Synergy among Differentially Regulated Repressors of the Ribonucleotide Diphosphate Reductase Genes of *Saccharomyces cerevisiae*

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The Ssn6/Tup1 general repression complex represses transcription of a number of regulons through recruitment by regulon-specific DNA-binding repressors. Rox1 and Mot3 are Ssn6/Tup1-recruiting, DNA-binding proteins that repress the hypoxic genes, and Rfx1 is a Ssn6/Tup1-recruiting, a DNA-binding protein that represses the DNA damage-inducible genes. We previously reported that Rox1 and Mot3 functioned synergistically to repress a subset of the hypoxic genes and that this synergy resulted from an indirect interaction through Ssn6. We report here cross-regulation between Rox1 and Mot3 and Rfx1 in the regulation of the RNR genes encoding ribonucleotide diphosphate reductase. Using a set of strains containing single and multiple mutations in the repressor encoding genes and lacZ fusions to the RNR2 to –4 genes, we demonstrated that Rox1 repressed all three genes and that Mot3 repressed RNR3 and RNR4. Each repressor could act synergistically with the others, and synergy required closely spaced sites. Using artificial constructs containing two repressor sites, we confirmed that all three proteins could function synergistically but that two Rox1 sites or two Rfx1 sites could not. The significance of this synergy lies in the ability to repress gene transcription strongly under normal growth conditions, and yet allow robust induction under conditions that inactivate only one of the repressors. Since the interaction between the proteins is indirect, the evolution of dually regulated genes requires only the acquisition of closely spaced repressor sites.

Transcriptional repression in eukaryotes is an active process mediated through complexes that organize chromatin and/or interact with the basal transcriptional machinery (3, 24, 41, 45, 47). The evolutionarily conserved Ssn6/Tup1 general repression complex of *Saccharomyces cerevisiae* is one such example. Tup1 interacts with the N-terminal domain of histones H3 and H4 and, for at least some Ssn6/Tup1 repressed genes, positions a nucleosome over the TATA box to block access by the general transcriptional machinery (7, 12, 55). In addition, Tup1 recruits a histone deacetylase, presumably to lock this nucleosome in place (13, 50, 52). In the absence of a positioned nucleosome, the general repression complex still can repress transcription through a chromatin-independent mechanism (20, 32, 33, 38, 40). Tup1 has been demonstrated to interact with a number of subunits of the mediator complex which may play a role in this latter repression mechanism. For some genes, both mechanisms function, and repression can only be genetically disabled through mutations in both the histones and the mediator components (27, 45, 54).

Ssn6 and Tup1 are expressed constitutively and have no intrinsic DNA-binding activity (29, 45). The gene or regulon specificity of repression is controlled through specific DNA-binding proteins that recruit the general repression complex. Thus, for example, Rox1 represses the hypoxic genes (2, 35); Mig1 and -2, the glucose-repressed genes (48, 51); Rfx1 (Clr1), the DNA damage-repair genes (22); and α2-Mcm1, the a mating type genes (29, 31, 37, 46). In each case the transcription, activity, or cellular compartmentalization of these DNA-binding repressors is regulated to control when repression is effected (10, 22, 31, 34, 35, 36, 46).

Our studies have focused on the regulation of the hypoxic genes (reviewed in references 28, 56, and 57). These genes are repressed aerobically by Rox1, which binds to Ssn6 to recruit the general repression complex. *ROX1* expression is dependent upon heme, the synthesis of which requires molecular oxygen. Hence under hypoxic conditions, heme levels are low, Rox1 is not made, and the Ssn6/Tup1 complex is not recruited to the hypoxic genes. Consequently, these genes are derepressed. Despite the large protein complex that localizes to the regulatory region of Rox1-repressed genes, including Ssn6/Tup1, positioned nucleosomes, deacetylases, and perhaps the general transcription machinery, repression is dependent on the number and quality of the Rox1-binding sites; a base pair change in a Rox1 site that caused a fewfold decrease in Rox1 binding in vitro resulted in a proportional decrease in repression in vivo (9). Recently, an additional DNA-binding protein, Mot3, was found to augment repression by Rox1. Strong hypoxic repression sites consist of a combination of Rox1 and Mot3 sites (27, 30, 35, 44). For example, the *ANB1* gene is repressed 250-fold, 8-fold by operator B which consists of two Rox1 sites, and 76-fold by operator A, which contains two Rox1 sites flanking a Mot3 site (27). The *HEM13* gene is repressed 50-fold by three Mot3 sites plus one Rox1 site (30). We have termed this repression combinatorial repression. Combinatorial repression can be additive or synergistic. Additive repression occurs when the two repressors act independently. While Rox1 can repress independent of Mot3, Mot3 can only repress independently through multiple sites, with each bound Mot3 molecule acting independently of the others. Synergy is defined as the two repressors combining to repress gene expression to a greater extent than the additive effects of the single proteins acting...
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tations must increase to compensate for limiting oxygen concen-
fore, this enzyme falls in the class of those whose concentra-
RNR enzyme requires molecular oxygen (4, 17), and, there-
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er conditions that relieve only one repressor’s activity, thus

MATERIALS AND METHODS

Strains and cell growth. The yeast strains used in the present study are

TABLE 1. Yeast strains

| Strain     | Genotype                  | Source or reference |
|------------|---------------------------|--------------------|
| RZ53-6 derivatives | MATa trp1 ura3 ade1       | 2                  |
| RZ53-6     | MATa trp1 ura3 ade1 rox1::LEU2 | 2                  |
| RZ53-6Δrox1| MATa trp1 ura3 ade1 rox1::LEU2 | 8                  |
| RZ53-6Δmot3| MATa trp1 ura3 ade1 mot3::KanMX | 27                 |
| RZ53-6Δmot3Δrox1| MATa trp1 ura3 ade1 rox1::LEU2 mot3::KanMX | 27                 |

