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Dissecting the *cis* and *trans* Elements That Regulate Adjacent-Gene Coregulation in *Saccharomyces cerevisiae*

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The relative positions that genes occupy on their respective chromosomes can play a critical role in determining how they are regulated at the transcriptional level. For example, a significant fraction of the genes from a variety of coregulated gene sets, including the ribosomal protein (RP) and the rRNA and ribosome biogenesis (RRB) regulons, exist as immediate, adjacent gene pairs. These gene pairs occur in all possible divergent, tandem, and convergent orientations. Adjacent-gene pairing in these regulons is associated with a tighter transcriptional coregulation than is observed for nonpaired genes of the same regulons. In order to define the *cis* and *trans* factors that regulate adjacent-gene coregulation (AGC), we conducted a mutational analysis of the convergently oriented RRB gene pair *MPP10-YJR003C*. We observed that coupled corepression of the gene pair under heat shock was abrogated when the two genes were separated by an actively expressed RNA polymerase (Pol) II transcription unit (the *LEU2* gene) but not when the inserted *LEU2* gene was repressed. In contrast, the insertion of an RNA Pol III-transcribed tRNA (Thr) gene did not disrupt the coregulated repression of *MPP10* and *YJR003C*. A targeted screen of mutants defective in regulating chromosome architecture revealed that the Spt20, Snf2, and Chd1 proteins were required for coupling the repression of *YJR003C* to that of *MPP10*. Nucleosome occupancy assays performed across the *MPP10* and *YJR003C* promoter regions revealed that the mechanism of corepression of the gene pair was not related to the repositioning of nucleosomes across the respective gene promoters.

One of the essential regulatory challenges that all cells face is the need to maintain an internal homeostasis as well as to react appropriately to changing external environments. Cells monitor their surroundings for a wide range of factors, including nutritional levels, temperature, and osmolarity changes, and they respond to changing conditions by regulating the activity of appropriate metabolic pathways. Given that these metabolic pathways can encompass hundreds of gene products, cells require mechanisms to quickly, effectively shift their use of large classes of genes simultaneously. Often this level of control is affected at the level of transcription, and depending on the particular environmental cue, a cell may either activate or repress hundreds to thousands of genes simultaneously (1, 2). Transcriptional reprogramming can be accomplished on many levels, including through the regulation of transcription factors, through nucleosome modifications, through chromatin remodeling, and even through changing the subnuclear localization of specific genes (3, 4, 5, 6, 7).

Among the more complex, important, and tightly regulated metabolic pathways are those that are involved in the production of ribosomes. Ribosome biogenesis can represent a substantial fraction of the total cellular economy, and it depends on the coordinated activity of hundreds of genes (8). For example, in budding yeast, the proper synthesis and assembly of the 79 ribosomal proteins (RPs) and the 4 highly processed rRNAs that constitute a ribosome involves 137 RP genes, some 150 rRNA gene repeats, as well as over 200 rRNA and ribosome biogenesis (RRB, or *ribi*) genes (9, 10). All three of these large sets of genes are tightly coregulated under changing conditions, including activation by nutrient replenishment and repression by temperature or osmolarity shocks. Since an actively dividing yeast cell needs to produce roughly 2,000 of the 3.6-MDa ribosomes per minute, it is an important and nontrivial task to effectively regulate the overall synthesis and relative stoichiometry of the numerous ribosome biogenesis factors (8).

Genome-wide expression analysis in *S. cerevisiae* has revealed that the expression profiles of hundreds of ribosome biogenesis-related genes are rapidly altered in response to stressors or nutrient availability (1, 11). This response is mediated in part by highly conserved signal transduction pathways that ultimately control the expression levels of the RRB, RP, and rRNA gene sets. The TORC1 and ras/PKA/cyclic AMP pathways converge on the Sch9p kinase and Sfp1 that targets the rRNA and ribosome biosynthesis (RRB) transcriptional repressors Stb3p, Dot6p, and Tod6p (12, 13, 14, 15). These proteins in turn recruit the Rpd3L histone deacetylase complex to the promoters of RRB genes and mediate the budding yeast stress response (16). TOR kinase signaling also regulates the activity of the RP promoter binding transcription factor Fhl1 through controlling the subcellular location of the corepressor Crf1 (17). Furthermore, TOR also regulates the transcription of the rRNA genes by RNA polymerase (Pol) I through the displacement of the Rrn3 transcription factor from ribosomal DNA (rDNA) promoters (18, 19). Altogether, these pathways ultimately target a large fraction of the genome (20, 21).

We previously reported that there is an additional level of transcriptional control for the members of the RP and RRB regulons

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that depends on the relative positions of the genes on the chromosomes. A significant fraction of the RP and RRB genes exist as immediately adjacent gene pairs, and this arrangement results in a tighter transcriptional coordination than those of genes within the same regulons that are not paired (10, 22). This phenomenon of adjacent-gene pairing extends to other large coregulated gene sets, including those related to DNA damage response, carbohydrate metabolism, nitrogen metabolism, and heat shock response (22). Importantly, more than half of the adjacent gene pairs are found in tandem or convergent orientations, suggesting that their coregulation is not simply a consequence of bidirectional promoters, like that described for the *GAL1-GAL10* genes (23). Whereas it has long been appreciated that adjacent genes are more closely coregulated than nonadjacent genes, the precise mechanisms by which it is achieved had not been elucidated (24). The coupled regulation of adjacent genes has been proposed to be significant on a genomic scale, including in the so-called neighboring gene effect, where deletions in one gene yield phenotypes associated with the disruption of its neighbor (25).

In this study, we have taken a mutational approach to identify both *cis* and *trans* factors that regulate the coordinated repression of the convergently oriented *MMP10-YJR003C* RRB gene pair following a heat shock. We have found that the coupled coregulation of the *MPP10* and *YJR003C* genes depends less on their relative positions than it does on whether or not they are separated by an actively expressed RNA Pol II transcription unit. Furthermore, the coupled repression of *YJR003C* depends upon the activity of Chd1, the SWI/SNF complex member Snf2, and the SAGA complex member Spt20. While these *trans* factors include chromatin remodelers, we did not observe a correlation between transcriptional repression and changes in the nucleosome occupancy profiles at the *MPP10* or *YJR003C* promoter.

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** *leu2Δ1trp1Δ63 ura3-52*) was used as a wild type and is the parent strain used to generate the various mutants. The insertions in the intergenic region of *MPP10* and *YJR003C* were generated using the two-step, *delitto perfetto* method (28), targeting the integration of the *LEU2* gene in either orientation between *MPP10* and *YJR003C*. 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 nucleosome-scanning assay (NSA).

Updating of RRB gene annotations. The lists of the predicted membership of the RRB regulon (10, 26) were tabulated and updated for gene function annotations according to the Saccharomyces Genome Database (SGD; http://www.yeastgenome.org/) as of 15 November 2013.

