Suppression of Expression Between Adjacent Genes Within Heterologous Modules in Yeast

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ABSTRACT Recent studies have shown that proximal arrangement of multiple genes can have complex effects on gene expression. For example, in the case of heterologous gene expression modules, certain arrangements of the selection marker and the gene expression cassette may have unintended consequences that limit the predictability and interpretability of module behaviors. The relationship between arrangement and expression has not been systematically characterized within heterologous modules to date. In this study, we quantitatively measured gene expression patterns of the selection marker (KlURA3 driven by the promoter, pKlURA) and the gene expression cassette (GFP driven by the galactose-inducible GAL1 promoter, pGAL1) in all their possible relative arrangements in Saccharomyces cerevisiae. First, we observed that pKlURA activity depends strongly on the relative arrangement and the activity of pGAL1. Most notably, we observed transcriptional suppression in the case of divergent arrangements: pKlURA activity was reduced when pGAL1 was inactive. Based on our nucleosome occupancy data, we attribute the observed transcriptional reduction to nucleosome repositioning. Second, we observed that pGAL1 activity also depends on the relative arrangement of pKlURA. In particular, strains with divergent promoters showed significantly different pGAL1 activation patterns from other strains, but only when their growth was compromised by lack of uracil. We reasoned that this difference in pGAL1 activation patterns arises from arrangement-dependent pKlURA activity that can affect the overall cell physiology (i.e., cell growth and survival in the uracil-depleted condition). Our results underscore the necessity to consider ramifications of promoter arrangement when using synthetic gene expression modules.

Stable expression of a heterologous gene module in prokaryotic or eukaryotic systems is widely used for various biological applications, ranging from basic gene expression studies to gene therapies (Buckholz and Gleeson 1991; Terpe 2006; Jana and Deb 2005; Desai et al. 2010; Romanos et al. 1992; Frommer and Ninnemann 1995). The typical design of a heterologous module combines two genetic components: a selection marker and a gene expression cassette that are adjacentively integrated into the genome of the desired system. The selection marker, consisting of a gene that is constitutively driven by its own promoter, allows the cell to survive a condition or environment that is otherwise deleterious. The gene expression cassette, consisting of a promoter driving the expression of the heterologous gene, can be customized to a specific goal of study. For example, the gene expression cassette may contain a reporter gene that detects gene expression, a therapeutic gene that corrects a mutated gene (Cavazzana-Calvo et al. 2000), or a pharmaceutical gene that can be produced en masse (Buckholz and Gleeson 1991). Traditionally, the gene expression cassette and the selection marker, each with its own intended function, are designed to be closely positioned, and the efficiency of heterologous gene expression is thought to be primarily determined by the strength of the promoter within the gene expression cassette.

However, the traditional view of heterologous gene expression is becoming increasingly challenged by studies demonstrating that transcriptional activity from the same promoter can be differentially affected by adjacent promoters. This phenomenon, known as promoter interaction, may be important in determining gene expression levels, particularly because proximal arrangement of genes can lead to unexpected gene expression dynamics (Shearwin et al. 2005; Mazo et al. 2007). For example, expression of a gene can be inhibited by...
adjacent transcriptional activity through a mechanism known as transcriptional interference. In transcriptional interference, RNA polymerase (RNAP) initiated from one promoter can occlude proper initiation of RNAP on the adjacent promoter (Adhya and Gottesman 1982), or RNAPs transcribing adjacent genes toward each other can lead to their collision (Callen et al. 2004; Prescott and Proudfoot 2002; Martens et al. 2004). These modes of transcriptional interference have been systematically studied with mathematical models (Palmer et al. 2009; Sneppen et al. 2005) and with synthetic gene circuits (Buetti-Dinh et al. 2009).

Interestingly, transcriptional interference alone is not sufficient to explain gene expression patterns of adjacent genes in eukaryotic genomes. For example, two gene expression cassettes inserted into the same locus in mammalian cells were shown to be more correlated than when inserted at different genomic sites, suggesting spatial extension of gene activation along the genome (Raj et al. 2006). Furthermore, a large fraction of adjacent genes in nature are shown to be temporally correlated (Batada et al. 2007; Michalak 2008; Purmann et al. 2007). In particular, divergent (pointing away from each other) genes show greater correlation in their temporal dynamics (coexpression) and in noise than those in serial (pointing in the same direction) or convergent (pointing toward each other) genes (Trinklein et al. 2004; Wang et al. 2011), suggesting arrangement-dependent coexpression between adjacent genes.