RZ53-6-related strains

| MZZ233-20 | MATa trp1 ura3 ade1       | This study          |
| MZZ233-2  | MATa trp1 ura3 ade1 mot3::KanMX | This study          |
| MZZ233-13 | MATa trp1 ura3 ade1 rfx1::kanMX | This study          |
| MZZ233-6  | MATa trp1 ura3 ade1 mot3::KanMX | This study          |
| MZZ255-6  | MATa trp1 ura3 ade1 rox1::LEU2 rfx1::kanMX | This study          |
| MZZ269-8  | MATa trp1 ura3 ade1 rox1::LEU2 mot3::KanMX | Yeast Deletion     |
| BY4741Δrfx1| MATa leu2 ura3 met15 rfx1::kanMX | Consortium         |

independently. Synergy results when Rox1 and Mot3 bind to
closely spaced sites. This synergy does not result from an inter-
between the two proteins but rather through each repressor’s interaction with the Snf6/Tup1 complex (30). Chro-
matin immunoprecipitation experiments demonstrated that
Rox1 bound to DNA independently of Mot3 and enhanced
Mot3 binding to DNA strengthen its interaction through Snf6/Tup1 (30).

This model requires no hypoxic specificity on the part of
Mot3 when it functions through synergistic interactions with
Rox1. Indeed, although Mot3 is repressed under hypoxia (44),
constitutive expression of Mot3 did not result in any repression of
\( \text{ANBI} \) during hypoxia (35). Since synergy requires no direct
interaction between Rox1 and Mot3, and if Mot3 cannot re-
press alone through a single site, we posited that Mot3 could func-
tion in the same capacity at other Snf6/Tup1 regulons. The
Mot3 binding site, \( (A/C/T)\text{AGG}(C/T)\text{A} \), is only 6 bp and de-
generate (19), so that it has a 50% chance of appearing once
every 340 bp in a random sequence. Consequently, we only
scanned the regulatory regions of Snf6/Tup1 repressed genes
with well-characterized regulon-specific DNA binding sites,
searching for Mot3 sites close to those sites. We found prom-
ising hits in the regulatory region of DNA damage-inducible
\( \text{RNR} \) and 4 genes encoding two of the proteins of ribonucle-
otide diphosphate reductase (\( \text{RNR} \)) (15, 21, 49). To our sur-
prise, we also found Rox1 binding sites in these two genes as
well as in \( \text{RNR2} \), encoding another \( \text{RNR} \) subunit (14, 23). In
the present study, we demonstrate that these genes are regu-
lated by oxygen through Rox1 and Mot3 and that both Mot3
and Rox1 can function synergistically with Rfx1. In hindsight,
these results are not surprising; the formation of the active
\( \text{RNR} \) enzyme requires molecular oxygen (4, 17), and, there-
fore, this enzyme falls in the class of those whose concentra-
tions must increase to compensate for limiting oxygen con-
centrations under hypoxia. The results reported here indicate that
synergy between repressors allows substantial derepression un-
YCp(33)AZ-M3Rfw was constructed by subcloning the ANB1-lacZ HindIII-KpnI fragment from Ycp(33)AZ-M3Rfs into the corresponding site of YIpplac211. This plasmid contained a unique BglII site within the ANB1 sequences 3’ to the lacZ coding sequence. The plasmid was digested with this enzyme and transformed into yeast to integrate the entire plasmid into the ANB1 locus.

β-Galactosidase and immunoblot assays. β-Galactosidase assays were performed on pooled transformants (30). Transformants were selected on SC-uracil plates and, after several days of growth, the colonies on each plate were pooled and used to start overnight cultures. Cells were grown in SC-uracil medium to mid-exponential phase, and the enzyme assays were carried out as described previously (26). The data presented represent at least three independent assays with the standard deviations shown.

For immunoblots, cells were grown on selective media to mid-exponential phase, 1.5-ml samples were chilled, and the cells were harvested by centrifugation, resuspended in 40 μl of sodium dodecyl sulfate gel sample buffer, and boiled for 5 min. The samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblots were carried out as described previously (1). The blots were probed with monoclonal antibody against the HA epitope (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After the bands were visualized, the blot was stripped and reprobed with antisera against eIF5A (prepared by Alexander Kastaniotis). The ratio of HA to eIF5A was determined by using ImageQuant software (Amersham Biosciences, Piscataway, NJ).

RESULTS

Mot3 and Rox1 repress the RNR genes. RNR2, -3, and -4 encode three of the four proteins of RNR (14, 15, 21, 23, 49).

![FIG. 1. Arrangements of repressor sites in the RNR genes. The upstream regions of the RNR2, RNR3, and RNR4 genes are presented. The sequences are numbered in negative integers from the first base preceding the ATG translational initiation codon. 1, 5'-wards to −800. The Rox1 sites are indicated as open boxes with a diagonal stripe, Mot3 sites are indicated as gray boxes, the strongly binding Rfx1 sites are designated as black boxes and the weakly binding Rfx1 sites are indicated as open boxes.](Image)
It has been reported that the expression of these genes is repressed by Rfx1 (Crt1) and Sn6/Tup1 under normal growth conditions (16, 22). DNA damage induces the phosphorylation of Rfx1, which results in the loss of DNA-binding activity and the derepression of these genes (22). Each Rfx1-repressed gene contains at least two widely spaced binding sites for Rfx1, which results in the loss of DNA-binding activity and derepression of these genes (22). The synergy factor was calculated by dividing the fold repression of wild-type cells by the product of the fold repression of the rfx1Δ and rox1Δ mutants.