Identification of putative *trans* regulators of RRB and RP gene expression. The data set from the 165-gene deletion chromatin interaction study (29) was analyzed to identify mutants that preferentially disrupted RRB and RP gene expression. The number of genes disrupted ($P < 0.05$) in the RRB, RP, paired RRB, and paired RP gene sets were determined, and the significance of the disruption was calculated by a hypergeometric probability density function:

$$P = 1 - \sum_{t=0}^{k-1} \frac{\binom{K}{x_t}\binom{N-K}{n-x_t}}{\binom{N}{n}}$$

TABLE 1 Yeast strains used in this study

Strain	Genotype	Source or reference
YMM13	*MAT***a** *leu2Δ1 trp1Δ63 ura3-52*	10, 26
YMM514	*MAT***a** *leu2Δ1 trp1Δ63 ura3-52 (ΔPACΔRRPE)MPP10*	27
YMM554	*MAT***a** *leu2Δ1 trp1Δ63 ura3-52 MPP10.LEU2*	This study
YMM555	*MAT***a** *leu2Δ1 trp1Δ63 ura3-52 MPP10.tRNA-Thr*	This study
YMM556	*MAT***a** *leu2Δ1 trp1Δ63 ura3-5 MPP10.Ty1.tRNA-Thr*	This study
YMM559	*MAT***a** *leu2Δ1 trp1Δ63 ura-52 MPP10.LEU2*	This study
YMM557	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 yjr003cΔ::KANr*	Open Biosystems
YMM593	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 snf2Δ::KANr*	Open Biosystems
YMM565	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 chd1Δ::KANr*	Open Biosystems
YMM594	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 isw1Δ::KANr*	Open Biosystems
YMM566	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 isw2Δ::KANr*	Open Biosystems
YMM595	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 asf1Δ::KANr*	Open Biosystems
YMM596	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 swr1Δ::KANr*	Open Biosystems
YMM562	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 spt20Δ::KANr*	Open Biosystems

where P is the probability, K is the total number of genes disrupted, k is the number of genes in the subset disrupted, n is the number of genes in the subset, and N is the total number of genes with measured P values in the original experiment.

Culture conditions for heat shock response. Strains were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) medium to early to mid-log phase (optical density at 600 nm of 0.40 to 0.90). A heat shock time course was induced by growing cultures at 30°C and transferring cells to 37°C medium (1).

RNA preparation and expression analysis. Aliquots of yeast were obtained across a time course and washed at 4°C to remove the medium, and RNA was obtained by a hot acid phenol extraction (30) 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.

Mapping of nucleosome positions. Nucleosome positions were mapped using the nucleosome-scanning assay as described in reference 31. Aliquots of 100 ml of cells were fixed with 2% formaldehyde for 30 min, and then the reaction was quenched with 125 mM glycine for 10 min. The cells were then washed once in Tris-buffered saline (TBS) buffer and spheroplasted with Zymolyase 20T for 40 min (until approximately 85%

TABLE 2 Oligonucleotides used in this study

Name	Forward primer (5′–3′)	Annealing site	Reverse primer (5′–3′)	Annealing site	Use
ACT1qRT	ATCGTTATGTCCGGTGGTACC	1196	TGGAAGATGGAGCCAAAGC	1281	qPCR
KANqRT	CACCGGATTCAGTCGTCACTCATGG	559	GGCAAGATCCTGGTATCGGT CTGCGATTCC	684	qPCR
EBP2qRT	AACGCTACCTTACAGAAACG	957	TCCGTTAGGCCTGCCTCTATCGAA	1122	qPCR
MPP10qRT	CGAGGAGGAGGAGGCTATTTAT	674	CTTCCTCATCCGCAAATAAGTC	844	qPCR
YJR003CqRT	ACCACCATTGACCCATACTCTC	147	GACCACTTCCATCAGTTCATCA	447	qPCR
SAG1-NSA-1	GGTTACTTTGAGCACACGGCTTTG	−634	GATACCGGACAATTGGCTTCCTG	−475	NSA
SAG1-NSA-2	CTCATCTCAGGGAACGAAAATTG	−566	CATAGGTTGAAATCATGAGAGAAG	−32	NSA
SAG1-NSA-3	CTCAGCTGAGCTCGGTTCGATC	−410	GAAGAAAAAAAGAGCCAGGATG	−272	NSA
YJR003C-NSA-1	GTACAGTTAGTACATTGAGTC	−346	GACGGAAAAGGATAAGAACTAG	−205	NSA
YJR003C-NSA-2	CATCCTGGCTCTTTTTTTCTTC	−294	CTATGATAAAATCTGCGGTG	−182	NSA
YJR003C-NSA-3	CTAGTTCTTATCCTTTTCCGTC	−224	GAAACAGCCTTCGGGTAATG	−105	NSA
YJR003C-NSA-5	CATTACCCGAAGGCTGTTTC	−124	GAATGGCGGTTAGCTGTTAAG	−5	NSA
YJR003C-NSA-6	CACCATGAAAGAGTTCGATGAG	−64	GAGAGAGTAAAACCTCTTGTTAG	57	NSA
YJR003C-NSA-7	CTTAACAGCTAACCGCCATTC	−26	GCTTTTTGATTATGTTCTTTC	92	NSA
YJR003C-NSA-8	CATTCTGCGGCTACGTTATCTAAC	15	GATGACGAATTGGATCGAAAG	129	NSA
YJR003C-NSA-9	ATAGAAAGAACATAATCAAAAAGC	68	CATTCTGCGATAGAGAGTATG	181	NSA
YJR003C-NSA-10	CTTTCGATCCAATTCGTCATC	108	CATATTGTGTACCATGGCCGCATC	243	NSA
YJR003C-NSA-11	CATACTCTCTATCGCAGAATG	160	CAACGCCGTAGTCAAGATCAC	280	NSA
YJR003C-NSA-12	GATGCGGCCATGGTACACAATATG	219	GTTTGATGGCGGGAAGTGAAG	343	NSA
YJR003C-NSA-13	GTGATCTTGACTACGGCGTTG	259	CAAGAGCTTTGTACTCTTCCTG	383	NSA
MPP10-NSA-1	TTATGACTATCATTCCTATCGCAAAG	−640	GAAGGCCTTTCGCAGCTCTTC	−506	NSA
MPP10-NSA-2	GTATTGGACGTTCTGATGAATGTG	−584	GCTATCAAAACGAAGACAAC	−460	NSA
MPP10-NSA-3	GAAGAGCTGCGAAAGGCCTTC	−526	GTAAAACACAAACCGGCCCCCAG	−400	NSA
MPP10-NSA-4	GTTGTCTTCGTTTTGATAGC	−479	GTTACGTGACAAGCCACTCTCTC	−313	NSA
MPP10-NSA-5	CTGGGGGCCGGTTTGTGTTTTAC	−422	CACCAACACCTAATGTGGACAAC	−272	NSA
MPP10-NSA-6	GAGAGAGTGGCTTGTCACGTAAC	−335	CAGAAGTACAGAGCTAATG	−197	NSA
MPP10-NSA-7	GTTGTCCACATTAGGTGTTGGTG	−294	GAAAAGGCGGTGAATATTTTATG	−124	NSA
MPP10-NSA-8	CATTAGCTCTGTACTTCTG	−215	CGTGCACACATCATTTATTCATAAC	−24	NSA
MPP10-NSA-9	CGTAATATACATATTTTCGTGTAG	−179	GACATTATACGACTTTCCTTGGGTC	5	NSA
MPP10-NSA-10	CTGGCCGCCGGCATGCGAG	−119	CAATACTCCAAAGAGTTCTGAC	24	NSA
MPP10-NSA-10.5	GAAACAGTGTTTTGTTATGAATAAATG	−61	GCTTTAACATCTTTAGAAGTGGC	86	NSA
MPP10-NSA-11	GTGCACGAAAGACCCAAGGAAAG	−30	CTTACAGGTATTGATAACTGAATC	117	NSA
MPP10-NSA-12	GTCAGAACTCTTTGGAGTATTG	3	CATCAACAGTGATTTCGTCCAG	166	NSA
MPP10-NSA-13	GATTCAGTTATCAATACCTGTAAG	94	GATCACCATCAATACTATCAAGAA	229	NSA
MPP10-NSA-14	CTGGACGAAATCACTGTTGATG	145	GGCGTGACAACATCTTTGAGTTCTTG	269	NSA
MPP10-NSA-15	TTCTTGATAGTATTGATGGTGATC	206	CTTTCACTTGATCTCCTCCGTC	340	NSA
HSP104-NSA-1	GAAACCGTGGATGTTCAGGAC	−700	CGCCTTTGAATCGATGACAAT	−588	NSA
HSP104-NSA-2	CACCGAGCCGGGGAAATTCG	−657	GTCGTCGATCCAGTCCATTTC	−546	NSA
HSP104-NSA-3	ATTGTCATCGATTCAAAGGCG	−608	GCCCTTGGAGTTTGGATTCTTG	−501	NSA
HSP104-NSA-5	CAAGAATCCAAACTCCAAGGGC	−522	GAATAAATAAGTGAATAGGTAG	−413	NSA
HSP104-NSA-6	GGTTTAAAAACCTTCTGCACCA	−474	CGATGGAGGGTTCAATGTTAAT	−358	NSA
HSP104-NSA-7	CTACCTATTCACTTATTTATTC	−434	TAACCCTTCTAGAAAATTCTGG	−279	NSA
HSP104-NSA-8	ATTAACATTGAACCCTCCATCG	−379	CTTTGAGATGGGCCCCCTGTTG	−203	NSA
HSP104-NSA-9	CCAGAATTTTCTAGAAGGGTTA	−300	GTTTGCGCCCCTTTGCCTTTTAC	−161	NSA
HSP104-NSA-11	GTAAAAGGCAAAGGGGCGCAAAC	−183	TTGCTGATTCGATTCAAGGG	−52	NSA
HSP104-NSA-12	GGCATTGTAATCTTGCCTCAATTCC	−132	CTGTATATTTTATGGTACGTGTAG	−5	NSA
HSP104-NSA-13	CCCTTGAATCGAATCAGCAA	−71	GCCAACGTCAAAATCGTTAGAGCCC	53	NSA
HSP104-NSA-14	CTACACGTACCATAAAATATACAG	−29	GTATAGGTTGTAATTGTGGATG	100	NSA
HSP104-NSA-15	GGGCTCTAACGATTTTGACGTTGGC	29	CTGATCCATCTTCTGGCGTTTC	142	NSA
HSP104-NSA-16	CATCCACAATTACAACCTATAC	79	GATCATAGTCGTAACGGCCC	190	NSA
HSP104-NSA-17	GAAACGCCAGAAGATGGATCAG	121	GCAGGTTGCTGTTGAGGAATTC	245	NSA
HSP104-NSA-18	GGGCCGTTACGACTATGATC	171	CCCCAAAGCATAACTTGGAG	279	NSA
HSP104-NSA-19	GAATTCCTCAACAGCAACCTGC	224	GCGCTATAAATGAGTCCTTCTG	337	NSA

