*-acting mutations, such as deletion of *SWI6* or mutation of *TRR1*, have little effect on endogenous gene expression, but have strong effects on reporter gene expression. Although other models are envisionable to explain the disparity, one plausible explanation is that the endogenous genes are subject to feedback inhibition. In other words, in wildtype cells, when transcription of the G1/S genes is triggered by a START-dependent process, the subsequent accumulation of G1/S proteins triggers a process that turns the endogenous genes back off. In *trr1* cells, where G1/S genes are activated more quickly following START, G1/S proteins accumulate more quickly, and the genes are turned off more quickly. In *swi6* cells, where the G1/S genes are moderately active in early G1, a feedback mechanism prevents them from being further activated following START. In contrast to the endogenous genes, the MCB reporter genes probably lack the *cis*-acting information for feedback inhibition, and therefore *trans*-

acting mutations affecting the rapidity or efficiency of gene induction following START have much stronger effects on reporter gene activity.

How might *rom1* mutations activate MCB elements? Thioredoxin reductase regenerates reduced thioredoxin (Trx) from oxidized thioredoxin using NADPH as electron donor. Diminished levels of reduced Trx in *rom1* mutants could lead to oxidation of regulatory thiols in proteins that either directly or indirectly control G1/S gene transcription. 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 NFkB (Matthews *et al.*, 1992), Fos/Jun (Xanthoudakis and Curran, 1992), glucocorticoid receptor (Silva and Cidlowski, 1989), and the MyoD-interacting protein E2A (Benezra, 1994). In these vertebrate examples, protein oxidation is correlated with loss of either DNA binding or transcriptional activity. However, in bacteria, oxidation of the OxyR regulatory protein is associated with enhanced transcriptional activity (Storz *et al.*, 1990). A direct role for thioredoxin in redox control of transcription has been suggested for NFkB (Matthews *et al.*, 1992). An indirect role for thioredoxin has been suggested for Fos/Jun regulation, where the proximal redox effector protein is thought to be Ref1 (Xanthoudakis and Curran, 1992). Outside the realm of transcription factors *per se*, thioredoxin has been implicated in the folding or conformational regulation of several eukaryotic and prokaryotic proteins (reviewed by Buchanan *et al.*, 1994).

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. It also functions as the electron donor during reduction of

sulphur from the level of sulfate to sulfite by adenosine 3'-phosphate 5'-phosphosulfate (PAPS) reductase.

In light of the activities of thioredoxin, two models suggest themselves in explaining how low thioredoxin reductase levels in *rom1* mutants activate MCB elements. The first is that inadequate levels of reduced thioredoxin protract S phase and that S phase protraction may disproportionately expand the cell cycle compartment compatible with MCB element activation. Such a model was proposed by Muller (1994), to explain the elevation of *RNR1* and *RNR2* mRNA in cells in which thioredoxin genes *TRX1* and *TRX2* were deleted. One problem with the model is that we saw strong MCB reporter gene activation by *rom1* mutations without observing any disproportionate increase in the duration of any cell cycle compartment.

A second model for thioredoxin involvement in G1/S transcriptional regulation (tentatively named the G1/S REDOX switch model) is outlined in Figure 20. According to the model, MCB genes are inactive in non-G1/S cells because regulatory thiols on a cell cycle control protein are maintained in a reduced state by an adequate supply of reduced Trx. Reduced Trx levels are adequate because, except for the cysteine biosynthesis step catalyzed by PAPS reductase, there is little high turnover oxidation of thioredoxin. However, after replication origins are triggered at G1/S, the cell deoxynucleoside triphosphate (dNTP) pools are quickly consumed through incorporation into DNA. Muller (1994) estimated the dNTP pools in budding yeast to be only 1% 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.

Figure 20. Model for thioredoxin serving as a link between replication initiation and gene induction at G1/S. In non-G1/S cells, reduced thioredoxin is plentiful and thioredoxin-dependent protein thiols are reduced. At G1/S, the increased flux of substrates through the ribonucleotide reductase enzyme depletes the supply of reduced thioredoxin, and thioredoxin-dependent thiols on cell cycle control proteins become oxidized, triggering MCB element activation.