To test the effects of Mot3 and Rox1 on the repression of these RNR genes, lacZ fusions were constructed to the regulatory region of each gene, and plasmids carrying these fusions were transformed into a set of congenic strains with single deletions in each of the repressor genes, the three combinations of double deletions, and the triple deletion. The levels of repression were determined under repressing conditions (aerobic and non-DNA damage) by β-galactosidase activity assays, and the results are presented in Tables 3 to 5. The data clearly confirmed the predictions above; all three gene fusions were repressed by Rox1 as well as Rfx1, and that the repressors functioned additively or synergistically, we analyzed the data in the three possible pairwise combinations. For example, for the RNR3-lacZ data presented in Table 4, the possible interactions between Mot3 and Rfx1 were determined in the absence of Rox1 by comparing the data in the rox1Δ deletant (the combined Mot3-Rfx1 effect) to that in the rox1Δ rfx1Δ deletant (the Mot3 effect alone) and in the rox1Δ mot3Δ deletant (the Rfx1 effect alone) and in the triple deletant (complete derepression). The fold repression for this set of comparisons was calculated by dividing the β-galactosidase activity in the fully derepressed triple deletant by that in the rox1Δ cells, the rox1Δ mot3Δ deletant, or the rox1Δ rfx1Δ deletant. We then

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**TABLE 3. Repression of the RNR2 gene**

| Genotypea | Mean β-galactosidase activity ± SD | Fold Rfx1-Rox1 repression (synergy factor)b |
|-----------|----------------------------------|------------------------------------------|
| Wild type | 33 ± 5.1                          | 45 (1.8)                                 |
| mot3Δ     | 35 ± 5.1                          | 2.3                                      |
| rfx1Δ     | 638 ± 73                          |                                          |
| rox1Δ     | 139 ± 22                          | 11                                       |
| rox1Δ rfx1Δ | 1,490 ± 117                      |                                          |
| rox1Δ mot3Δ rfx1Δ | 1,520 ± 44             |                                          |

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**TABLE 4. Repression of the RNR3 gene**

| Genotypea | Mean β-galactosidase activity ± SD | Fold repression (synergy factor)b |
|-----------|----------------------------------|---------------------------------|
| Wild type | 0.66 ± 0.2                       | 436 (44)                       |
| mot3Δ     | 2.7 ± 0.7                        | 54 (1.2)                       |
| rfx1Δ     | 22 ± 5.7                         | 9.3                            |
| rox1Δ     | 13 ± 0.7                         | 4.7                            |
| rox1Δ mot3Δ | 253 ± 20                       | 9.3                            |
| rox1Δ rfx1Δ | 127 ± 9.0                       | 2.1                            |
| rox1Δ mot3Δ rfx1Δ | 568 ± 27                   | 2.1                            |
| rox1Δ rfx1Δ mot3Δ | 1,80 ± 71                      | 2.1                            |

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**TABLE 5. Repression of the RNR4 gene**

| Genotypea | Mean β-galactosidase activity ± SD | Fold repression (synergy factor)b |
|-----------|----------------------------------|---------------------------------|
| Wild type | 27 ± 6.6                          | 20 (5.7)                       |
| mot3Δ     | 50 ± 3.8                          | 1.7 (1.3)                      |
| rfx1Δ     | 591 ± 120                         | 7.5 (2.8)                      |
| mot3Δ rfx1Δ | 135 ± 8.9                       | 1.3                            |
| rox1Δ     | 794 ± 43                          | 0.7                            |
| rox1Δ mot3Δ | 1,350 ± 43                       | 0.7                            |
| rox1Δ mot3Δ rfx1Δ | 377 ± 16                     | 2.7                            |
| rox1Δ rfx1Δ mot3Δ | 1,010 ± 49                     | 2.7                            |

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*a The strains used were MZ233-20 (wild type), MZ233-2 (mot3Δ), MZ233-13 (rfx1Δ), RZ53-6 (rox1Δ), MZ233-6 (mot3Δ rfx1Δ), MZ255-6 (rox1Δ rfx1Δ), and MZ269-8 (rox1Δ mot3Δ rfx1Δ). The cells were transformed with YCp33/RNR2Z and grown aerobically in SC-uracil.

*b The fold repression was calculated as described in Table 3, except that the values for each of the single and double mutants indicated were divided into that for the rox1Δ mot3Δ rfx1Δ strain. The synergy factor was calculated as described in the text.
calculated the synergy factor, defined as the fold repression by Mot3 alone (enzyme activity in \( \text{rox1}^\Delta \text{mot3}^\Delta \text{rfx1}^\Delta \text{rox1}^\Delta \text{rfx1}^\Delta \) cells) times the fold repression by Rfx1 alone (enzyme activity in \( \text{rox1}^\Delta \text{mot3}^\Delta \text{rfx1}^\Delta \text{rox1}^\Delta \text{mot3}^\Delta \) cells) divided into the fold repression by both repressors (enzyme activity in \( \text{rox1}^\Delta \text{mot3}^\Delta \text{rfx1}^\Delta \text{rox1}^\Delta \text{mot3}^\Delta \) cells). This comparison assumes that independent, or additive, repression should be measured using the product of the effects of Rfx1 and Mot3 alone rather than the sum, because we envision repression through a single repressor occurring whenever the repressor is bound. Therefore, the fraction of time a gene can be expressed is the fraction of time when neither repressor is bound, or the product of the fold repression by the two repressors alone. This is a conservative estimate of additive repression, and we feel more confident assigning synergy using this assumption. A synergy factor of 1.0 represents additive repression, whereas a value significantly higher than 1 indicates synergy. For the example above, the synergy factor was 4.7, indicating a synergistic interaction between Mot3 and Rfx1 at \( \text{RNR3} \).

