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# Adjacent Gene Pairing Plays a Role in the Coordinated Expression of Ribosome Biogenesis Genes *MPP10* and *YJR003C* in *Saccharomyces cerevisiae*[▽]

James T. Arnone and Michael A. McAlear*

*Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, Connecticut 06459*

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**The rRNA and ribosome biogenesis (RRB) regulon from *Saccharomyces cerevisiae* contains some 200 genes, the expression of which is tightly regulated under changing cellular conditions. RRB gene promoters are enriched for the RRPE and PAC consensus motifs, and a significant fraction of RRB genes are found as adjacent gene pairs. A genetic analysis of the *MPP10* promoter revealed that both the RRPE and PAC motifs are important for coordinated expression of *MPP10* following heat shock, osmotic stress, and glucose replenishment. The association of the RRPE binding factor Stb3 with the *MPP10* promoter was found to increase after glucose replenishment and to decrease following heat shock. Similarly, bulk histone H3 clearing and histone H4K12 acetylation levels at the *MPP10* promoter were found to increase or decrease following glucose replenishment or heat shock, respectively. Interestingly, substitutions in the PAC and RRPE sequences at the *MPP10* promoter were also found to impact the regulated expression of the adjacent RRB gene *YJR003*, whose promoter lies in the opposite orientation and some 3.8 kb away. Furthermore, the regulated expression of *YJR003C* could be disrupted by inserting a reporter cassette that increased its distance from *MPP10*. Given that a high incidence of gene pairing was also found within the ribosomal protein (RP) and RRB regulons across different yeast species, our results indicate that immediately adjacent positioning of genes can be functionally significant for their coregulated expression.**

Cell growth and division are tightly regulated processes that depend upon the proper sensing and integration of environmental cues, coupled with the appropriate balancing of metabolic pathways necessary to maintain viability and to meet the complex biosynthetic demands of cellular duplication. In the budding yeast *Saccharomyces cerevisiae*, favorable conditions (i.e., when carbon and nitrogen sources are plentiful) lead to the activation of the Ras/PKA and TOR pathways (reviewed in references 31 and 43), which promote cell growth and division, in part, through their activation of the Sch9 kinase and Sfp1 (4, 8, 35). These pathways ultimately target and activate factors that alter the expression of up to 40% of the genome, including the upregulation of over 1,300 gene targets (23, 39). Broadly defined, there is a global upregulation of genes whose products increase the translational capacity within the cell (43). Conversely, environmental stresses downregulate these same pathways, thereby allowing cells to divert resources to maintain homeostasis (11).

One of the major targets of this global regulation is the metabolic machinery responsible for the production of ribosomes. Dividing cells have an increased demand for protein production, and this demand is met by a concomitant increase in ribosome production (21, 28). Rapidly growing yeast cells produce approximately 2,000 of the roughly 4.5-MDa ribosomes every minute (26, 41), which requires the 137 genes that encode the 79 ribosomal proteins (RPs), some 200 rRNA and ribosome biogenesis (RRB) genes (38), and the rRNA genes themselves. All three RNA polymerases are required for this process, and together, ribosome production accounts for some 60% of the total transcription events in a dividing yeast cell (41). In addition to the bulk requirement for the synthesis of these varied gene products, cells need to carefully coordinate the levels of production, since ribosomes require equimolar amounts of the RPs and rRNAs. One of the major points of regulation of this pathway is at the level of transcription.

It has long been recognized that the members of the RP regulon are transcriptionally regulated as a group in response to changing cellular conditions (reviewed in reference 40). RP promoters have been characterized and found to be enriched for binding sites for Abf1 or Rap1, and they are bound by the transcriptional activators Fhl1, Ifh1, Hmo1, and Sfp1 (reviewed in reference 21). Previously, we identified and characterized the genes from the distinct RRB regulon (also known as the Ribi regulon), members of which include the proteins involved in the production and modification of the 4 rRNAs as they are assembled into ribosomes. Expression of the members of the RRB regulon is tightly regulated across changing cellular growth conditions (16, 37, 38) in a manner that is related to, yet distinct from, that involved in the RP regulon. There are two highly conserved motifs within the promoter regions of members of the RRB gene set: the polymerase A and C (PAC) motif (6) and the rRNA-processing element (RRPE) (14, 33, 37, 38). These motifs have been found to regulate the transcription of RRB and reporter genes in plasmid-based reporter constructs (8, 30, 37). Neither motif on its own appeared to confer any basal level of transcription; however, both motifs can increase transcription in combination with an upstream activating sequence (30). Recently, a series of screens has

* Corresponding author. Mailing address: Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT 06459-0175. Phone: (860) 685-2443. Fax: (860) 685-2141. E-mail: mmcalear@wesleyan.edu.

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TABLE 1. Yeast strains used in this study

| Strain | Promoter of *MPP10* | Genotype | Source |
|---|---|---|---|
| YMM13 | Wild type | MAT**a** *leu2Δ1 trp1Δ63 ura3-52* | 37 |
| YMM547 | ΔRRPE | MAT**a** *leu2Δ1 trp1Δ63 ura3-52* | This study |
| YMM549 | ΔPAC | MAT**a** *leu2Δ1 trp1Δ63 ura3-52* | This study |
| YMM514 | ΔRRPE ΔPAC | MAT**a** *leu2Δ1 trp1Δ63 ura3-52* | This study |
| YMM539 | Wild type | MAT**a** *his3-1 leu2-0 met15-0 ura3-0 STB3-TAP::HIS3* | Open Biosystems |
| YMM553 | Wild type | MAT**a** *leu2Δ1 trp1Δ63 ura3-52 MPP10::KAN$^r$::URA3* | This study |

identified the long sought after binding factors for the RRPE and PAC motifs. Stb3 (Sin three binding protein) has been identified as the RRPE binding factor, and increased Stb3 binding to RRPE-containing promoters is associated with increased expression (23). Pbf1 (Tod6) and Pbf2 (Dot6) were identified by two independent groups as the PAC binding factors (9, 44). Both factors have been shown to play nonredundant roles in the transcriptional repression of RRB genes; however, strains bearing deletions in both genes still maintain an attenuated transcriptional repression in response to stress (24).

One of the remaining challenges involved in understanding how ribosome production is controlled lies in connecting the environmental sensing pathways to the *cis*- and *trans*-acting factors that regulate RP and RRB gene expression. Presumably, changing cellular conditions (i.e., nutrient changes or stress) will trigger the relevant signal transduction pathways to engage mechanisms to coordinately and appropriately regulate the expression of the hundreds of RRB and RP genes. For example, when starved yeast cells are prompted to enter a growth phase by the addition of glucose, their genome undergoes significant changes that modulate transcription levels, including the modification of histone tails (10). Decreasing the acetylation of histone proteins can result in the repression of transcription, and increasing acetylation increases expression (12, 19). The global increase in the acetylation of histone 4 and histone 3 residues is due to the activities of two lysine acetyltransferases, Esa1 and Gcn5 (10). Likewise, the transcriptional repression achieved during stress involves the activity of the Rpd3 histone deacetylase (HDAC). Rpd3 is recruited to the promoters of repressed genes during stress, and its activity is required for transcriptional repression of RRB genes during the stress response (1, 15). Targeted deacetylation of a promoter can create a repressed transcriptional domain within a euchromatic region, which can then allow rapid reactivation of transcription (25). Specific changes in histone acetylation can allow for the differential binding of factors such as the Swi/Snf complex and TFIID, which lead to the recruitment of RNA polymerase II and increased transcription (19). In this way, these changes can modulate the expression of genes that allow rapid growth, including the genes whose transcripts result in the production of ribosomes (21, 43).