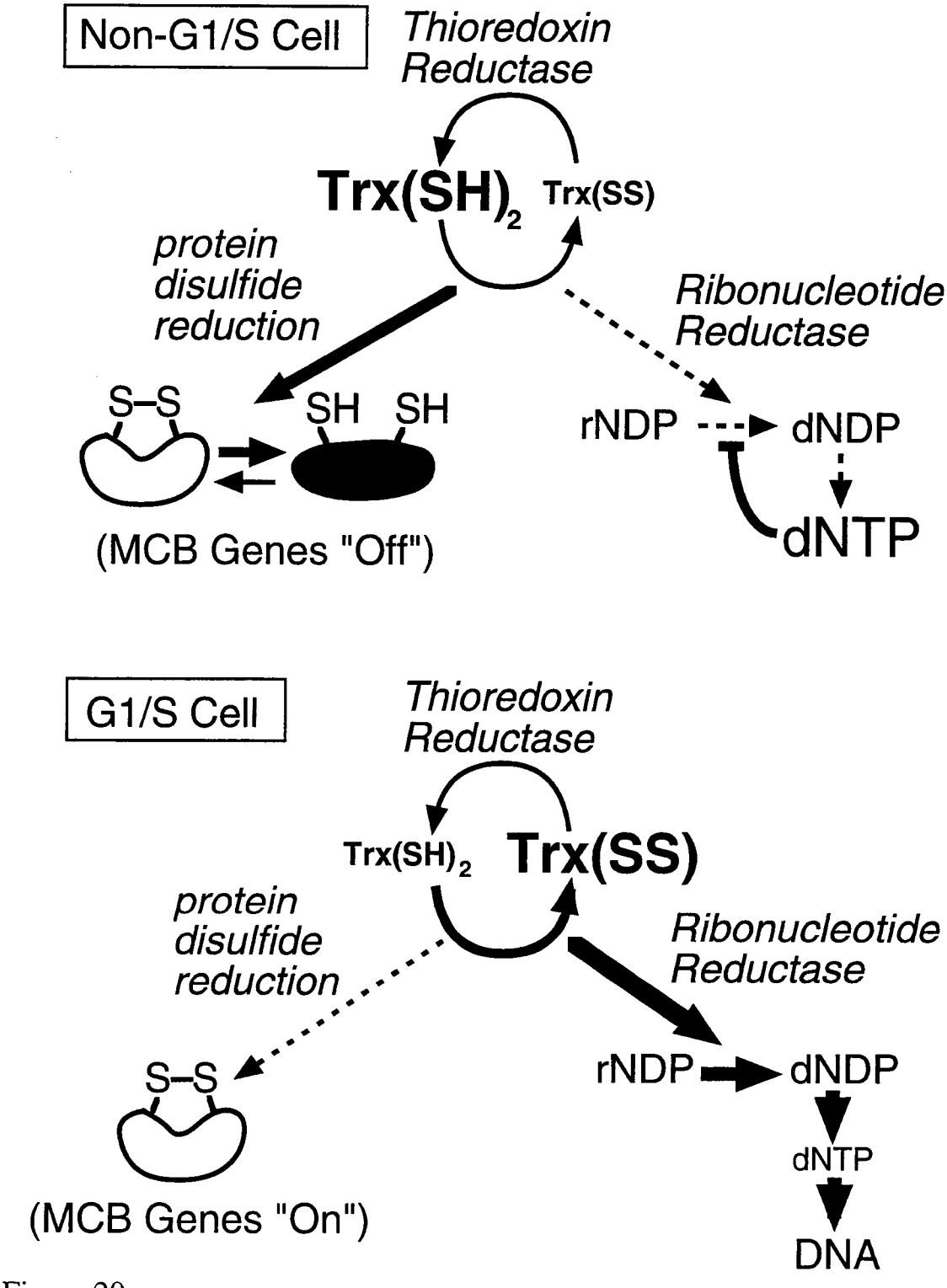
Non-G1/S Cell
Thioredoxin Reductase
Trx(SH)2
Trx(SS)
protein disulfide reduction
Ribonucleotide Reductase
S–S
SH SH
rNDP
dNDP
dNTP
(MCB Genes "Off")
G1/S Cell
Thioredoxin Reductase
Trx(SH)2
Trx(SS)
protein disulfide reduction
Ribonucleotide Reductase
S–S
rNDP
dNDP
dNTP
DNA
(MCB Genes "On")

Figure 20.

Proteins with thioredoxin-dependent thiols would become oxidized, resulting in 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 re-accumulate, 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 interesting to note that the *TRR1* gene itself contains an upstream MCB element and is maximally expressed at G1/S (Figure 19B).