Expression of the \( \text{RNR2-lacZ} \) (Table 2) was repressed by Rfx1 and Rox1, but not by Mot3, as predicted above from the location of binding sites. The lack of a Mot3 effect was clearly evident in that the \( \beta \)-galactosidase activity was the same in the wild-type and \( \text{mot3}^\Delta \) cells and in the \( \text{rfx1}^\Delta \) and \( \text{mot3}^\Delta \text{rfx1}^\Delta \) mutants. Rox1 alone repressed expression of \( \text{RNR2} \) 2.3-fold, the ratio of enzyme activity in \( \text{rox1}^\Delta \text{rfx1}^\Delta \) to that in the \( \text{rfx1}^\Delta \) cells, and Rfx1 alone repressed expression 11-fold, the ratio of enzyme activity in \( \text{rox1}^\Delta \text{rfx1}^\Delta \) to that in the \( \text{rox1}^\Delta \) cells. The synergy factor was calculated as 1.8, a value that is suggestive, but not conclusive, that these repressors act synergistically at this gene.

As expected from previously reported results (15, 22), the expression of the \( \text{RNR3-lacZ} \) was most strongly repressed, showing a nearly 1,800-fold difference between the enzyme activity in wild-type cells and cells carrying the triple deletion (Table 4). This repression was achieved by synergy between Mot3 and Rfx1 (a synergy factor of 4.7) and Rox1 and Rfx1 (a synergy factor of 44) but not between Rox1 and Mot3 (a synergy factor of 1.2). Any one of the repressors alone effected weak repression as seen in the double mutants; for example, Rfx1 alone repressed \( \text{RNR3} \) expression only about twofold (the ratio of enzyme activity in the triple deletant to that in the \( \text{rox1}^\Delta \text{mot3}^\Delta \) strain). Interestingly, the strongest repression by a single repressor is the effect by Mot3, 9.3-fold in the \( \text{rox1}^\Delta \text{rfx1}^\Delta \) mutant, and this probably resulted from the three adjacent sites close within 100 bp of the start of translation.

\( \text{RNR4-lacZ} \) expression was repressed by all three repressors. Synergy was clearly indicated between Rox1 and Rfx1, but we have some reservations about the calculation of synergy involving Mot3. The enzyme activity measured in the triple deletant was somewhat lower than that determined for the \( \text{rox1}^\Delta \text{rfx1}^\Delta \) strain, which resulted in a fold repression of less than 1, making the synergy factor calculation suspect. Although the reason for this discrepancy is not known, the fact that there is only one Mot3 site adjacent to an Rfx1 site suggests that Mot3 can act synergistically with Rfx1 but not independently; hence, no repression in the \( \text{rox1}^\Delta \text{rfx1}^\Delta \) mutant. This Mot3 effect is the same as that observed in \( \text{ANB1} \) where Mot3 could not repress in the absence of Rox1 (27, 30).

It is interesting that despite the different effects of the three repressors, the \( \text{RNR2} \) and \( \text{RNR4} \) fusions appear to be expressed at very similar levels in wild-type cells and in the triple deletion. These genes encode the small subunit of the RNR enzyme (14, 21, 23, 49) and are probably required in equivalent concentrations in the cell. The \( \text{lacZ} \) fusions used in the present study contain the upstream region and one or six codons of the beginning of the coding sequences of the \( \text{RNR} \) genes only; the 3’ noncoding and transcriptional termination signals are those of the \( \text{ANB1} \) gene. Thus, the levels of enzyme activity measured from these fusions may accurately represent the relative levels of transcription of the native genes.

It should be noted that, in cases in which synergy was clearly evident, the binding sites for the interacting repressors were within 30 bp of each other. When sites were 40 bp or greater apart, synergy was not seen, as in the case of the Mot3 and Rox1 sites in \( \text{RNR3} \). However, the strength of the synergy was not predictable. Other factors, such as the intervening sequences, the location of undefined activation sequences, and the distance of the sites from the TATA box, may play roles in the strength of the synergy.

Finally, to address the concern that the results obtained with these \( \text{RNR-lacZ} \) fusions might be influenced by changes in the plasmid copy number in the different mutant strains, we integrated the \( \text{RNR3-lacZ} \) fusion into the genomic \( \text{RNR3} \) locus in the wild-type and seven mutant strains. The \( \beta \)-galactosidase assays were repeated, and the results are presented in Table 6. Clearly, there were differences in the levels of expression of \( \text{RNR3-lacZ} \). In most, but not all cases, the measured enzyme activity was lower. The levels of repression calculated were also not identical, but the synergy values calculated for the three pairwise interactions proved to be consistent. Using a centromeric plasmid, the synergies calculated for Rfx1-Mot3 and Rox1-Mot3 were 4.7 and 1.2, respectively, whereas the corresponding values obtained with the integrated plasmid were 8.6 and 1.2, respectively. While the synergy factors calculated for Rfx1-Rox1 were very different (44 for experiments with the centromeric plasmid and 152 for those with the integrated reporter), both indicate strong synergy. Overall, despite quantitative differences, the conclusions with both constructs are identical.
Derepression of the RNR genes results in resistance to DNA damage. Mutations in RFX1 or other genes that increase RNR levels or activity increase cell resistance to DNA-damaging agents (5, 22). To visualize this effect for the combinations of repressor mutations, we arrayed cells on a plate containing a gradient of NQO. As seen in Fig. 2, the greatest resistance was observed with any set of mutants containing the rfx1Δ allele, which not only causes derepression of RNR2 to -4 but also other genes whose products may help protect the cell from NQO (53). Clearly, the rox1Δ allele alone also increased NQO resistance, and resistance was further enhanced in combination with the rfx1Δ allele. No significant affect was observed with the mot3Δ allele.