An interesting observation that came out of the initial characterization of the RRB regulon was the discovery that RRB genes are disproportionately found as adjacent gene pairs (38). Others have noted that adjacent genes that are involved with the same cellular processes are frequently coexpressed (3). Coexpression of adjacent genes could be correlated with the presence of shared upstream activating sequences that were presumed to exert a regional effect, sometimes across a considerable distance (5). However, the significance of these observations and the mechanisms involved need to be further investigated experimentally.

In the present work, we studied the roles that the RRPE and the PAC elements play *in vivo* in the regulated expression of the adjacent RRB gene pair *MPP10-YJR003C*. We observed that the PAC and RRPE motifs from the *MPP10* promoter were not only important for the regulated expression of the *MPP10* gene under changing cellular conditions, they were also important for the coordinated regulation of the convergently oriented adjacent gene *YJR003C*. Coregulation of the *MPP10* and *YJR003C* genes could also be interrupted by separating the two genes with the pCORE reporter cassette. The high incidence of adjacent-gene pairing within the RRB and RP regulons across divergent yeast species suggests that relative gene positioning may play an important role in their transcriptional coregulation.

## MATERIALS AND METHODS

**Yeast strains.** A complete list of all strains used in this study, as well as their relevant genotypes, is included in Table 1. Strain YMM13 (MAT**a** *leu2Δ1 trp1Δ63 ura3-52*) was used as a wild type and is the parent strain used to generate the *MPP10* promoter mutants. The mutations in the promoter of *MPP10* were generated using the *delitto perfetto* method (32), where the promoter of *MPP10* was replaced by homologous recombination with a PCR fragment that contained homology to the region flanking the *KAN$^r$* and *URA3* genes. This cassette was subsequently replaced by a homologous region of the *MPP10* promoter, introducing an XhoI restriction site into the RRPE motif (YMM547), an AatII restriction site into the PAC motif (YMM549), or both restriction sites (YMM514). Strain YMM553 was generated using the *delitto perfetto* method, this time targeting the intergenic region between *MPP10* and *YJR003C*. Strain YMM539 was purchased from Open Biosystems.

A complete list of the oligonucleotide primers used in this study is provided in Table 2. The primers are named according to their targeted gene, the strand and position that they anneal to (W or C), and whether they were used for mRNA expression studies (quantitative reverse transcription [qRT]) or chromatin immunoprecipitation (ChIP) assays. ChIP analysis of genomic coding regions was performed using the same primers as for the qRT analysis.

**Culture conditions for environmental response.** Strains were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) medium either to early to mid-log phase (optical density at 600 nm [$OD_{600}$] = 0.40 to 0.90) for stress induction or for 72 h (past the diauxic shift) for glucose replenishment. A heat shock time course was induced by growing cultures at 30°C and transferring cells to 37°C medium (11). An osmotic shock was induced by growing cultures at 30°C and adding sorbitol to a final concentration of 1 M (11). Glucose replenishment was performed by adding glucose to a final concentration of 2% (11, 23).

**Calculating the statistical significance of gene adjacency.** The rRNA and ribosome biogenesis regulon in *S. cerevisiae* was defined as described previously (38) and consists of 188 genes. We searched for homologues in *Candida albicans* and *Schizosaccharomyces pombe* and identified 168 and 97 RRB genes, respectively. Ribosomal proteins were defined as all genes whose products are considered structural components of the ribosome (both cytosolic and mitochondrial). There were 180 (*S. cerevisiae*), 118 (*C. albicans*), and 166 (*S. pombe*) genes identified in each species. The total number of genes used in the calculations

TABLE 2. Oligonucleotides used in this study

| Name | Sequence (5′–3′) | Annealing site relative to ATG |
|---|---|---|
| ACT1qRTW | ATCGTTATGTCCGGTGGTACC | 1,196–1,217 |
| ACT1qRTC | TGGAAGATGGAGCCAAAGC | 1,259–1,281 |
| EBP2qRTW | AACGCTACCTTACAGAAACG | 957–977 |
| EBP2qRTC | TCCGTTAGGCCTGCCTCTATCGAA | 1,098–1,122 |
| MPP10qRTW | CGAGGAGGAGGAGGCTATTTAT | 674–696 |
| MPP10qRC | CTTCCTCATCCGCAAATAAGTC | 822–844 |
| YJR003CqRTW | ACCACCATTGACCCATACTCTC | 147–169 |
| YJR003CqRTC | GACCACTTCCATCAGTTCATCA | 425–447 |
| ACT1ChIPW | ATAGGATCTTCTACTACATGAG | −85–−63 |
| ACT1ChIPC | GTGCAATTCTTCTTACAGTTAA | 24–46 |
| EBP2ChIPW | CTGCCTAAATACAGATGAGATG | −98–−66 |
| EBP2ChIPC | CAACTCCTTCAACTTGAAACCT | 8–30 |
| MPP10ChIPW | CACCGCCTTTTCTGTACTGGCC | −135–−113 |
| MPP10ChIPC | ACGACTTTCCTTGGGTCTTTCG | −25–−3 |
| YJR003CChIPW | CCTCTTGTTAGATAACGTAGCC | −65–−43 |
| YJR003CChIPC | TCACCATGAAAGAGTTCGATGA | 23–45 |

included all verified genes in each organism but did not include those that are considered dubious open reading frames. This left us with a total of 5,797 (*S. cerevisiae*), 6,017 (*C. albicans*), and 4,970 (*S. pombe*) genes. The probability that there would be $j$ adjacent genes within a regulon of $M$ genes was as follows:

$$1 - \sum_{k=0}^{j} \left( \frac{M!}{k!(M-k)!} \right) (p^k (1-p)^{M-k})$$

where $p$ is equal to $(M/N)(2 - M/N)$ and $N$ is the total number of genes present within each species. The functional $P$ values were calculated in Mathematica.

**RNA preparation and expression analysis.** A complete list of oligonucleotides used in this study is provided in Table 2. Aliquots of yeast were obtained across a time coursed and washed at 4°C to remove the medium, and RNA was obtained by a hot acid phenol extraction (2) with the following modifications. Samples were extracted twice with phenol and once with chloroform and then ethanol precipitated prior to resuspension in diethyl pyrocarbonate (DEPC) water. Ten micrograms of RNA was cleared of genomic contaminants by treatment with DNase I according to the manufacturer's instructions (DNA-free; Ambion) and were checked by PCR using primers directed to the *ACT1* coding region. cDNA was generated with oligo(dT) primers using the Retro-script kit according to the manufacturer's instructions (Ambion). Linear conditions were identified by the titration of cDNA template for PCR, followed by native PAGE. Quantitative PCR (qPCR) was then performed across the time course, and the products were analyzed by native PAGE stained with Sybr Gold (Invitrogen). Images were obtained on either a Typhoon or a Storm phosphorimager scanner (Molecular Dynamics) and quantified using the manufacturer's ImageQuant software. Each expression profile represents the normalized average (to *ACT1*) of at least six qPCRs from at least two independent RNA extractions ± standard error (SE).