of cells had spheroplasted). Spheroplasts were then washed twice and resuspended in 1.7 ml of MNase digestion buffer. Aliquots (360 μl) were then digested with limiting concentrations of MNase I (New England BioLabs) for 40 min. The digestion reaction was stopped by the addition of Tris-EDTA (TE)-SDS buffer, and cross-links were reversed by incubating the samples overnight at 65°C in the presence of proteinase K (New England BioLabs). DNA was recovered by phenol-chloroform/isoamyl alcohol extraction and ethanol precipitation. RNA was removed from the sample by treating the samples with RNase A for 60 min, at which point the DNA was again extracted by phenol-chloroform/isoamyl alcohol extraction and ethanol precipitation. The DNA samples were air dried and then resuspended in TE buffer.

TABLE 3 Previously uncharacterized RRB gene members with annotated RRB functions

Systematic name	Standard name	Function and or pathway per SGD	Systematic name	Standard name	Function and/or pathway per SGD
YAL036C	*RBG1*	Ribosome-associated protein	YJL109C	*UTP10*	Processing of the 18S rRNA
YBL028C		Nucleolar protein	YJL122W	*ALB1*	Production of the 60S ribosomal subunit
YBL054W	*TOD6*	Ribosome biogenesis transcription factor	YKL082C	*RRP14*	Production of the 60S ribosomal subunit
YBR247C	*ENP1*	Production of the 40S ribosomal subunit	YKL099C	*UTP11*	18S rRNA single-subunit processome
YCR072C	*RSA4*	Production of the 60S ribosomal subunit	YKL143W	*LTV1*	Production of the 40S ribosomal subunit
YDL063C	*SYO1*	Production of the 60S ribosomal subunit	YLR002C	*NOC3*	Production of the 60S ribosomal subunit
YDR101C	*ARX1*	Production of the 60S ribosomal subunit	YLR276C	*DBP9*	Helicase, 27S rRNA processing
YDR365C	*ESF1*	Processing of the 18S rRNA	YLR401C	*DUS3*	Dihydrouridine synthase, RNA modification
YER049W	*TPA1*	Translation factor	YML093W	*UTP14*	18S rRNA single-subunit processome
YGL099W	*LSG1*	Production of the 60S ribosomal subunit	YNL110C	*NOP15*	Production of the 60S ribosomal subunit
YGR103W	*NOP7*	Production of the 60S ribosomal subunit	YNL132W	*KRE33*	Production of the 40S ribosomal subunit
YGR145W	*ENP2*	Production of the 40S ribosomal subunit	YNR053C	*NOG2*	Production of the 60S ribosomal subunit
YGR283C		Ribosome-associated methyltransferase	YOL124C	*TRM11*	Guanosine methyltransferase
YHL039W	*EFM1*	Elongation factor methyltransferase	YOR091W	*TMA46*	Ribosome-associated protein
YHR196W	*UTP9*	18S rRNA single-subunit processome	YPL012W	*RRP12*	Ribosomal subunit export factor
YIL096C		Production of the 60S ribosomal subunit	YPL093W	*NOG1*	60S ribosomal subunit biogenesis
YIL127C	*RRT14*	rRNA biogenesis factor	YPR143W	*RRP15*	Production of the 60S ribosomal subunit

The digestion reactions were then visualized on a 1.0% agarose gel, and the sample that resulted in the generation of mononucleosome-sized fragments was subsequently analyzed by real-time PCR. Real-time PCR was performed on an Applied Biosystems 7300 instrument utilizing Sybr green chemistry (Life Biosystems) and analyzed using the manufacturer's software. Twenty-μl reactions from each chromatin preparation were run in triplicate, and outliers were removed based on the manufacturer's criteria prior to analysis. The ratio of nucleosome-protected to nucleosome-depleted regions within the GAL locus was used as a control, and nucleosome positioning data were determined as previously described (32).

RESULTS

The RRB regulon membership predictions were accurate. The RRB regulon was originally defined as a set of 65 transcriptionally coregulated genes that were enriched for the PAC and RRPE promoter motifs and whose products were suggested to play a role in the rRNA and ribosome biogenesis pathways (26). A full 30 of the original set of 65 RRB genes were uncharacterized at the time, and they were known only as unannotated open reading frames. By analyzing genome-wide expression profiles of yeast cells progressing through multiple changing environmental conditions, as well as through the analysis of gene promoter sequences, we expanded the predicted membership of the RRB regulon to include some 188 genes (10). This expansion indicated that the RRB regulon is at least as important in overall ribosome biogenesis as the 137-member ribosomal protein (RP) regulon and the large, 150-member tandem array of rRNA gene repeats. The expanded RRB gene set also included as-yet uncharacterized genes, as well as genes for which other non-RRB functions had been ascribed. To investigate the degree to which the RRB regulon membership assignments accurately predicted activities in the rRNA and ribosome biogenesis pathways, we reevaluated the known functions and annotations of the gene set (Table 3). We found that of the predicted 38 RRB genes for which there were previously no known functions, 34 (89%) have subsequently been shown to play a role in rRNA or ribosome biogenesis. Furthermore, of the 70 predicted RRB genes that had reported functions in pathways other than that of rRNA and ribosome biogenesis, 53 (76%) have subsequently been shown to exhibit additional RRB-consistent activities (Table 4). Therefore, the predictions of the RRB regulon membership were accurate, indicating that a similar approach could be useful in the identification and characterization of other coregulated gene sets in other metabolic pathways and species.