**RNR2 and -3 are derepressed under anaerobiosis.** If Rfx1 and Mot3 repress the expression of RNR2 to -4, these genes should be induced under anaerobic growth conditions when Rfx1 and Mot3 are not present in the cell. To test this prediction, wild-type and mutant cells carrying either RNR2-lacZ or RNR3-lacZ fusions were grown for at least 17 h anaerobically to mid-exponential phase and assayed for the accumulation of β-galactosidase (Table 7). Comparison of the activity in wild-type cells grown aerobically and anaerobically shows that RNR2-lacZ expression was derepressed 7-fold and RNR3-lacZ expression was derepressed 31-fold. Aerobic repression was due to Rfx1 for RNR2 and Rfx1 plus Mot3 for RNR3, as evident from the equivalent aerobic and anaerobic expression of these genes in the rfx1Δ and the rfx1Δ mot3Δ mutants, respectively. On the other hand, Rfx1 alone still repressed anaerobically, as determined by the 11-fold repression of RNR2-lacZ both aerobically and anaerobically (rxx1Δ rfx1Δ rxx1Δ mot3Δ). These results indicate that Rfx1 is not regulated either positively or negatively by oxygen (and, by inference, Rox1 and/or Mot3); this repressor gave the same levels of repression both aerobically and anaerobically.

If Rfx1 still repressed RNR2 and -3 under anaerobic conditions, then further induction should result from the addition of a DNA-damaging agent during anaerobic growth. This is the case as seen in Fig. 3. Cells were grown aerobically and then divided into two cultures. Anaerobic growth was initiated in both cultures by bubbling nitrogen through the flasks in the absence or presence of 1.3 μM NQO. It is clear that the addition of NQO at the onset of anaerobiosis resulted in a much more dramatic induction than anaerobiosis alone, which was barely visible on the scales of these plots.

**Rox1 and Mot3 levels and activity are not regulated by DNA damage.** The apparent synergy between Rfx1 and Rox1 or Mot3 could result if these repressors indirectly regulated each other. While we ruled out regulation of Rfx1 activity by Rox1 and/or Mot3, it was still possible that the deletion of RFX1 somehow decreased the expression of Rox1 and Mot3, which would then result in derepression of RNR2-4 and the apparent loss of synergy. To test the effect of Rfx1 on Mot3 and Rox1 accumulation, we performed immunoblots with epitope-tagged forms of these proteins which are fully active in repression (27, 35). The mot3Δ and mot3Δ rfx1Δ mutants were transformed with a plasmid carrying the MOT3HA allele, and the rox1Δ and rox1Δ rfx1Δ mutants were transformed with a plasmid carrying the ROX1/HA allele. As can be seen in Fig. 4, there was no difference in the levels of Mot3HA (Fig. 3A) or Rox1HA (Fig. 3B) in RFX1 or rfx1Δ cells. Furthermore, the addition of NQO to RFX1 cells did not alter the levels of either Rox1HA or Mot3HA over a 3-h period. The blots were also probed for the translation factor eIF5A as a loading control. The relative levels of Mot3HA or Rox1HA, normalized to that of eIF5A, are presented below each lane.

Although Rox1 and Mot3 levels did not fall upon induction of the DNA damage response, and although no change in the migration pattern of either protein was noted in the immuno-

**TABLE 7. Anaerobic expression of RNR2 and -3**

| Genotype | RNR2-lacZ | RNR3-lacZ |
|----------|-----------|-----------|
|          | Aerobic    | Anaerobic |
|          | activity ≥ 5D | activity ≥ 5D |

| Genotype   | RNR2-lacZ | RNR3-lacZ |
|------------|-----------|-----------|
|            | Aerobic    | Anaerobic |
|            | activity ≥ 5D | activity ≥ 5D |

| Genotype   | RNR2-lacZ | RNR3-lacZ |
|------------|-----------|-----------|
|            | Aerobic    | Anaerobic |
|            | activity ≥ 5D | activity ≥ 5D |

The strains used were MZ233-20 (wild type), MZ233-13 (mot3Δ), MZ233-10 (mot3Δ rfx1Δ), RZ53-6Δroxl (rox1Δ), RZ56-6Δrox1Δ mot3Δ, MZ233-6 (mot3Δ rfx1Δ), MZ255-6 (rox1Δ rfx1Δ), and MZ209-8 (rox1Δ mot3Δ rfx1Δ).

The values for aerobic activity are taken from Tables 3 and 4.

**FIG. 2.** Mutations in the repressor genes increased cell resistance to DNA damage. Wild-type and mutant cells were shifted onto a gradient plate containing NQO from 0 to 2.6 μM (right to left) as described in Materials and Methods. The cells were MZ233-20 (WT), MZ233-13 (mot3Δ), MZ233-10 (mot3Δ rfx1Δ), RZ53-6Δroxl (rox1Δ), RZ56-6Δrox1Δ mot3Δ (rox1Δ mot3Δ), MZ233-6 (mot3Δ rfx1Δ), MZ255-6 (rox1Δ rfx1Δ), and MZ209-8 (rox1Δ mot3Δ rfx1Δ).
concentration of 1.3
Materials and Methods). The results are plotted as Miller units versus the hours of anaerobic growth either in the absence (+NQO) or presence (-NQO) of the DNA-damaging agent.

To investigate this possibility, we measured RNR-lacZ induction by NQO in wild-type and rfx1Δ cells. If Rox1 and Mot3

A model system for characterizing synergy. Past experience suggested that synergistic interactions in repression can be

FIG. 3. Anaerobiosis and DNA damage induced RNR2Z and -3Z expression. MZ233-20 (wild-type) cells were transformed with either YCp(33)RNR2Z (A) or YCp(33)RNR3Z (B), and a 50-ml culture was grown aerobically to mid-exponential phase in SC-uracil supplemented with 0.2% Tween 80 and 20 μg of ergosterol/ml. A sample was taken for time zero, and then the cultures were divided into four flasks. A final concentration of 1.3 μM NQO was added to two of the flasks. Nitrogen was bubbled through the cultures, and time points were taken (see Materials and Methods). The results are plotted as Miller units versus the hours of anaerobic growth either in the absence (-NQO) or presence (+NQO) of the DNA-damaging agent.