**Chromatin immunoprecipitation.** Yeast cultures were grown as described above, and chromatin was immunoprecipitated as described in reference 34 with the following modifications. Cells were cross-linked in 1% formaldehyde for 30 min at room temperature and quenched with 333 mM glycine for 15 min. Samples were washed twice in cold phosphate-buffered saline (PBS) and resuspended in high-salt lysis buffer (50 mM HEPES-KOH, 500 mM NaCl, 1% Triton X-100, 0.1% Na deoxycholate, 0.1% SDS, 1 mM EDTA) supplemented with protease inhibitors (Protease Inhibitor Cocktail Set 1; Calbiochem). The cells were lysed by vortexing them with an equal volume of glass beads, and chromatin was sheared by sonication to an average size of approximately 400 to 600 bp. Cellular debris was cleared by centrifugation, and the lysate was transferred to a fresh tube.

Lysates of 50 μl were precleared with magnetic protein A beads (New England Biolabs) prior to immunoprecipitation. Histones were immunoprecipitated with antibodies specific for total (bulk) histone H3 (Abcam; catalog no. AB1791), H4K12Ac (Abcam; catalog no. AB1761), or H4K16Ac (Millipore; catalog no. 07-329) and STB3p-TAP was immunoprecipitated with an anti-TAP tag antibody (Open Biosystems; catalog no. CAB1001) at 4°C overnight. Immune complexes were harvested by incubating them with protein A magnetic beads, washed twice with high-salt lysis buffer and once with wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% Na deoxycholate, 1 mM EDTA), and once with TE (50 mM Tris, pH 8.0, 10 mM EDTA). DNA was eluted into 30 μl elution buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1.0% SDS), and cross-links were reversed by incubation at 65°C overnight.

Samples were purified by extraction twice with phenol-chloroform–isoamyl alcohol and once with chloroform and then ethanol precipitated with glycogen as a carrier. Samples were then resuspended in Tris-EDTA (TE) and diluted 100-fold. Linear conditions were identified as described above. Enrichment of a fragment was determined by calculating the abundance in the immunoprecipitated fraction and subtracting the presence in the no-antibody control and is presented as a ratio with the abundance in the whole-cell lysate. Each graph is the normalized average (to a corresponding fragment of the *ACT1* promoter or coding region) of 3 or 4 qPCRs performed on 2 or 3 independent ChIP experiments ± standard deviation (SD).

## RESULTS

**The RRPE and PAC motifs are not essential for basal *MPP10* expression.** The RRB regulon consists of some 200 transcriptionally coregulated genes, and their products function at various levels of the rRNA and ribosome biosynthesis pathways. Interestingly, an unusually large fraction (15%) of the RRB genes exist as immediately adjacent gene pairs, including pairs of genes that are present in divergent, tandem, and convergent gene orientations (37). RRB gene expression is tightly regulated in response to changing cellular conditions, and RRB gene promoters are enriched for the RRPE and PAC promoter motifs. In order to determine the roles that these promoter motifs play in regulating RRB gene expression *in vivo*, we created a series of isogenic yeast strains that contained mutations in one or both of these motifs. We chose the adjacent RRB gene pair *MPP10* and *YJR003C* for this analysis because it allowed us to simultaneously investigate the functional significance, if any, of the immediate adjacency of coregulated and divergent RRB gene pairs. The two genes exhibit similar expression levels, and the *MPP10* gene promoter contains sequences matching the PAC and RRPE motif consensus sequence between bases 63 and 94 upstream of the initiator ATG, but the *YJR003C* promoter does not (Fig. 1). To investigate the activities of these promoter sequences, the *delitto perfetto* approach (32) was used to engineer substitutions into the RRPE and PAC motifs, respectively, from within the promoter of the *MPP10* gene. Each of the strains was created by alternately integrating and then excising the pCORE cas-

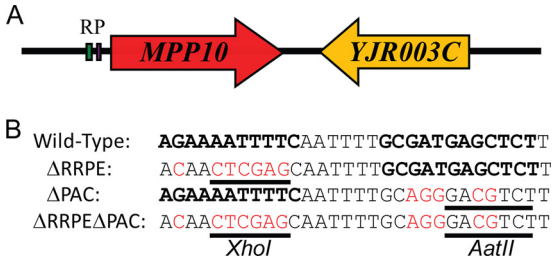


FIG. 1. Mutant construction at the *MPP10-YJR003C* locus. (A) Relative positions of the RRPE (R) and PAC (P) motifs with respect to the *MPP10-YJR003C* gene pair. (B) Sequences of the respective promoter regions from bases −95 to −62 upstream of the coding region for *MPP10*. The intact promoter motifs are highlighted in boldface, mutated bases are in red, and the restriction sites are underlined.

sette and plating it on the appropriate medium. The final strains contained short substitutions within the RRPE and PAC motifs that also introduced new DNA restriction sites. The integrity of the promoter motif substitutions was verified by restriction digests of PCR products that spanned the promoter regions.

The *MPP10* gene encodes an essential protein component of the U3 snoRNP, and disruption analysis indicated that Mpp10 functions in processing of the 18S pre-rRNA at sites A0, A1, and A2 (7). Given that the strains harboring the RRPE and PAC substitutions were viable, the two promoter elements were not essential for basal *MPP10* expression. We did, however, observe that strains bearing substitutions in the PAC and RRPE motifs increased the generation time from 90 min for the wild-type strain (YMM13) at 30°C to 95 min for strains YMM547 (ΔRRPE) and YMM514 (ΔRRPE ΔPAC), and to 115 min for strain YMM549 (ΔPAC). mRNA levels were also measured in log-phase cell cultures by reverse transcription followed by quantitative PCR (RT-qPCR) to quantify the relative steady-state levels of expression of *MPP10*, *YJR003C*, *EBP2*, and *ACT1*. The basal expression of *MPP10* and *YJR003C* (compared to the *ACT1* control) was diminished in the mutant strains, but on average by only 2- to 3-fold (data not shown).

**The RRPE and PAC motifs from the *MPP10* promoter function in the regulated expression of both *MPP10* and *YJR003C* *in vivo*.** It has long been recognized that the members of the RRB regulon are transcriptionally coregulated across many changing cellular conditions, including heat shock, osmotic stress, and glucose replenishment (11, 37). Because the RRB gene promoter sequences presumably regulate this response to changing cellular conditions, we monitored *MPP10* and *YJR003C* expression levels in our RRPE and PAC substitution strains. The expression level of the *ACT1* gene was measured as an internal control, and we also monitored the expression of *EBP2*, an independent RRB gene containing both RRPE and PAC promoter motifs. To measure the characteristic induction of expression following glucose replenishment, the strains were grown in YPD medium for 72 h, followed by the addition of glucose. Expression levels were monitored at 10-min intervals following glucose addition by RT-qPCR, and they were normalized to *ACT1* levels. In the wild-type strain, we could observe the characteristic boost in expression for the three RRB genes *EBP2*, *MPP10*, and *YJR003C* beginning at 10 min (Fig. 2A). This induction of expression was eliminated for the *MPP10* gene in each of the three strains containing either single or double RRPE and PAC substitutions, indicating that these motifs do play a critical role *in vivo*. Interestingly, these short substitutions within the *MPP10* RRPE and PAC promoter motifs were also found to impact the activation of the adjacent *YJR003C* gene, to the point of eliminating induction altogether in the ΔPAC and ΔRRPE ΔPAC strains (Fig. 2B to D). These changes in regulated expression were not due to a global impact on RRB gene regulation, because in each strain the characteristic transient upregulation of expression was maintained for the *EBP2* gene.