Defining the *cis* elements required for adjacent-gene coregulation (AGC). When we first identified and characterized the membership of the RRB regulon, we noticed that a significant fraction (roughly 15%) of the RRB genes were located on the chromosomes as immediate, adjacent gene pairs. Significant levels of adjacent-gene pairing were subsequently found in other coregulated gene sets in budding yeast and in the RRB and RP regulons across divergent eukaryotes (22). Additionally, the sets of paired genes were found to be more tightly coregulated across multiple changing growth conditions than were those of the unpaired genes of the same regulons. In order to define the *cis* elements that control the coregulated expression of the adjacent gene pairs, we initiated a mutational analysis of the convergently transcribed gene pair *MPP10-YJR003C* (26). We created a mutant yeast strain that contained discrete substitutions within the PAC and RRPE promoter motifs of the *MPP10* gene, and we monitored the gene expression profiles following heat shock (27). We found that the *MPP10* promoter substitutions were sufficient to disrupt the regulated expression of not only *MPP10* but also of the adjacent *YJR003C* gene, even though the promoter for *YJR003C* lies some 3.5 kbp away and is oriented in the opposite direction (Fig. 1). We also altered the relative positions of the two genes through the insertion of a *URA3 kanMX4* pCORE cassette that separated the two genes by an additional 3.8 kbp. In this case, the heat shock-induced repression of the *MPP10* gene remained intact, but it did not extend to the separated *YJR003C* gene (27).

In order to further define the DNA sequence elements that play a role in mediating adjacent gene coregulation, we created and tested new sets of mutants that altered the positions and relationships of the *MPP10* and *YJR003C* genes. Given that sequences from the promoter of the *MPP10* gene were able to direct the expression pattern of the adjacent *YJR003C* gene, we tested whether the *MPP10* promoter could similarly exert a regulatory influence on an exogenous gene that replaced *YJR003C*. To do this, we took advantage of a genome-wide deletion library in

TABLE 4 RRB gene members with newly annotated RRB functions

Systematic name	Standard name	Function and/or pathway per SGD	Systematic name	Standard name	Function and/or pathway per SGD
YAL025C	*MAK16*	Production of the 60S ribosomal subunit	YKL021C	*MAK11*	Production of the 60S ribosomal subunit
YAL036C	*RBG1*	Ribosome-associated protein	YKL191W	*DPH2*	Modifies histidine residues in translation elongation factor 2
YBL024W	*NCL1*	tRNA methyltransferase	YKR056W	*TRM2*	tRNA methyltransferase
YBR034C	*HMT1*	Methyltransferase of ribosomal protein Rps2p	YKR092C	*SRP40*	Pre-ribosomal assembly and transport
YBR267W	*REI1*	Production of the 60S ribosomal subunit	YLR009W	*RLP24*	Production of the 60S ribosomal subunit
YCL037C	*SRO9*	Ribosome-associated protein	YLR249W	*YEF3*	Translational elongation factor eEF1B subunit
YCR055C	*PWP2*	35S pre-rRNA processing	YLR401C	*DUS3*	Dihydrouridine synthase
YDL201W	*TRM8*	tRNA methyltransferase	YMR014W	*BUD22*	Production of the 40S ribosomal subunit
YDR060W	*MAK21*	Production of the 60S ribosomal subunit	YMR131C	*RRB1*	Production of the 60S ribosomal subunit
YDR101C	*ARX1*	Production of the 60S ribosomal subunit	YMR309C	*NIP1*	Subunit of eukaryotic translation initiation factor 3
YDR120C	*TRM1*	tRNA methyltransferase	YNL062C	*GCD10*	tRNA methyltransferase
YDR165W	*TRM82*	tRNA methyltransferase	YNL110C	*NOP15*	Production of the 60S ribosomal subunit
YDR299W	*BFR2*	Component of 90S preribosomes	YNL119W	*NCS2*	tRNA uridine modification
YDR465C	*RMT2*	Ribosomal protein Rpl12 methyltransferase	YNL175C	*NOP13*	Preribosomal complex nucleolar protein
YDR496C	*PUF6*	Production of the 60S ribosomal subunit	YNL247W		Cysteinyl-tRNA synthetase
YER126C	*NSA2*	Production of the 60S ribosomal subunit	YNL308C	*KRI1*	Production of the 40S ribosomal subunit
YGL099W	*LSG1*	Production of the 60S ribosomal subunit	YNR053C	*NOG2*	Production of the 60S ribosomal subunit
YGL111W	*NSA1*	Production of the 60S ribosomal subunit	YNR054C	*ESF2*	Involved in pre-18S rRNA processing
YGR162W	*TIF4631*	Production of the 60S ribosomal subunit	YOL124C	*TRM11*	tRNA methyltransferase
YGR245C	*SDA1*	Production of the 60S ribosomal subunit	YOR206W	*NOC2*	Production of the 60S ribosomal subunit
YHR052W	*CIC1*	Production of the 60S ribosomal subunit	YOR243C	*PUS7*	5S rRNA pseudouridine synthase
YHR070W	*TRM5*	tRNA methyltransferase	YOR272W	*YTM1*	Production of the 60S ribosomal subunit
YHR170W	*NMD3*	Production of the 60S ribosomal subunit	YPL093W	*NOG1*	Production of the 60S ribosomal subunit
YIR012W	*SQT1*	Production of the 60S ribosomal subunit	YPL146C	*NOP53*	Production of the 60S ribosomal subunit
YIR026C	*YVH1*	Production of the 60S ribosomal subunit	YPL212C	*PUS1*	tRNA export protein
YJL125C	*GCD14*	tRNA methyltransferase	YPL226W	*NEW1*	Production of the 40S ribosomal subunit
YJR041C	*URB2*	Ribosome biogenesis protein			

which nonessential genes were deleted and replaced with a *kanMX4* marker that is driven from the TEF promoter from *Ashbya gosypii*. The *yjr003c*Δ::*kanMX4* deletion mutant was subjected to a 37°C heat shock and monitored by reverse transcription-PCR (RT-PCR) for the expression levels of *MPP10*, *kanMX4*, *EBP2*, and *ACT1* as an internal reference control (Fig. 1). The *MPP10* gene exhibited the classic heat shock repression response, as did the RRB control gene *EBP2*. However, the exogenous *kanMX4* gene was not repressed during the heat shock, indicating that it was not subject to repression via the adjacent *MPP10* promoter.