FIG. 4. Rox1 and Mot3 levels were not regulated by Rfx1 or NQO. (A) An immunoblot was carried out with protein extracts from MZ233-2 (a mot3Δ strain designated wild type [WT] here because it carried the MOT3HA allele on a plasmid) and MZ233-6 (a mot3Δ rfx1Δ strain designated rfx1Δ here because it carried the MOT3HA allele on a plasmid) cells transformed with YCp(23)MOT3HA, and MZ233-2 (lane C) cells transformed with YCp(22)MOT3. Cells were grown aerobically in SC-tryptophan medium. DNA damage was induced in MZ233-2 cells transformed with YCp(23)MOT3HA by the addition of NQO to a final concentration of 1.3 μM, and samples were taken at the times indicated. The blots were probed with monoclonal antibody against the HA epitope (Mot3-HA) and then stripped and reprobed with polyclonal antibody to eIF-5A. The relative levels of Mot3HA compared to the wild type are indicated below each lane, calculated as follows. The density of Mot3HA signal in each lane was divided by that for eIF5A in that lane. Then, this normalized level was divided by that for Mot3HA in the WT lane. 

(B) An immunoblot was carried out as in panel A with RZ53-6Δrnx1 and MZ255-6 transformed with YCp(22)ROX/HA (WT and Δrnx1, respectively), and RZ53-6Δrnx1 transformed with YCp(23)ROX1 (designated C). NQO induction was performed with the RZ53-6Δrnx1 transformed with YCp(33)ROX/HA for the times indicated. The relative levels of Rox1HA, calculated as described for panel A, are indicated below each lane.

retained full repression activity during NQO induction, then there would be no increase in RNR-lacZ expression in the rfx1Δ strain. As can be seen in Fig. 5, this was the case; the expression of the three genes remained unchanged in the rfx1Δ mutant and the rox1Δ mot3Δ rfx1Δ control (rox1Δ rfx1Δ for RNR2, which is not repressed by Mot3) compared to the robust induction in wild-type cells. To compare the induction profiles, we normalized the enzyme activity to the fold induction, with the uninduced levels as 1.0. (It should be noted that the un-normalized levels were quite different for each strain as seen in Tables 3 to 5, and although the fold induction levels in the rfx1Δ and double and triple mutants remained close to 1.0 throughout induction as expected, the absolute levels were quite high.)
respect to each other, and a construct with a deletion of 5 bp between the two sites (changing the side of the helix to which the proteins bound). Synergy was evident in all cases (data not shown). This lack of orientation or spacing requirements again reinforces the conclusion that Rox1 and Mot3 do not interact directly, but through a larger, more flexible complex.

Rfx1-repressed genes contain at least one strong and one weak binding site. We constructed reporter genes containing directly, but through a larger, more flexible complex.

It is evident from the data that the combined repression by Rox1 and Rfx1 was greater than that expected from additive repression alone; the synergy factor was about 3 independent of the orientation of the Rox1 site. Furthermore, although the orientation of the Rox1 site did appear to have a significant effect on repression, the fold repression was about three times greater for the Rox1 site in the inverted orientation both in the absence (r1f1Δ cells) and in the presence (wild-type cells) of Rfx1; there was no orientation dependence of the synergy factor. This observation also suggests that the two repressors do not interact directly. Thus, all of the evidence suggests that synergy involving Rox1, Rfx1, and Mot3 functions through indirect interactions.

**FIG. 5.** Rox1 and Mot3 retained repression activity during DNA damage. MZ233-20 (WT), MZ233-13 (rfx1Δ), and MZ255-6 (rox1Δrfx1Δ) cells were transformed with YCp(33)RNR2Z (A), and MZ233-20 (WT), MZ233-13 (rfx1Δ), and MZ269-8 (rox1Δrox3Δrfx1Δ) cells were transformed with YCp(33)RNR3Z (B) or YCp(33)RNR4Z (C). The transformants were grown to mid-exponential phase in SC-uracil medium at 30°C with vigorous shaking. β-Galactosidase assays were performed with samples taken before (time zero) and at the indicated times after the addition of NOO to a final concentration of 1.3 μM. The β-galactosidase values were normalized to fold induction by dividing the activity at time zero into the activity at the indicated times for each strain.

| Genotype | Mean β-galactosidase activity (fold repression, synergy factor) ± SD |
|----------|---------------------------------------------------------------|
|          | Mot3-Rox1   | Mot3-Rfx1 weak     | Mot3-Rfx1 strong |
| Wild type| 8.8 ± 1.8 (36, 6.3)                                       |
| mot3Δ    | 118 ± 34 (2.7)                                           |
| mot3Δ    | 148 ± 13 (2.1)                                           |
| mot3Δ    | 316 ± 17 (1.0)                                           |
| Wild type| 34 ± 8.7 (13, 2.3)                                       |
| mot3Δ    | 167 ± 15 (2.6)                                           |
| rfx1Δ    | 199 ± 20 (2.2)                                           |
| mot3Δ    | 434 ± 15 (1.0)                                           |
| rfx1Δ    | 458 ± 21 (1.0)                                           |

*a* The strain series transformed with YCp(33)AZ-M3R1 were RZ23-6 (wild type), RZ33-6mot3 (mot3), RZ33-6rox1 (rox1Δ), and RZ33-6mot3 (mot3Δ rox1Δ). The strain series transformed with YCp(33)AZ-M3Rw and YCp(33)AZ-M3Rfs plasmids were MZ233-20 (wild type), MZ233-2 (mot3); MZ233-13 (rfx1Δ), and MZ233-6 (mot3Δ rfx1Δ).

*b* The fold repression and synergy factor were calculated as described in Table 3.