In order to test the roles that the RRPE and PAC promoter motifs may play in the regulated repression of RRB genes, the same strains were monitored for changes in expression levels following heat shock. The strains were grown at 30°C in YPD and shifted to 37°C, and aliquots of cultures were taken for RT-qPCR analysis for up to 30 min (Fig. 2E to H). Again, the characteristic decline in expression levels was observed for all three RRB genes (*EBP2*, *MPP10*, and *YJR003C*) in the wild-type strain (Fig. 2E) and for the *EBP2* gene in all four different strains. Substitutions within the RRPE and PAC promoter motifs from *MPP10* were found to significantly abrogate the heat shock-induced repression of the *MPP10* gene either singly or when combined, indicating that these motifs are important for regulating gene repression, as well as activation. Notably, once again, we observed that the substitutions in the *MPP10* promoter also impacted the regulated repression of the adjacent gene, *YJR003C*, this time most profoundly in the ΔRRPE and ΔRRPE ΔPAC strains (Fig. 2F to H).

Hyperosmotic stress is also known to trigger the regulated repression of RRB genes through the activation of signaling pathways that are distinct from the heat shock response (13). To test whether the RRPE and PAC promoter motifs may be involved in this pathway as well, the four strains were grown to mid-log phase and then sorbitol was added to a final concentration of 1 M. Relative expression levels were monitored by RT-qPCR for up to 1 h after the hyperosmotic shock. The *EBP2*, *MPP10*, and *YJR003C* gene expression levels were all found to fall upon osmotic shock, as did the *EBP2* expression profile in all four strains. The RRPE and PAC *MPP10* promoter motif substitutions were found to disrupt this regulated decrease in expression, either on their own or in combination, for both the *MPP10* and *YJR003C* genes (data not shown). Thus, the PAC and RRPE *MPP10* promoter motifs not only play an important role *in vivo* for the regulated induction and repression of the *MPP10* gene, they also play important roles in the regulated expression of the adjacent RRB gene, *YJR003C*, whose promoter is orientated in the opposite direction and is situated some 3.8 kb away.

**Disruption of the adjacency of the *MPP10-YJR003C* gene pair interferes with their transcriptional coregulation.** In order to test whether the transcriptional coregulation of the *MPP10-YJR003C* gene pair was related to their immediate adjacency, we engineered a strain that separated the two genes. To do this, we used the pCORE cassette from the *delitto perfetto* approach to introduce a 3.2-kb *KAN*$^r$*-URA3* construct at a position midway between the stop codons of the *MPP10* and *YJR003C* genes (Fig. 3A). The integrity of the disrupted

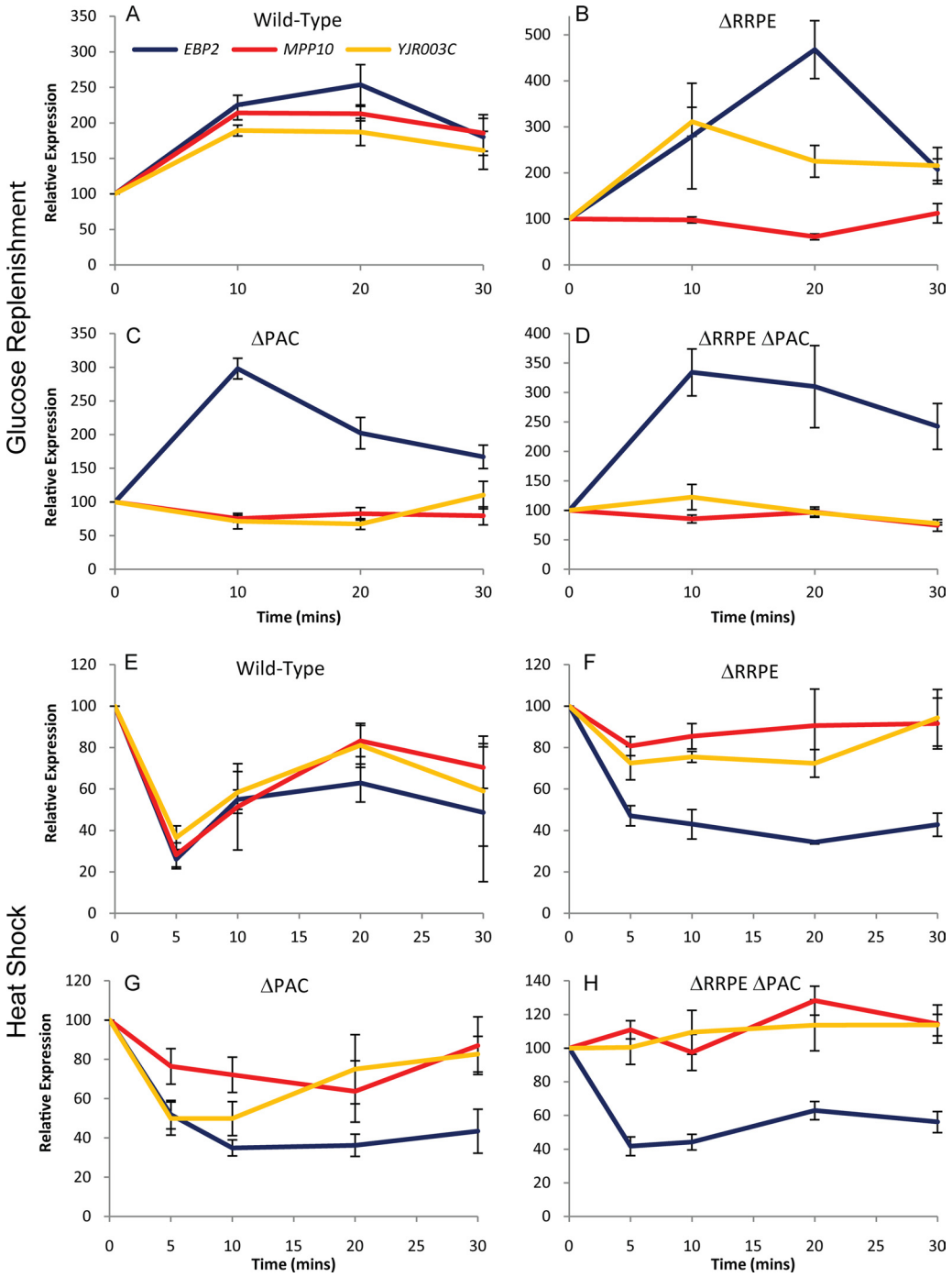


FIG. 2. RRPE and PAC motifs in the promoter of *MPP10* mediate the transcriptional response of both *MPP10* and *YJR003C* to changing environmental stimuli. Gene expression profiles were monitored after glucose replenishment following the diauxic shift in wild-type (A), ΔRRPE (B), ΔPAC (C), and ΔRRPE ΔPAC (D) and following a 30°C to 37°C heat shock in wild-type (E), ΔRRPE (F), ΔPAC (G), and ΔRRPE ΔPAC (H) *MPP10* promoter strains. The error bars indicate standard errors.

strain was verified by selective plating and PCR analysis, and it was subjected to expression analysis under changing cellular conditions. Whereas the *MPP10* and *EBP2* genes exhibited similar and characteristic repression responses to heat shock and osmotic shock, the expression profile of the *YJR003C* gene was significantly disrupted when it was separated from the *MPP10* gene (Fig. 3B and C). The response to glucose replenishment was also measured in this strain, and whereas the *EBP2* and *MPP10* activation profiles were very similar, the activation profile of the *YJR003C* gene was somewhat diminished (Fig. 3D). Therefore, the regulated expression of *YJR003C*, particularly with regard to stress-induced repression, not only depends on the PAC and RRPE motifs from the *MPP10* gene promoter, it also depends upon its immediate