The creation of the pCORE- and *kanMX4*-associated mutants described above involved the use of exogenous, constitutively expressed genes and promoters that are not native to budding yeast: the *kanMX4* gene is derived from bacteria, and the *URA3* gene comes from *K. lactis*. To further characterize the sequence elements that play a role in adjacent gene coregulation, we engineered mutants that involved more native and potentially regulatable gene insertions. We used the pCORE-based *delitto perfetto* approach to engineer the native *LEU2* gene and promoter from budding yeast between *MPP10* and *YJR003C* (28). The advantage of the *LEU2* insertion construct is that the *LEU2* gene could be repressed or induced by growing the cells in media containing or lacking leucine, respectively (33). We created the *LEU2* insertion strain, subjected it to heat shock, and monitored the transcript levels. When the strain was grown in synthetic complete (SC)-leucine media (i.e., under conditions where the *LEU2* gene was expressed), we again observed that the *YJR003C* gene was no longer subjected to heat shock repression. However, when the strain was grown in SC or YPD medium containing leucine, the regulated repression of *YJR003C* remained intact (Fig. 2A and B; also see Fig. S1 in the supplemental material). We monitored the expression levels of the *LEU2* gene under the two conditions and observed that *LEU2* expression levels were consistent across the time course and were 4.5-fold lower in YPD than they were in SC-leucine (see Fig. S2). To test whether these findings were related to the orientation of the *LEU2* insertion, we created another strain harboring the *LEU2* insertion in the opposite direction. We observed the same effect, namely, that the coregulated repression of *YJR003C* after heat shock remained intact under conditions where the intervening *LEU2* gene was not expressed but not when the *LEU2* gene was expressed (Fig. 2C and D).

Given that the insertion of an active, native RNA Pol II-transcribed gene was able to uncouple the regulated corepression of the *MPP10* and *YJR003C* genes, we sought to determine whether this phenomenon extended to genes that are expressed by a different RNA polymerase. We integrated an RNA Pol III-transcribed tRNA (Thr) gene between *MPP10* and *YJR003C* by the *delitto perfetto* method and monitored the expression of the genes following heat shock (Fig. 2E and F). We observed that the integration of this 0.7-kbp insert did not abrogate the regulated repression of *MPP10* or *YJR003C*. We also integrated a larger tRNA (Thr) construct between *MPP10* and *YJR003C* which contained an associated Ty element, a construct that was previously found to exhibit nucleosome boundary activity (34, 35). Again, the tRNA(Thr) Ty insert did not uncouple the regulated repression of *YJR003C* from *MPP10*. Thus, it appears that the adjacent coregulation of the

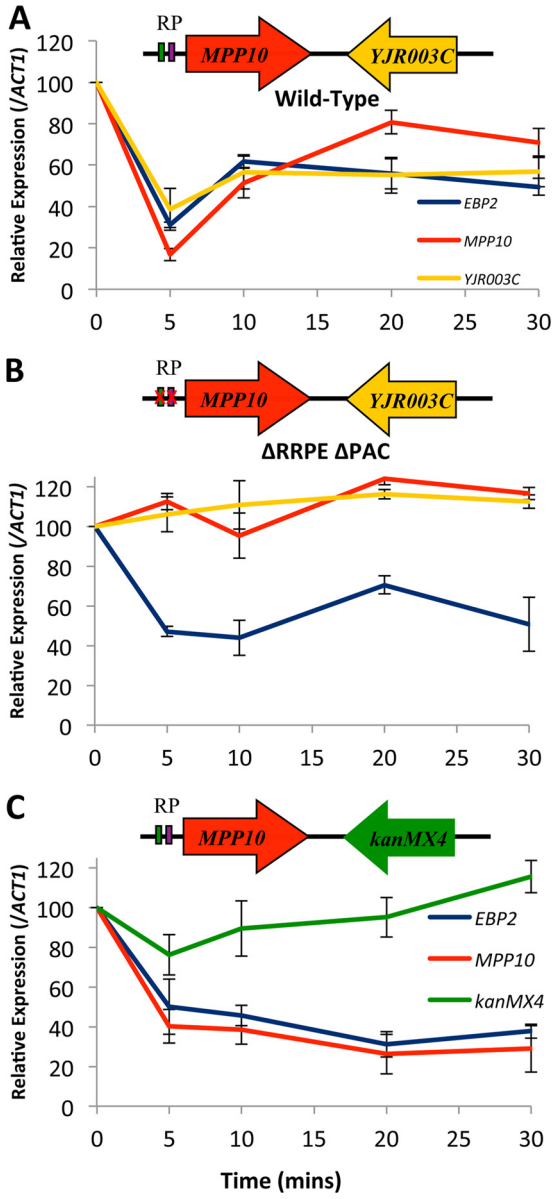

FIG 1 Relative expression profiles of RRB genes following heat shock. Strains were grown in YPD media to early log phase, subjected to a 37°C heat shock, and monitored for their expression profiles at the *EBP2*, *MPP10*, *YJR003C*, and *kanMX4* genes by RT-PCR. (A) YMM13 (WT); (B) YMM514 Δ *RPPE*/Δ*PAC*; (C) YMM557 *yjr003c*Δ::*kanMX4*.

MPP10 and *YJR003C* genes depends less upon the distance that separates them than it does on whether or not they are separated by an active, RNA polymerase II-transcribed gene.

***Trans*-acting factors related to chromatin remodeling mediate AGC.** We reasoned that in addition to depending on *cis*-acting DNA sequence elements, AGC will depend on the activity of *trans*-acting factors to couple the coregulated gene expression. In order to identify factors that may play a role in mediating AGC, we used a bioinformatics approach to survey a large gene expression data set that targeted 165 nonessential genes that have been implicated to function in regulating gene expression and chromatin architecture (29). The members of this extensive gene deletion study included factors that function in nucleosome remodeling (SWI/SNF, RSC, and INO80), histone assembly (FACT and CAF-1), histone modification (COMPASS, Rpd3L/S, NuA4, and SAGA), and transcription factors and transcriptional coactivators (Mediator). We screened the 165-gene knockout data set for mutants that preferentially disrupted the expression patterns of RRB and RP genes, as these factors would represent likely candidates for controlling their regulation (Table 5). For each knockout strain, we determined whether the expression profile of each gene in the genome deviated significantly ($P < 0.05$) from that in the wild-type strain. We then analyzed the sets of disrupted genes to determine whether they were significantly enriched for members of the RRB or RP regulons. We found that a substantial fraction of the 165 mutants did indeed preferentially disrupt the expression of the RRB genes over the other genes in the genome ($P < 0.005$), including mutants associated with the SWI/SNF, SAGA, RSC, NuA4, and Mediator complexes. Other mutants that were not associated with larger complexes also preferentially disrupted the RRB gene expression, including, as would be expected, the RRB promoter-associated PAC motif binding factor Tod6 (Table 5). The wide range of mutants that were found to affect RRB gene expression may be related to the fact that the expression of the RRB gene set is tightly controlled under a wide range of changing conditions, and that it may be subjected to multiple forms of regulation. We also identified a smaller set of mutants that exhibit preferential ($P < 0.05$) disruptions in RP gene expression, including members of the SAGA, CAF-1, and SET3 complexes. To determine whether any of the 165 mutants specifically play a role in AGC, we identified those mutants that preferentially ($P < 0.05$) disrupted the expression of any of the RRB or RP genes that were members of an adjacent pair. This analysis identified components of the same transcriptional regulators, including members of the SWI/SNF, SAGA, COMPASS, and Mediator complexes. Overall, this analysis suggests that the coordinated control of the RRB and RB genes involves multiple classes of transcriptional regulators, and that the same classes of transcriptional regulators control the expression of both the paired and nonpaired gene sets.