The β-galactosidase fusion plasmids used were: YCp(33)AZ-M3R1 containing the Mot3-Rox1 sites; YCp(33)AZ-M3Rw containing the Mot3-Rfx1 weak sites; and YCp(33)AZ-M3Rfs containing the Mot3-Rfx1 strong sites. The inserts are presented in Table 2.
Neither Rox1 nor Rfx1 can function in synergy with itself. Previous evidence indicated that neither Rox1 nor Mot3 could act synergistically with itself. While the data for Mot3 were obtained in a comprehensive study involving sites in HEM13 (30), the data for Rox1 were compiled from several different studies (9, 27, 30). To test the synergy between two Rox1 sites from the two sites was additive. It should be noted that the deletions did not alter the calculated synergy factor; repression of Rox1 molecules bound. As seen in Table 10, these deletions did not alter the calculated synergy factor; repression from the two sites was additive. It should be noted that the 10-bp deletion did reduce repression about twofold. Perhaps the close proximity of the sites lead to an interference in synergy. To ensure that the lack of synergy was not due to the relative location of the Rox1 sites, we also assayed for synergy in two constructs were transformed into a moto3Δ and moto3Δ rfx1Δ strains. For a single Rfx1 site, the plasmid YCP(33)AZ-M5Rfw, containing the same Rfx1 site and a Mot3 site, was transformed into the same strains. Since both strains contained the moto3Δ deletion, the lacZ expression from the two plasmids would provide the fold repression for one and two Rfx1 sites. RFX1 cells carrying the plasmid with two Rfx1 sites contained 239 ± 14 Miller units, whereas rfx1Δ cells carrying the same plasmid contained 459 ± 28 Miller units, a 1.9-fold repression. The plasmid carrying only one Rfx1 site gave a 2.6-fold repression (Table 5). Thus, two weak Rfx1 sites positioned 10 bp apart could not even function additively in repression. Perhaps two Rfx1 molecules could not bind simultaneously or could not recruit additional Snf6/Tup1 molecules. In any event these results and those with two Rox1 and two Mot3 sites indicate that synergy appears to be limited to heterologous proteins.

### DISCUSSION

We previously reported two types of combinatorial repression by Rox1 and Mot3: additive and synergistic (30). In the former, a set of sites functioned independently, or additively, to repress transcription. An example of such repression was reported for HEM13, in which Mot3 could repress independently from a single site but, in combination with Rox1, enhanced Rox1 repression. This synergy was observed both in HEM13 and in ANB1 and resulted from an indirect interaction in which Rox1 and Mot3 interacted through the Snf6 subunit of the general repression complex. The indirect nature of synergy suggested that it might operate between Mot3 and/or Rox1 and other Snf6/Tup1 recruiting repressors. We demonstrated here that this is indeed the case; Mot3 and Rox1 interacted synergistically with Rfx1.

Rfx1 sites are most often present in pairs, with one strong and one weak site in the upstream region of genes (for examples, see Fig. 1). However, these sites are quite far apart. To determine whether two closely spaced Rfx1 sites could act combinatorially, YCP(33)AZ-2Rf was constructed containing two weak Rfx1 sites 10 bp apart (Table 2). This plasmid was transformed into the moto3Δ and moto3Δ rfx1Δ strains. For a single Rfx1 site, the plasmid YCP(33)AZ-M5Rfw, containing the same Rfx1 site and a Mot3 site, was transformed into the same strains. Since both strains contained the moto3Δ deletion, the lacZ expression from the two plasmids would provide the fold repression for one and two Rfx1 sites. RFX1 cells carrying the plasmid with two Rfx1 sites contained 239 ± 14 Miller units, whereas rfx1Δ cells carrying the same plasmid contained 459 ± 28 Miller units, a 1.9-fold repression. The plasmid carrying only one Rfx1 site gave a 2.6-fold repression (Table 5). Thus, two weak Rfx1 sites positioned 10 bp apart could not even function additively in repression. Perhaps two Rfx1 molecules could not bind simultaneously or could not recruit additional Snf6/Tup1 molecules. In any event these results and those with two Rox1 and two Mot3 sites indicate that synergy appears to be limited to heterologous proteins.

### TABLE 9. Combinatorial repression between Rox1 and Rfx1

| Genotype | Mean β-galactosidase activity (fold repression, synergy factor) ± SD |
|----------|---------------------------------------------------------------|
|          | Rox1-Rfx1weak | InvertedRox1-Rfx1weak |
| Wild type | 5.1 ± 0.5 (57.29) | 2.0 ± 0.2 (134.30) |
| rfx1Δ     | 46 ± 1.8 (6.3) | 58 ± 4.9 (4.6) |
| rfx1Δ     | 95 ± 11 (3.1) | 28 ± 2.2 (9.6) |
| rfx1Δ     | 29 ± 21 (1.0) | 26 ± 27 (1.0) |

### TABLE 10. Combinatorial repression between two Rox1 molecules

| Genotype | Mean β-galactosidase activity (fold repression) ± SD |
|----------|--------------------------------------------------|
|          | Wild type | Δ5 | Δ3′ | –5 | –10 |
| moto3Δ   | 38 ± 1.7 (9.7) | 75 ± 2.6 (3.7) | 190 ± 7.6 (2.2) | 33 ± 3.8 (9.7) | 100 ± 4.6 (5.7) |
| moto3Δ rfx1Δ | 374 ± 10.6 | 277 ± 60 | 418 ± 7.8 | 322 ± 29 | 573 ± 29 |

### Notes:

a. The strain series transformed with the YCP(33)AZ-R1Rf or YCP(33)AZ-1Rf were RZ53-6 (wild type), RZ53-6/H11032, in OpA and the 3′ motifs. These constructs were transformed into a wild-type plasmid, and 5 bp in the YCP(33)AZ-R1Rf containing the inverted Rfx1 and Rox1 sites. The inserts are shown in Table 2.