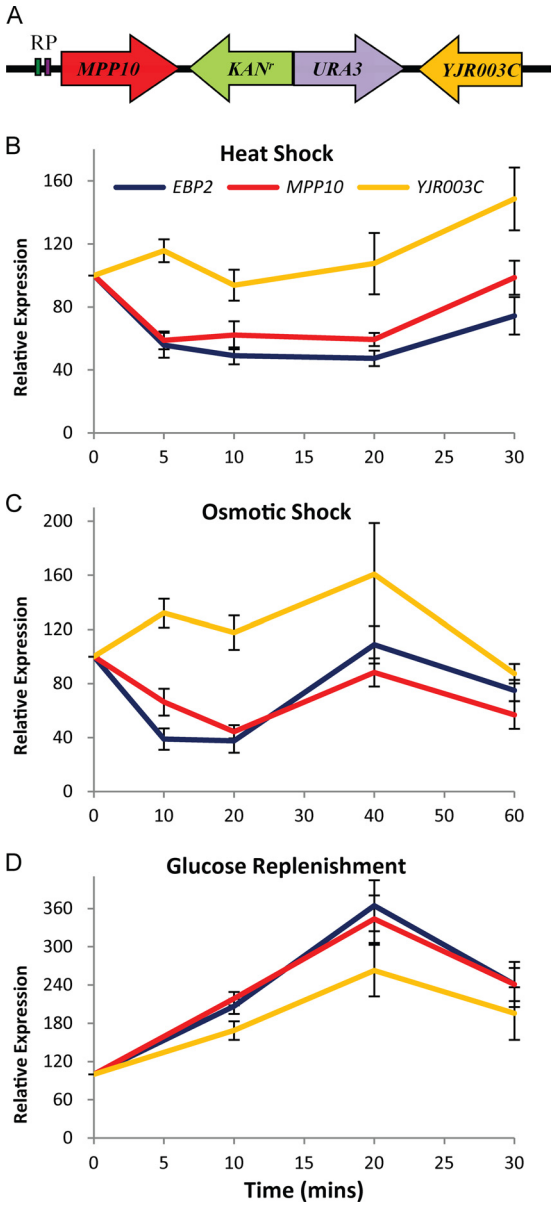


FIG. 3. Coregulation of the *MPP10-YJR003C* gene pair is disrupted when the two genes are separated. (A) Relative positions of the *KAN-URA3* disruption of the *MPP10-YJR003C* gene pair. (B to D) The transcriptional responses of the *EBP2*, *MPP10*, and *YJR003C* genes were monitored during heat shock (B), hyperosmotic shock (C), and glucose replenishment (D). The error bars indicate standard errors.

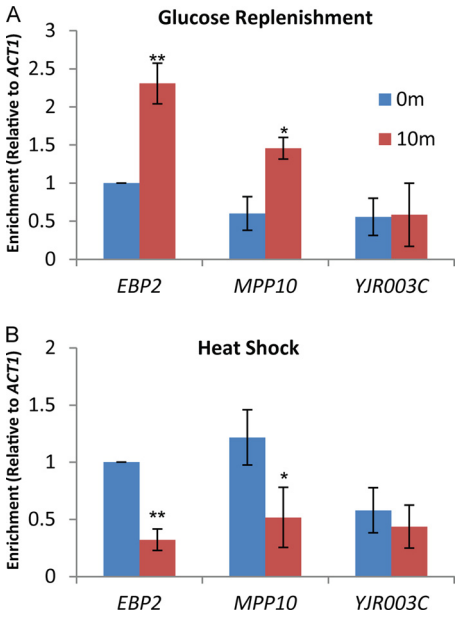


FIG. 4. Stb3 is recruited to RRPE-containing promoters during glucose replenishment and diminished following heat shock. Stb3 binding at the *EBP2*, *MPP10*, and *YJR003C* promoters was monitored by ChIP analysis at 0 and 10 min (0m, 10m) intervals during glucose replenishment (A) and heat shock (B) (*, $P < 0.05$; **, $P < 0.01$; calculated by *t* test). The error bars indicate standard deviations.

positional adjacency to *MPP10*. The insertion of the 3.2-kb fragment of the pCORE cassette (which contains two transcription units) appears to uncouple the coregulation of the *YJR003C* gene from that of *MPP10* and the standard RRB gene response, even though the promoter sequences, coding region, and 150 bp of the 3′ untranslated regions of *YJR003C* were unchanged.

**Binding of Stb3p to RRPE-containing promoters varies in response to cellular stresses.** Although the RRPE and PAC promoter motifs were first described many years ago (6, 14), it is only recently that their respective binding proteins have been identified: Pbf1 and Pbf2 for PAC and Stb3 for RRPE (23, 44). In order to assess the extent to which the regulated expression of RRB genes is potentially related to the binding properties of these motif binding factors, we monitored Stb3 binding to RRB promoters under changing cellular conditions (Fig. 4). A TAP-tagged Stb3 strain (Open Biosystems) was grown and subjected to glucose replenishment or heat shock for 10 min, and ChIP followed by qPCR was used to measure occupancy of the promoter regions at *ACT1*, *MPP10*, *YJR003C*, and *EBP2*. There was a significant (greater than 2-fold) increase in the occupancy of Stb3 at the RRPE-containing *EBP2* and *MPP10* promoters in response to glucose replenishment (Fig. 4A) and an opposite, greater-than-2-fold decrease in Stb3 binding to the same promoters following heat shock (Fig. 4B). The occupancy at the *YJR003C* and *ACT1* promoters—which do not contain RRPE motifs—was also monitored, and Stb3 binding was low and did not change significantly. Thus, changes in Stb3 binding were observed for those promoters that contained RRPEs, and increased binding of Stb3 was associated with increased levels of gene expression.

**The RRPE and PAC motifs are required for the histone acetylation changes observed in response to cellular stresses.** One of the important consequences associated with the identification of Pbf1, Pbf2, and Stb3 as the PAC and RRPE binding factors is their independent links to histone-modifying protein complexes, including the Sin3-Rpd3 complex (18, 29). Stb3 was identified in a 2-hybrid screen using Sin3 as bait (18), and Pbf1/Pbf2 were both identified by mass spectrometry in immunoprecipitation experiments as being associated with Rpd3 complexes (29). In genome-wide experiments, the Rpd3L HDAC was shown to be important for the transcriptional repression of RRB genes (including *EBP2*, *MPP10*, and