In order to test directly whether the candidate chromatin modifier complexes identified above function in mediating AGC, we screened a select panel of mutants for potential defects in the coregulation of the *MPP10* and *YJR003C* genes after a heat shock. We reasoned that mutations in putative coordinating *trans* factors mimic the phenotype that we observed in the *cis* mutants, namely, that the regulation of *MPP10* gene would proceed normally but the regulated repression of *YJR003C* would be compromised (Fig. 3). We chose mutants from the SAGA complex (*spt20*Δ), from the SWI/SNF complex (*snf2*Δ), and from other regulators, including chromatin remodelers (*chd1*Δ, *isw2*Δ, and *swr1*Δ) and a nucleosome assembly factor (*asf1*Δ). Each of the mutants was subjected to a heat shock, and the relative expression levels of the *MPP10*, *YJR003C*, *EBP2*, and *ACT1* genes were determined by RT-PCR. Interestingly, we did observe an uncoupling defect in the *spt20*Δ, *chd1*Δ, and *snf2*Δ mutants, since in these strains the regulated repression of *MPP10* remained intact but that of the *YJR003C* gene was lost. However, we did not see the same effect in other mutants, indicating that the activities of the Isw1, Swr2, and Asf1 proteins are not required for mediating this case of AGC. Thus, the coordinated repression of the *MPP10* and *YJR003C* genes appears to depend on the activity of the *trans* factors Snf2, Chd1, and Spt20.

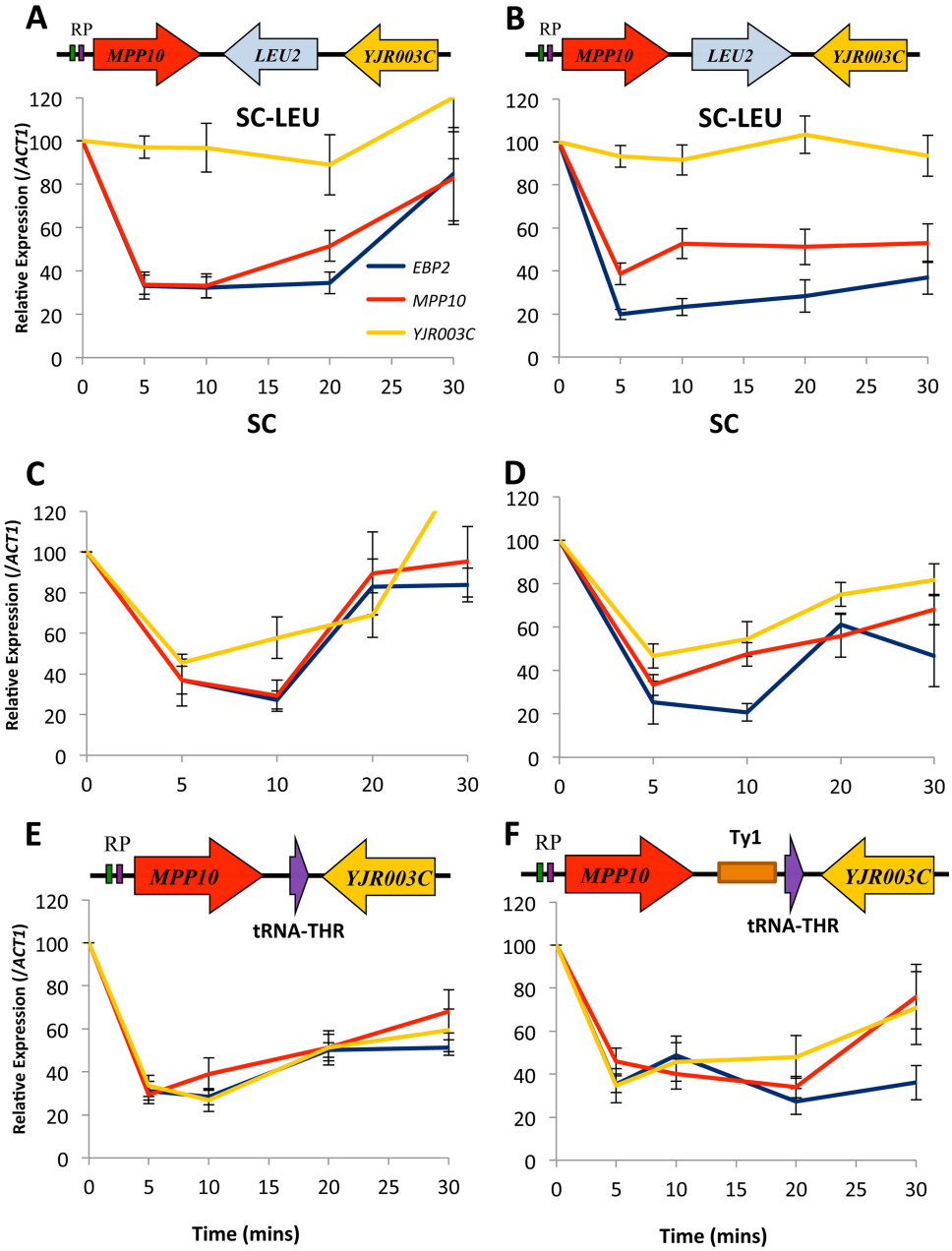

FIG 2 Insertions of active RNA Pol II-transcribed genes can abrogate AGC. Strains were grown in either SC-leucine (A and B), SC media (C and D), or YPD (E and F), subjected to a 37°C heat shock, and monitored for their expression profiles at the *EBP2*, *MPP10*, and *YJR003C* genes by RT-PCR. The profiles of the leftward-oriented *MPP10.LEU2* insert (YMM554) are represented in panels A and C, and the profiles of the rightward-oriented *MPP10.LEU2* insert (YMM559) are presented in panels B and D. (E) YMM555 *MPP10. tRNA* (*Thr*); (F) YMMM556 *MPP10.Ty tRNA* (*Thr*).

Adjacent-gene coregulation does not appear to be mediated by nucleosome repositioning. Given that the Snf2 and Chd1 proteins are components of chromatin remodeler complexes, one possible mechanism whereby AGC could be mediated is through the repositioning of nucleosomes within the respective gene promoters. Typically, the promoters of actively transcribed genes contain nucleosome-depleted regions (NDRs) that favor the association of RNA polymerase II and the initiation of transcription, and the dynamic repositioning of nucleosomes in NDRs can play an important role in regulating gene expression. To investigate whether this aspect of chromatin management plays a role in the regulated repression of the *MPP10* and *YJR003C* genes, we monitored the positions of the nucleosomes in the respective gene promoters before and after heat shock by a micrococcal nuclease sensitivity assay. As a control, we monitored the nucleosome occupancy of the *HSP104* promoter, since it has been shown previously that a temperature shift-induced activation of the gene is associated with the displacement of a particular nucleosome in its promoter (Fig. 4). We compared our nucleosome occupancy profiles with those that have been previously reported for the *HSP104* gene (36), and we did observe the displacement of a nucleosome located approximately 150 bp upstream of the *HSP104* transla-

TABLE 5 List of chromatin architecture-related deletion mutants that preferentially disrupt the expression of the indicated gene sets

Disrupted gene(s)							
RRB ($P < 0.005$)		RP ($P < 0.05$)		RRB gene pairs ($P < 0.05$)		RP gene pairs ($P < 0.05$)	
Mutant	Complex	Mutant	Complex	Mutant	Complex	Mutant	Complex
SPT20, *ADA2*, *GCN5*, *HFI1*	SAGA/ADA	*SPT7*, *GCN5*	SAGA/ADA	*SPT20*	SAGA	*MED2*, *MED15*	MEDIATOR
MED2, *MED9*, *MED15*, *MED16*	MEDIATOR	*MSI1*, *CAC1*	CAF-1	*SWD3*, *SDC1*	COMPASS	*SNF6*	SWI/SNF
NPL6, *RSC1*, *RSC2*, *RSC30*	RSC	*SET3*	SET3			*IES3*	INO80
SNF2, *SNF5*, *SNF6*, *SWI3*, *SNF12*	SWI/SNF	*RRT109*				*NOT3*	CCR4/NOT
CCR4, *NOT4*, *CAF130*, *CAF40*	CCR4/NOT						
EAF1, *EAF6*, *EAF7*	NuA4						
TOD6, *LEO1*, *SAS4*, *HIR1*, *JHD2*, *RTT109*, *SSN6*, *TUP1*, *CPR1*, *HST1*							

tional start site. The position of this nucleosome overlaps the positions of the heat shock response (HSE) and stress response promoter elements (STRE), which are the binding sites for the Hsf1 and Msn4/Msn2 transcriptional activators, respectively. The clearing of this nucleosome allows for the binding of these transcriptional regulators and the induction of transcription after heat shock.