b. The fold repression was calculated as the enzyme activity in the moto3Δ cells divided by the enzyme activity in the moto3Δ cells for each plasmid.
and R. Zitomer, unpublished results). Ribonucleotide reductase is comprised of a large and a small subunit (25). Rnr2 and -4 form the small subunit in yeast, and Rnr2 contains a distinct tyrosyl radical center required for catalysis (5, 17). Formation of the tyrosyl radical requires molecular oxygen and is facilitated by Rnr4 (5, 17). Hence the reason for the regulation of RNR2 and -4 by Rox1 and Mot3 may be to increase the levels of protein to drive radical formation under hypoxic conditions. (It should be noted that there is a hypothetical 138 codon open reading frame 155 bp upstream from RNR2 which, if authentic, would share a regulatory region with RNR2.) The large subunit is composed of Rnr1 homodimers under normal growth conditions, whereas Rnr3 represents an alternative large subunit protein induced during DNA damage (15). The role that Rnr3 might play under hypoxia is unclear, but Rnr1-Rnr3 heterodimers are more active in vitro (11), and the hypoxic induction of Rnr3 may function to increase RNR activity as formation of the holoenzyme becomes compromised.

The synergy among differentially regulated repressors reported here has great biological impact. It allows strong repression of a gene through the action of two (or three) weakly acting repressors, yet the loss of repression by only one allows substantial derepression. Thus, multiple repressors functioning at a single gene do not have to be coordinately regulated to achieve robust induction in response to only one signal; the loss of synergy achieves that end. For the repressors studied here, Rox1 and Mot3 synthesis are regulated positively by oxygen, while Rfx1 activity is negatively regulated by DNA damage. We presented evidence here that there was little, if any, cross-regulation; Rox1 and Mot3 were fully functional during DNA damage, and Rfx1 retained full activity during hypoxia. Nonetheless, during DNA damage, a substantial part of Rox1 and Mot3 repression of the RNR genes was lost because the synergy with Rfx1 was lost. RNR4 regulation presents a good example of this phenomenon as seen in the data in Table 5. RNR4 expression was repressed 37-fold by the combination of the three repressors (comparing the triple deletion to the wild type). Rfx1 alone could repress expression only 2.7-fold (comparing the triple deletion to the roxlΔ mot3Δ mutant). Yet the loss of Rfx1 repression that might mimic DNA damage resulted in a 22-fold induction (comparing the wild type). Because of the loss of the combined synergy between Rfx1 and Rox1 and between Rfx1 and Mot3, nearly full induction was achieved. Similarly, under hypoxic conditions, simulated by the loss of Rox1 and Mot3 in the roxlΔ mot3Δ mutant, RNR4 was induced 14-fold due to the inability of Rfx1 to strongly repress in their absence. Furthermore, since synergy is indirect, functioning through Sn6, the evolution of synergy in this system did not even require the acquisition of direct interactions among these proteins. The only requirement for the introduction of this type of synergy in a system is the acquisition of closely spaced binding sites. Thus, we anticipate that many other examples of this biological phenomenon will be discovered. It should be noted, however, that these systems can be quite well disguised; elegant studies of Rfx1 repression of the RNR genes gave no clue that the robust repression by Rfx1 actually required the presence of two other proteins at the genes (22).

To explore the nature of synergy further, we devised artificial constructs in which we could control the number and relative positions of the repressor binding sites with respect to each other while holding the upstream activation sites, TATA box, and all other upstream sequences constant. Using single binding sites for each repressor, these constructs demonstrated the synergy between pairwise combinations. Interestingly, Mot3 acted synergistically equally well with strong and weak Rfx1 sites, suggesting that Mot3 was not simply helping Rfx1 bind better to weak sites. This conclusion fits well with our model of Rox1-Mot3 synergy in which Mot3 helps Rox1 recruit Ssn6; perhaps Mot3 is functioning in the same capacity with Rfx1. In addition, synergy is independent of the orientation of the asymmetric Rox1 and Mot3 sites and the Rox1 and Rfx1 sites, reinforcing the model that synergy does not require direct contact between the DNA-binding repressors. Interestingly, synergy was not previously observed between closely spaced Mot3 molecules or, in the present study, between closely spaced Rox1 or Rfx1 molecules. Thus, it appears that synergy is limited to heterologous repressors. This conclusion supports our previously proposed model in which the synergy arises from the sharing of the Ssn6/Tup1 complexes by Rox1 and Mot3. The requirement for heterology in this model arises because the two repressors must contact different surfaces of Ssn6.

There are at least two other examples in the literature of two different Ssn6/Tup1 recruiting repressors regulating the same genes. Proft and Serrano (39) reported that the ENA1 gene was repressed by Sko1, a repressor of osmotically induced genes, and Mig1/Mig2, repressors of glucose-repressed genes. The binding sites for Sko1 and Mig1/Mig2 are 20 bp apart. While the authors did not address the question of additive or synergistic repression directly, data presented in Table 3 concerning the expression of an ENA1-lacZ fusion in wild-type, sko1Δ, mig1Δ mig2Δ, and triple sko1Δ mig1Δ mig2Δ cells allow the calculation of a synergy factor of 3.1. Thus, it appears that Sko1 can act synergistically with Mig1 and Mig2. In the second example, the Sn6/Tup1 recruiting repressors Rim101, a repressor of meiotic genes, and Nrg1, a repressor of glucose-repressed genes, were demonstrated to repress the divergently transcribed DIT1-DIT2 genes (42). Both repressors bound to a 40-bp fragment between the two genes. This fragment was inserted upstream of the CYC1-lacZ gene, and expression studies presented in Fig. 7 of reference 42 allow the calculation of a Synergy Factor of 1, indicating that Nrg1 and Rim101 did not act synergistically. These two examples suggest that some, but not all Ssn6/Tup1 recruiting repressors can act synergistically.

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