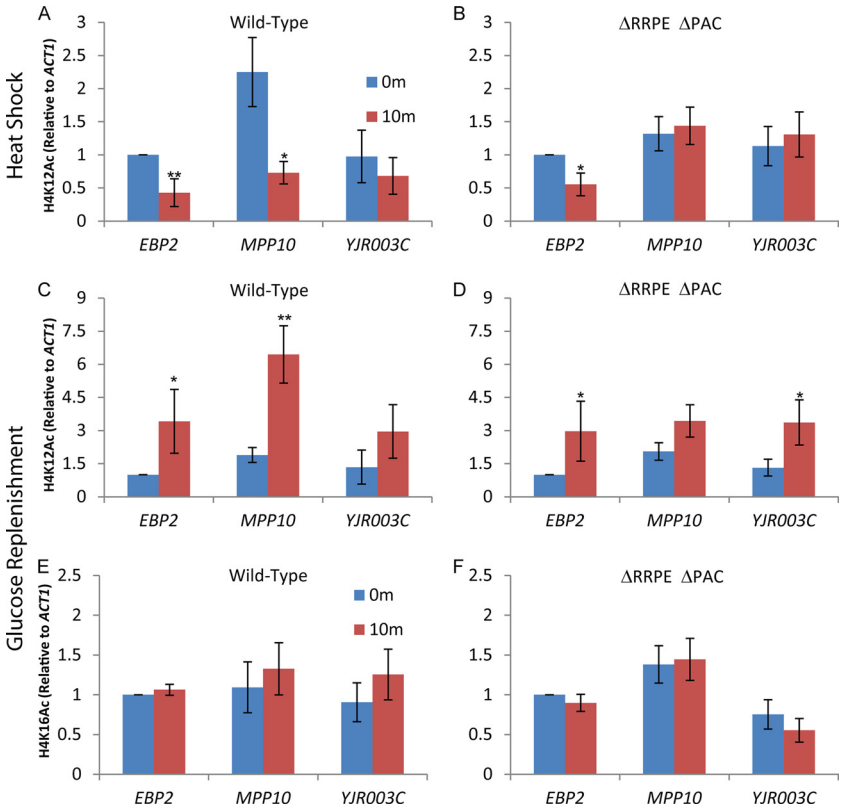


FIG. 5. The RRPE and PAC motifs are necessary for the changes in the acetylation of histone 4 K12, but not histone 4 K16, during the response to changing environmental stimuli. The relative levels of histone H4K12ac were monitored by ChIP analysis at the *EBP2*, *MPP10*, and *YJR003C* promoters following a 10-min heat shock in yeast with wild-type (A) and ΔRRPE ΔPAC (B) *MPP10* promoter strains. The relative levels of histone H4K12ac were monitored by ChIP analysis at the *EBP2*, *MPP10*, and *YJR003C* promoters in cultures following 10 min of glucose replenishment in yeast with wild-type (C) and ΔRRPE ΔPAC (D) *MPP10* promoter strains. The relative levels of histone H4K16ac were monitored by ChIP analysis at the *EBP2*, *MPP10*, and *YJR003C* promoters in cultures following 10 min of glucose replenishment in yeast with wild-type (E) and ΔRRPE ΔPAC (F) *MPP10* promoter strains (*, $P < 0.05$; **, $P < 0.01$; calculated by *t* test). The error bars indicate standard deviations.

*YJR003C*) during the stress response, and histone deacetylation by Rpd3L occurs preferentially at histone 4 lysine 12 (H4K12) and lysine 5 (H4K5) residues (1, 17). Therefore, one potential mechanism for regulating RRB gene expression could be through changes in histone modifications mediated through the recruitment of histone deactylases like Rpd3 via the PAC and RRPE binding factors, and decreased levels of acetylation would be expected to be correlated with decreased levels of gene expression. Since it is conceivable that the recruitment of histone-modifying enzymes to a gene promoter could result in the modification of histones at adjacent regions of DNA, including potentially adjacent promoters, we measured histone acetylation levels by ChIP analysis at the *MPP10-YJR003C* gene pair sequences in response to changing conditions.

We first measured H4K12 acetylation (H4K12ac) levels in the wild-type strain and observed that after 10 min of heat shock there was a significant, 2- to 3-fold decrease in the H4K12ac levels at the *EBP2* and *MPP10* promoters (Fig. 5A). This drop in acetylation levels is consistent with the stress-induced decrease in gene expression. However, even though *YJR003C* expression also falls in response to heat shock, we did not observe a significant change in the H4K12ac levels at its promoter. We performed a similar analysis of the strain YMM514, which lacked the PAC and RRPE motifs within the *MPP10* promoter, and found that while the H4K12ac decline observed for *EBP2* remained robust, the heat shock treatment did not elicit a decline in the H4K12ac status for the *MPP10* gene promoter (Fig. 5B). Again, the acetylation status of the *YJR003C* gene promoter—which contains neither RRPE nor PAC elements—was not significantly affected by heat shock in either strain. Therefore, the heat shock-induced changes in the acetylation status at the *MPP10* promoter are dependent on the presence of the PAC and RRPE motifs.

Similarly, we measured H4K12ac levels in response to glucose replenishment for the wild type and the ΔRRPE ΔPAC strain YMM514 (Fig. 5C and D). Glucose replenishment was associated with a roughly 3-fold increase in H4K12ac levels at the *EBP2* and *MPP10* promoters, a change that is consistent with the associated induction of expression of these genes. A less significant change in the acetylation levels was seen in the sequence of the *YJR003C* promoter. The ΔRRPE and ΔPAC substitutions in the mutant strain YMM514 appeared to significantly reduce the observed changes in H4K12ac levels in the *MPP10* promoter, whereas the magnitudes of the changes observed for the *EBP2* and *YJR003C* promoters were similar to those seen in the wild-type strain. Therefore, the RRPE and PAC motifs are important for mediating changes in the histone

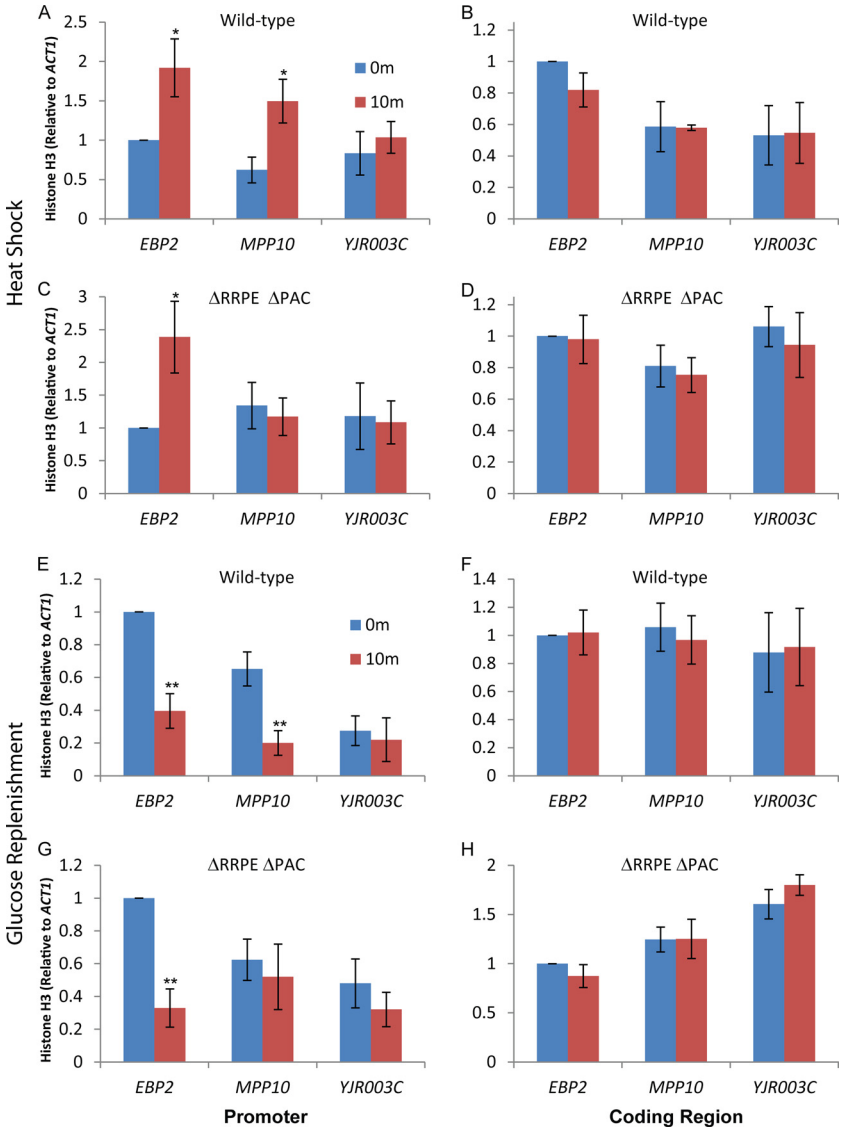


FIG. 6. The RRPE and PAC motifs are necessary for the changes in histone H3 occupancy seen in the promoters during the response to changing environmental stimuli. The relative levels of total histone H3 were monitored by ChIP analysis at the *EBP2*, *MPP10*, and *YJR003C* promoters and coding regions following a 10-min heat shock or glucose replenishment. (A, B, E, and F) Wild-type strains. (C, D, G, and H) ΔRRPE ΔPAC *MPP10* promoter strains. *, $P < 0.05$; **, $P < 0.01$; calculated by *t* test. The error bars indicate standard deviations.