One prediction of the second model is that *trr1* mutations would activate MCB reporter genes more rapidly because reduced thioredoxin levels would be more quickly exhausted in the absence of thioredoxin reductase. Consistent with this prediction *trr1* mutation advanced the onset of MCB gene activity (Figure 5C and 6B).

A second prediction of the model is that MCB/SCB gene transcription should be triggered after, and not before, replication origins fire. Following START, the exact timing of events such as initiation of budding, DNA replication, spindle pole body duplication, and induction of G1/S gene transcription are difficult to resolve. Most analyses suggest DNA synthesis begins at the time of bud emergence and that MCB gene mRNA levels begin to rise before bud emergence. Thus, for the second model to be tenable, either some cryptic DNA replication must be occurring prior to bud emergence, or G1/S gene transcription may initially be triggered by a replication-independent process and then reinforced by the replication-dependent depletion of reduced thioredoxin. Consistent with the later part of this prediction, when *cdc15*-blocked cells were

released into medium containing the RNR inhibitor HU, the increase in MCB gene mRNA that usually occurs at G1/S was greatly suppressed (Figure 19C). Inhibition of a thioredoxin-oxidizing enzyme prevented the reinforcement of G1/S gene transcription.

One attractive feature of the G1/S REDOX switch model is that it suggests a testable biochemical mechanism for linking the onset of DNA replication to induction of specific gene transcription at G1/S. Many testable predictions of the model can be envisioned. First, the model predicts that thioredoxin should become more highly oxidized at G1/S. This prediction can be tested by assaying the thioredoxin REDOX state during the cell cycle. Second, if RNR mediates the expected burst of thioredoxin oxidation, inhibition of RNR should block thioredoxin oxidation. This prediction can be tested by assaying the REDOX state as above. Third, if activation of RNR at G1/S requires DNA synthesis, inhibition of DNA replication should block thioredoxin oxidation and block MCB gene induction. This prediction can be tested by releasing synchronized yeast into medium with the DNA polymerase inhibitor aphidicolin and assaying the REDOX state as above. Fourth, if RNR could somehow be activated in the absence of replication, it should oxidize thioredoxin and activate MCB genes. This prediction might be tested by over-expressing RNR1 so that it could escape allosteric inhibition. Fifth, if RNR is activated at G1/S because replication consumes inhibitory dATP, RNR should be present but inactive prior to G1/S, the dNTP pools should transiently decrease at G1/S, and the rate of dNTP pool labeling by exogenous substrate should increase at G1/S. This prediction can be tested by dNTP pool level measurements.

Other predictions could be tested through genetic characterization of the thioredoxin/MCB repression pathway. The model predicts that thioredoxin oxidation during G1/S either directly or indirectly activates a MCB gene activator or inhibits a MCB gene inhibitor. Other members of the thioredoxin pathway could be genetically identified. Yet, when we initially screened for recessive MCB-activating mutations they all mapped to a single locus -*TRR1*. Either all the genes downstream from *TRR1* are redundant, like *TRX1* and *TRX2*, and therefore do not give a phenotype when singly mutated, or the genes are essential for viability and therefore non-lethal alleles are extremely rare. To search for possible redundant genes isolation and characterization of high copy suppressors of the *trr1* phenotype could be done (in Table 7, nine partial high copy suppressors of *trr1* were listed). To search for essential genes that act downstream in the thioredoxin pathway a screen for mutations that result in temperature-sensitive repression of MCB reporter genes could be done.

Finally, to further confirm that G1/S gene transcription may initially be triggered by a replication-independent process and then reinforced by the replication-dependent depletion of reduced thioredoxin, DNA synthesis could be de-coupled from START. Schowb and Nasmyth (1993) demonstrated that Δ*clb5* Δ*clb6* double deletion de-coupled DNA synthesis from START by 30 minutes. The prediction would be that in Δ*clb5*Δ*clb6* yeast G1/S gene transcription would initiate at moderate levels at START but only be reinforced 30 minutes later at the onset of DNA synthesis. Northern blot analysis of synchronized Δ*clb5*Δ*clb6* yeast MCB gene expression should show moderate MCB gene expression induction at START (budding index would verify START onset), and reinforcement of expression 30 minutes later at G1/S (FACS analysis would verify S onset).

Summarizing, in our search for additional gene products that participate in activating MCB genes following START, we discovered that thioredoxin reductase gene mutations derepress MCB reporter genes and advance the onset of endogenous MCB gene activation. This finding suggested for the first time a biochemical mechanism for linking the onset of DNA replication to induction of specific gene transcription at G1/S.

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