Nucleosome occupancy levels were also monitored across the *MPP10* and *YJR003C* promoters, and in each case we could detect

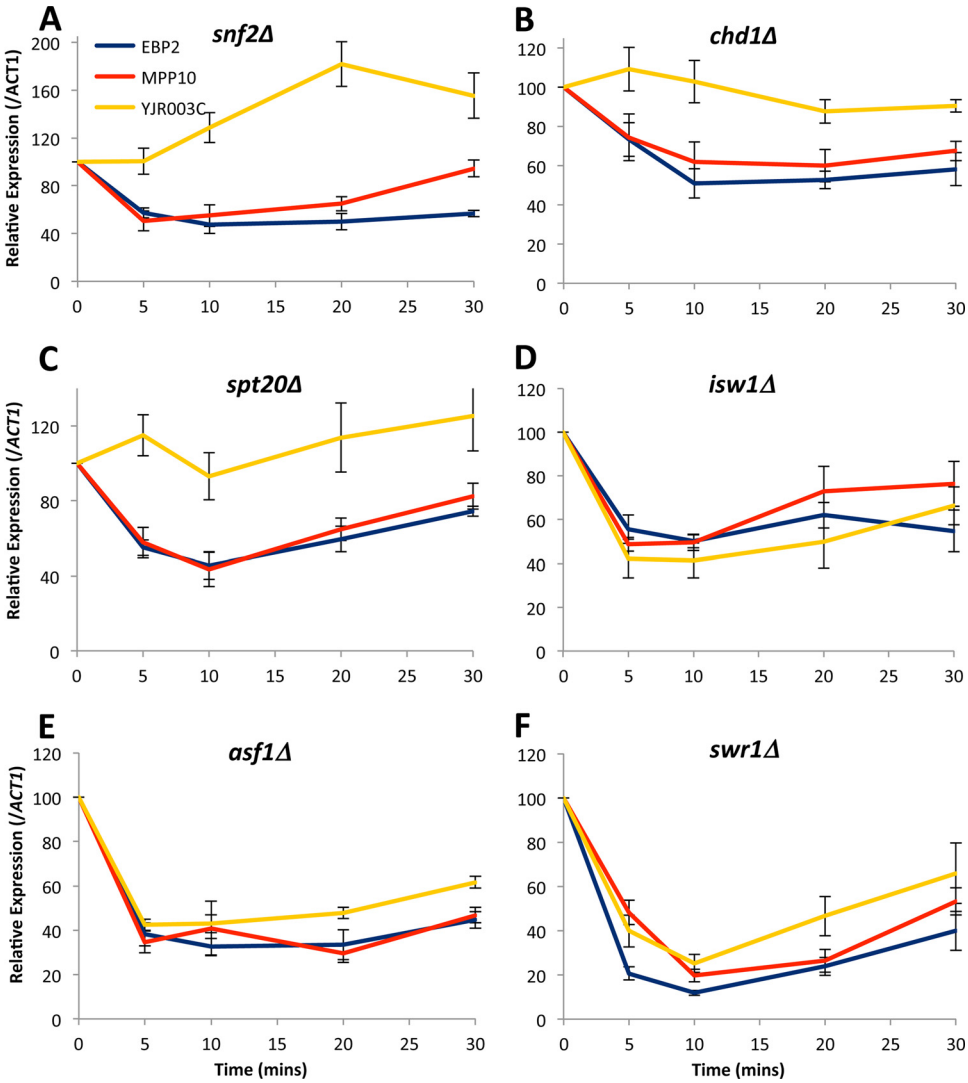

FIG 3 Mutations in *trans* factors abrogate AGC. The indicated yeast strains were grown in YPD media to early log phase, subjected to a 37°C heat shock, and monitored for their expression profiles by RT-PCR. (A) YMM593 *snf2*Δ; (B) YMM565 *chd1*Δ; (C) YMM562 *spt20*Δ; (D) YMM566 *isw1*Δ; (E) YMM595 *asf1*Δ; (F) YMM596 *swr1*Δ.

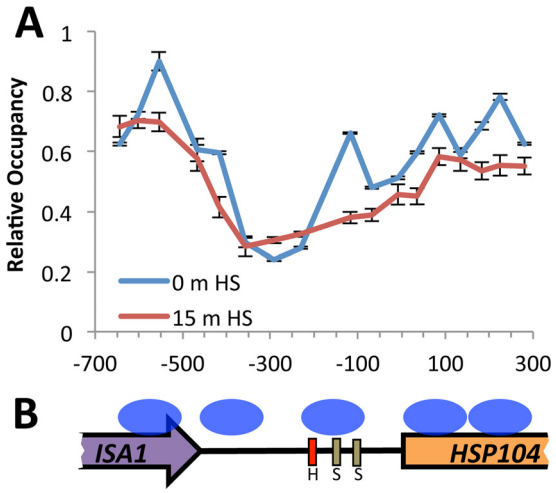

FIG 4 Stress response-associated nucleosome displacement occurs at the *HSP104* promoter. (A) Nucleosome positions were determined across the *HSP104* promoter by a nucleosome-scanning assay in the wild-type background before and after a 15-min heat shock at 39°C. (B) Previously published nucleosome positions at the *HSP104* promoter as determined by ChIP are represented as blue ovals (36). H, heat shock response element; S, stress response elements.

an NDR region that corresponded well with the nucleosome occupancy pattern detected previously (4). However, when the cells were subjected to a heat shock, we could detect no significant changes in the nucleosome occupancy profiles, as neither the *MPP10* nor the *YJR003C* promoter exhibited a significant alteration in nucleosome position (Fig. 5). We also tested the *MPP10* ΔPAC-ΔRRPE promoter mutant by this assay before and after heat shock and likewise found that this *cis* mutant showed no significant changes in the positions of its *MPP10* and *YJR003C* promoter-associated nucleosomes. As a positive control, we monitored the nucleosome occupancy profiles in the *snf2*Δ mutant, and as predicted, they were disrupted across the *MPP10* and *YJR003C* promoters (see Fig. S3 in the supplemental material). Thus, while factors associated with protein remodeler complexes are required for the coordinated repression of the adjacent *MPP10-YJR003C* gene pair, they do not appear to be affecting their control at the level of nucleosome repositioning.