acetylation status in response to changing cellular conditions, but these effects are greatest at the *MPP10* promoter itself, with little change observed at the *YJR003C* promoter. The direction of the changes in the H4K12ac levels we measured are consistent with the expectation that higher H4K12ac levels are associated with higher levels of expression.

In order to test the specificity of the observed histone acetylation changes, and as a control for a modification that was not expected to vary, we also monitored the strains for the acetylation of lysine residue 16 of histone 4 (H4K16). We observed that H4K16 acetylation (H4K16ac) status did not change in response to glucose replenishment, nor did it change in the strains bearing the RRPE and PAC substitutions (Fig. 5E and F). Thus, the observed changes in H4K12ac levels were not due to global changes in histone modification.

**Histone H3 occupancy changes at the *MPP10* promoter, but not at the *YJR003C* promoter, under changing cellular conditions.** Gene activation is typically associated with clearing of nucleosomes from promoter regions, and lower levels of gene expression are typically associated with higher levels of promoter nucleosome occupancy (20). In order to assess this aspect of the chromatin structure of the *MPP10* and *YJR003C* genes, we measured histone H3 occupancy levels by ChIP assays of both promoter and coding-region sequences under changing cellular conditions. In wild-type cells, the promoter sequences of the *EBP2* and *MPP10* genes showed increased H3 occupancy levels in response to heat shock (Fig. 6A), but H3 levels did not vary significantly over the coding regions of the genes (Fig. 6B). Consistent with the expression profiles, this increase in H3 promoter occupancy at the *MPP10* promoter

TABLE 3. Conservation of gene adjacency and orientation

| Species | Pair | Regulon size (genes) | No. of adjacent genes | *P* value | No. divergent | No. tandem | No. convergent |
|---|---|---|---|---|---|---|---|
| *S. cerevisiae* | RRB-RRB | 188 | 28 | $3.9 \times 10^{-7}$ | 5 | 5 | 4 |
| | RP-RP | 180 | 24 | $4.4 \times 10^{-5}$ | 4 | 6 | 3 |
| *C. albicans* | RRB-RRB | 168 | 46 | $2.3 \times 10^{-15}$ | 11 | 8 | 5 |
| | RP-RP | 118 | 25 | $1.4 \times 10^{-14}$ | 4 | 7 | 2 |
| *S. pombe* | RRB-RRB | 97 | 20 | $1.2 \times 10^{-10}$ | 6 | 3 | 1 |
| | RP-RP | 166 | 26 | $1.2 \times 10^{-5}$ | 5 | 7 | 1 |

was eliminated in the ΔRRPE ΔPAC strain (Fig. 6C). However, H3 occupancy levels did not appear to vary significantly after heat shock at the *YJR003C* promoter in either strain, and little variation was seen in the coding regions of the *EBP2*, *MPP10*, and *YJR003C* genes in the mutant strain (Fig. 6D).

We also monitored H3 occupancy levels in the strains and genes under activating conditions of glucose replenishment. We observed that the promoter regions of the *EBP2* and *MPP10* genes exhibited decreased levels of H3 occupancy upon glucose replenishment but that the H3 occupancy at the *YJR003C* promoter was not significantly changed (Fig. 6E). The observed changes in the *MPP10* promoter were dependent on the RRPE and PAC sequences, as the decrease was eliminated in the ΔRRPE ΔPAC strain (Fig. 6G). The H3 occupancy levels in the coding regions did not change appreciably for any of the genes, in either strain, under changing conditions (Fig. 6F and H). Therefore, the regulated expression of *MPP10* depends upon its own RRPE and PAC promoter sequences, and *MPP10* gene activation or repression is associated with expected histone H3 occupancy and histone H4K12 acetylation changes at the *MPP10* promoter. However, although the regulated expression of the *YJR003C* gene depends upon the *MPP10* RRPE and PAC promoter sequences, the mechanism whereby these motifs regulate *YJR003C* does not appear to be mediated by changes in the histone H3 occupancy or H4K12 acetylation status of the sequences in the *YJR003C* promoter.

**The gene sets involved in ribosome biogenesis are enriched for adjacent gene pairs.** To investigate the extent to which immediate gene pair adjacency may be important in other coregulated gene sets, we investigated the positions of genes across different regulons and different species. As previously reported, the 188 members of the RRB regulon were found to be widely dispersed across the yeast genome, yet 28 of them were found to exist as immediately adjacent gene pairs (Table 3). This is a highly significant enrichment ($P = 3.9 \times 10^{-7}$) and suggests that the physical arrangement of the RRB genes may play an important role in their function, potentially related to their transcriptional regulation. In terms of orientation, it was not the case that the majority of the gene pairs were arranged divergently, as one might expect for a bidirectional promoter; tandem and convergent gene pair orientations were represented roughly equally within this set of 28 genes. Also, because in no cases were the RRB genes found either as gene pairs separated by one other gene or as adjacent gene triplets, the bias appears to be for adjacent gene pairs.

In order to investigate the degree to which this curious paired arrangement of genes was specific to the RRB regulon in *S. cerevisiae*, we investigated the positions of RRB and RP regulon gene members in different yeast species. We found that there is also a significant enrichment for gene pairs within the RP regulon in *S. cerevisiae* (24 of 180 genes; $P = 4.4 \times 10^{-5}$). While because of an ancestral genome duplication the majority of the RP genes are present twice in the budding yeast genome, none of these 13 gene pairs (in this case there are two triplets) represent homologous gene partners. In terms of the relationship between the RRB and RP regulons, it was not the case that there was a bias toward gene pairing of RP with RRB members. Given the sizes of these two gene sets, one would expect by chance to find roughly 11 pairs of adjacent genes, and there were 7 such cases.

To gauge the broader evolutionary significance of this phenomenon, we looked for a similar arrangement of gene pairs in the distantly related *C. albicans* and in *S. pombe*. For this, we analyzed the sets of 118 genes from *C. albicans* and 166 genes from *S. pombe* that have been identified as structural components of the ribosome (cytoplasmic or mitochondrial). Because the memberships of RRB regulons have not been as well established in these organisms, we sought and identified homologues of the 188 RRB genes from *S. cerevisiae* and recovered sets of 168 and 97 genes from *C. albicans* and *S. pombe*, respectively. Again, we found that for both regulons in both species, there was significant enrichment for adjacent gene pairs, including an impressive 46 (23 pairs) of the 168 putative RRB genes in *C. albicans* ($P = 2.3 \times 10^{-15}$). The finding that over 1/4 of the RRB genes from *C. albicans* are found as adjacent pairs argues very strongly for their positional significance. There were infrequent examples of adjacent gene triplets in these regulons—no more than two for each gene set—but overwhelmingly, the arrangements were in gene pairs. In some cases, there was a bias toward divergent orientations (i.e., the RRB gene pairs in *S. pombe*), but tandem and convergent gene pairings were well represented. Thus, the members of the RRB and RP regulons exhibit significantly enriched paired gene distributions across widely divergent yeast species. Coupled with our detailed analysis of the *MPP10-YJR003C* gene pair, we therefore suggest that immediately adjacent gene pairing may play a significant and as yet underappreciated role in the transcriptional coregulation of genes within the ribosome biogenesis pathways.

## DISCUSSION

The advent of genome-wide technologies has allowed an increasingly sophisticated understanding of how eukaryotic

cells coordinately regulate the expression of large gene sets under changing conditions, including gene sets that play critical roles in controlling cell fate, cell growth, and cell division. Our analysis of the RRB and RP regulons in yeast contributes to that understanding and reveals that the physical locations of genes, and indeed, their immediate adjacency, can potentially be a relevant factor for their coordinated expression. The coregulated expression of two genes from a bidirectional promoter has been well characterized in budding yeast, and this arrangement is understood to be an efficient strategy to control the expression of genes that are involved in specific pathways (i.e., the *GAL* genes and the histone genes) (27, 42). Our observations extend this paradigm beyond divergently transcribed genes to include convergent and potentially tandemly oriented gene pairs. This strategy would still be more efficient than regulating genes individually; however, the implication is that coordinated expression of these adjacent gene pairs would involve mechanisms beyond the recruitment of RNA polymerase II to a single bidirectional promoter. Our observation that the coordinated expression is abrogated when the gene pairs are separated from one another reinforces the importance of the immediate adjacency and speaks to the relative paucity of coregulated gene pairs (i.e., RRB or RP regulon members) that are separated by a single unrelated gene. The disruption of adjacency may be related to an increased physical distance between the genes or due to the insertion of additional transcription units. The general importance of this phenomenon is bolstered by the observation that in a study of the 416 genes that exhibit cell cycle-dependent periodicity, approximately 25% exist as directly adjacent gene pairs (3). There are other observed cases of coregulated, convergent gene pairs where only one gene contains an identifiable upstream activating sequence (5). Therefore, the budding yeast genome may be comprised of many more locally coexpressed domains of gene pairs than expected, and the expression of both genes may depend on the sequences within a single promoter.

Previous genetic studies on the PAC and RRPE promoter motifs showed that both sequences play a role in regulating gene expression in response to changing growth conditions, but these studies were limited to plasmid-based or artificial reporter constructs (8, 30, 37). Specifically, both the RRPE and the PAC motifs were found to contribute to the activation of a plasmid-based construct in response to fresh medium (30). Our observations that the RRPE and PAC motifs from the *MPP10* promoter are critical for the regulated activation and repression of both the *MPP10* and *YJR003C* genes represents the first characterization of their activities in their normal chromosomal contexts. Whereas eliminating both motifs consistently altered the transcriptional response of the *MPP10* and *YJR003C* genes the most, the roles of the individual motifs were important and distinct. Mutations in the RRPE most greatly altered the heat and osmotic stress-induced repression of the RRB genes, and mutations in the PAC motif more greatly altered the activation response following glucose replenishment. Given that the two promoter motifs are typically close to one another within RRB gene promoters, it is reasonable to suggest that the PAC and RRPE binding factors may well interact with each other and cooperate in the regulated expression of the respective genes. As such, rather than the case where one factor (e.g., Stb3) may be an activator and the other (e.g., Pbf1 or Pbf1) a repressor, both factors may contribute to both activation and repression.

Our ChIP analysis of the binding properties of Stb3 is consistent with its suggested role as an RRPE binding RRB gene regulator. The binding of Stb3 to the RRPE-containing *EBP2* and *MPP10* promoters increases with conditions associated with gene activation and decreases with gene repression. Due to the limitations of the ChIP assay and the differences in the primer sequences used for amplification, it is difficult to compare the relative Stb3 binding levels between gene sequences, but importantly, the Stb3 binding levels remained low and did not change appreciably at the *YJR003C* promoter under the changing conditions. Our results are consistent with the independent finding that the binding of Stb3 to the RRPE-containing *NSR1* and *DBP10* promoters is increased in response to glucose replenishment and that an *stb3*Δ strain fails to increase the expression of RRB genes during this environmental change (23). However, a more recent investigation by the same group indicated that Stb3 may also be acting as a repressor, as overexpression of *STB3* results in decreased expression of growth-related genes, and this is alleviated by deletion of the *HOS2* histone deacetylase (22). In fact, these seemingly contradictory roles are both consistent with our observations that the RRPE sequence is involved in both transcriptional activation and repression of the same genes under different circumstances.

The induction of gene transcription in response to glucose replenishment is accompanied by global changes in histone acetylation that are, in part, dependent on the essential lysine acetyltransferase Esa1 (10). In contrast, stress-induced repression of gene expression is dependent upon the activity of the Rpd3 histone deacetylase (1). Rpd3 is found in a complex with the Sin3 protein, and recruitment of this complex results in a local region of deacetylation, particularly on histone 4 lysine 12 residues (17). Our results are consistent with a model in which the RRPE and PAC promoter motifs—presumably through their associated binding proteins (i.e., Stb3)—interact with the Rpd3 complex, thereby modifying the acetylation status of the gene promoters. The wild-type heat shock response yields a rapid decrease in H4K12ac levels at the promoters of the *MPP10* and *EBP2* genes, but little change was seen at the *YJR003C* promoter. This acetylation change at the *MPP10* promoter was lost in the strain that lacked the RRPE and PAC motifs. The reciprocal relationship was observed during glucose repletion, that is, H4K12 acetylation levels rose at the *MPP10* promoter in a manner that was dependent upon the RRPE and PAC promoter motifs. In neither case did the RRPE or PAC substitution substantially affect the acetylation status of the *YJR003C* gene promoter. When this is coupled with the ChIP data, the simplest model would be that glucose replenishment is associated with increased Stb3 binding at the *MPP10* promoter, as well as increased H4K12 acetylation levels and gene activation. Conversely, heat shock is associated with decreased binding of Stb3 at the *MPP10* promoter, lower levels of H4K12 acetylation, and gene repression.

The findings that promoter motifs from one gene can impact the regulated activation and repression of an adjacent gene whose promoter is oriented in an opposite direction and some 3.8 kb away is a novel one, and the mechanism by which this is effected needs further investigation. Among the possible mechanisms that may play a role here is the local looping of the

DNA whereby the promoter of the *YJR003C* gene could fold over and interact with factors at the *MPP10* promoter. Such a looping mechanism has been postulated to play a role in silencing at the *HMR* locus in budding yeast (36), and a similar mechanism could allow transcriptional regulators to influence expression at both genes. Presumably, there would be a distance constraint on such a looping mechanism, because the transcriptional coregulation between *MPP10* and *YJR003C* is not preserved when they are separated by the additional 3.2-kb pCORE cassette. Alternatively, there could be a local chromatin conformation that is set up to cover a region overlapping the promoters of the gene pairs, and the regulation of that structure could regulate activation or repression of the genes under changing cellular conditions. The physical basis for that potential local chromatin conformation is not known, except that it does not appear to be related to changes in bulk histone H3 occupancy or the acetylation status at histone 4 K12 or K16 residues.

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