DISCUSSION

The discovery and characterization of the RRB regulon (also known as the *ribi* regulon) considerably expanded our understanding concerning the classes and numbers of genes that contribute to ribosome biogenesis (10, 26, 37). Previously, it had been recognized that the expression levels of the set of 137 RP and 150 rRNA genes was subjected to tight regulatory control, albeit through different RNA polymerases (RNA Pol II for the RPs, RNA pols I and III for the rRNAs) (37, 38). The addition of the genes of the RRB regulon reveals that the overall ribosome biogenesis pathway is dependent on the coordinated expression of some 500 genes or more. This is a significant fraction of the entire yeast genome, and given that all cells must make their own ribosomes, it is reasonable to assume that all organisms will likewise contain similarly large classes of genes. Defining these gene sets will represent an important component of the gene annotation projects that are arising from the rapidly accumulating DNA sequence data sets of newly described species. Because ribosomes are highly conserved,

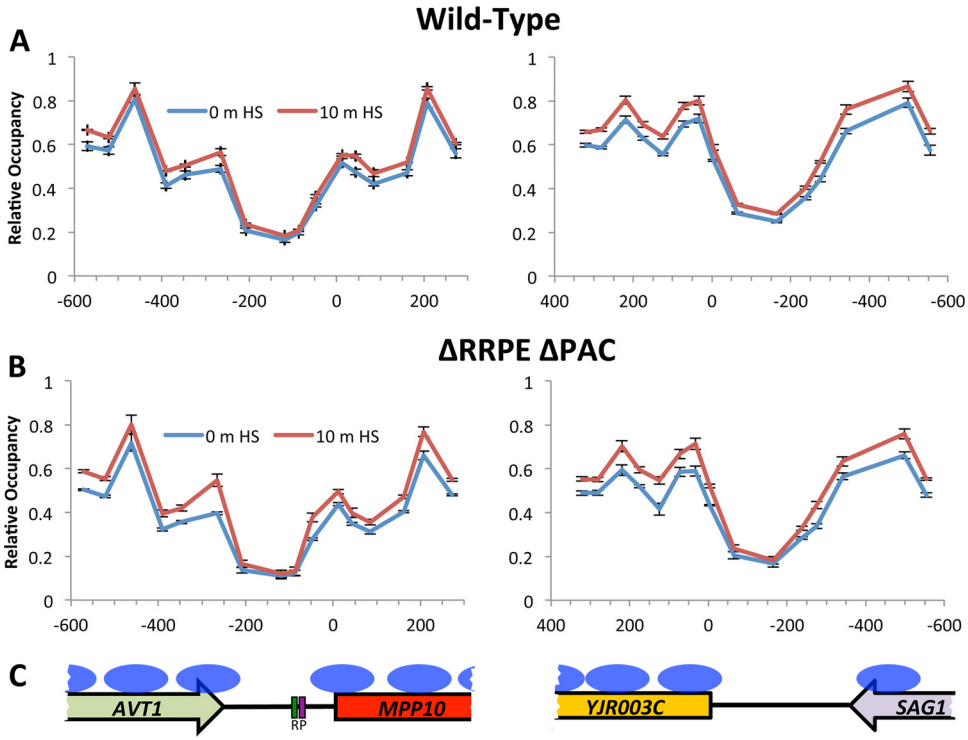

FIG 5 Nucleosome mapping at the *MPP10* and *YJR003C* promoter regions. Nucleosome positions were determined across both promoters by a nucleosome-scanning assay in the wild-type background (A) and in the YMM514 ΔRRPE ΔPAC background (B) before and after a 10-min heat shock at 37°C. (C) Previously published nucleosome positions at the *MPP10* and *YJR003C* promoters as determined by ChIP are represented as blue ovals (35).

it is relatively easy to identify rRNA and RP homologs in new species (39, 40). The validation of the approach that we used originally to predict the membership of the RRB regulon suggests that a similar approach could be successfully applied to identify RRB genes in new species, many of which may have limited other bases for gene annotations. Furthermore, the approach that we used is not limited to identifying genes associated with rRNA and ribosome biogenesis pathways, since the regulon membership was based solely on classifying genes through common promoter motifs and common transcriptional responses to changing conditions (10).

The observation that the coupled repression of the *MPP10* and *YJR003C* genes can be maintained even after they have been separated by the insertion of an exogenous 1.5-kbp DNA fragment indicates that the mechanism of coregulation is not strictly distance limited. It also argues against a model in which the two genes are coordinately regulated via colliding RNA polymerases or the interactions between overlapping 3′ untranslated region (UTR) transcripts, since it would be unlikely for the *MPP10* and *YJR003C* transcripts to extend across the additional intervening DNA. Furthermore, the finding that the coordinated repression of *YJR003C* was consistently abrogated when it was separated from the *MPP10* gene by an actively expressed RNA Pol II transcription unit (*LEU2 URA3 kanMX*) but not by an RNA Pol III transcription unit [*tRNA* (*Thr*)] indicates that disrupting the mechanism of coupling between the two genes is RNA polymerase promoter specific. This finding is consistent with the native positions of the nonpaired and paired RRB and RP genes; they were found as either isolated single genes or immediately adjacent pairs. We did not observe cases in which two RRB or two RP genes were separated by a single gene from outside the respective regulons. Again, since the relative activity, but not the relative orientation of the intervening *LEU2* transcription unit, was the determining factor in abrogating corepression, the disruption of AGC was unlikely to be mediated through the interactions of mRNA transcripts. That the inserted *LEU2* or *kanMX4* gene from the *yjr003c*Δ::*kanMX4* deletion strain did not fall under the repressive influence of the *MPP10* promoter indicates that the *YJR003C* promoter sequences are specifically receptive to repression. Defining those sequences should be as straightforward as it was to identify the relevant motifs in the *MPP10* promoter.

The identification of relevant *trans* factors also yields insight as to how AGC is mediated. The Spt20 protein is a structural component of the SAGA complex, a multisubunit histone acetyltransferase that interacts with the TATA-binding protein TBP and promotes the formation of the preinitiation complex (41, 42). Snf2 is a catalytic subunit of the SWI/SNF chromatin remodeling ATPase that can regulate gene expression by altering the positions of nucleosomes on DNA, including those that have been modified by SAGA (43, 44, 45). Chd1 is an ATP-dependent chromatin remodeling enzyme that regulates various aspects of transcription (44). It contains an Snf2/Swi2-type helicase domain and a C-terminal nucleosome-binding domain. All of these factors are known to be important transcriptional regulators, and they may contribute to AGC directly or through their association with or recruitment of other transcriptional regulators, including components of the basal transcription machinery.

One model for mediating AGC across the gene pairs is through the formation of short DNA loops that can form transiently in a transcription-dependent manner (46). Gene loops can physically bridge distant segments of DNA and, through the interactions of associated factors and complexes, bring the promoter and terminator regions of genes into close contact (46, 47, 48). Such interactions have the potential for impacting levels of gene expression, including gene silencing. The *HMR-E* and *HMR-I* silencers are separated by several kilobase pairs of DNA, yet they can be seen to physically and functionally interact *in vivo* (49). In the tandemly arranged *SNA3-INO1* gene pair, regulated inositol-induced repression of *SNA3* was mediated through Ino2/Ino4 binding proteins that recognize E-box consensus sequences not from within the *SNA3* promoter but from within the downstream (intergenic region) *INO1* promoter (50). Furthermore, a short, stable DNA loop linking the promoter and terminator regions of the *INO1* gene could be seen during activated transcription (51), and it was suggested to be formed through interactions between transcriptional activators and TFIIB. Interestingly, gene looping has even been shown to be important in the regulated expression of divergently transcribed genes, as in the case of the establishment of transcriptional memory at the *GAL1-GAL10* locus (46).

Therefore, one possibility is that the heat shock-induced corepression of *YJR003C* is mediated through a DNA loop that juxtaposes its promoter next to the promoter of *MPP10*. This physical association could transmit a repressive signal that is mediated through the recognition of the *MPP10* PAC and RRPE promoter motifs. Our analysis of the *trans* mutants suggest that the putative DNA looping arrangement between the *MPP10* and *YJR003C* promoters depends on the activity of the Snf2, Chd1, and Spt20 proteins, and our analysis of the *cis* mutants suggests that it cannot extend past another active RNA Pol II promoter. Together, the identification of relevant *cis* and *trans* elements that regulate AGC provides important insights and direction for further investigations as to how it is achieved at the molecular level.

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