Recent studies have proposed several mechanisms to account for coexpression between divergent genes. One mechanism is inherent bidirectional transcription from a single active promoter (Xu et al. 2009) that has been demonstrated in a large fraction of promoters in yeast. Consistent with this notion, coexpression in Arabidopsis thaliana is shown to be the strongest in gene pairs whose intergenic distance is less than 400 bp, indicating the presence of a single promoter in this region (Chen et al. 2010). Intriguingly, coexpression can also be strong in serial arrangements, which suggests that an additional mechanism may counteract transcriptional interference. An attractive mechanism is shared chromatin domains that switch between euchromatin and heterochromatin states, in which adjacent genes may be simultaneously expressed or repressed (Batada et al. 2007).

Transcriptional interference and coexpression mechanisms suggest that heterologous gene expression may be subject to complex gene regulation. For example, activity of the gene expression cassette may interfere with that of the selection marker and vice versa. Furthermore, because the selection marker is often essential for cell growth, its expression level is inevitably linked with the general cell physiology. Therefore, modulation of its activity by the activity of the adjacent gene may make it difficult to interpret and predict gene expression output.

In this work, we systematically studied promoter interaction within heterologous modules. To achieve this, we rearranged the selection marker and the gene expression cassette of a commonly used heterologous module in all possible configurations of relative arrangement and direction. With these modules, we first asked how the selection marker activity is affected by the relative arrangement and the activity of the gene expression cassette. We also asked how the activity of the gene expression cassette is affected by the relative arrangement of the selection marker. In addressing the first question, we observed that the selection marker can be significantly suppressed with the gene expression cassette, but only in the divergent arrangement. To elucidate the underlying mechanism, we obtained nucleosome occupancy data that suggest repositioning of nucleosomes as a potential explanation for transcriptional suppression. In addressing the second question, we also observed unique expression patterns in the gene expression cassette only in the divergent arrangement. Such unique expression patterns are likely attributable to the effect of the selection cell marker on the overall cell physiology.

**MATERIALS AND METHODS**

**Construction of gene circuits**

All heterologous modules were derived from the plasmid pFA6a-TRP1 (Longtine et al. 1998). The entire plasmid excluding the TRP1 gene was PCR-amplified with primers GGGGGATCCGGTCGACCGTGACATTACGTCGAGATATC and GGGGGATCGTCGACGGATCCTACGTCGAGATATC. The PCR-amplified fragment contains multiple enzyme-cutting sites including BamHI and EcoRI. The KIURA3 gene containing the 189-bp-long promoter region upstream of the KIURA3 coding region was PCR-amplified with primers GGGGGATCCGAATTCATGAGGAGAGAAGAAGGAC and GGGGGATCGTCGACGGATCCTACGTCGAGATATC. This KIURA3 amplicon also contains BamHI at one end and EcoRI at the other end, and these cutting sites were used to fuse the amplicon from pFA6a-TRP1 with KIURA3 to generate pFA6a-KIURA3. Then, we PCR-amplified pGAL1-GFP-yADH1 from pFA6a-TRP1-pGAL1-GFP (Longtine et al. 1998) with primers GGGGAATTCGGTACCATCATCGTATTTAGC and GGGGAATTACCATCAGCTTATATTTAGC. This pGAL1 promoter, derived from the GAL10 promoter elements (Johnston and Davis 1984) and contains the 542 bp GAL1 promoter region upstream of its start codon. The termination signal for the ADH1 gene (yADH1) is placed downstream of the GFP protein for transcription termination. The amplified fragment contains BamHI and EcoRI at both ends and was inserted into pFA6a-KIURA3, at BamHI site to generate divergent pFA6a-KIURA3-pGAL1-GFP or serial pFA6a-KIURA3-TRP1-pGAL1, and at EcoRI site to generate convergent pFA6a-pGAL1-GFP-KIURA3 or serial pFA6a-GFP-pGAL1-TRP1-KIURA3. All PCRs were performed with high-fidelity DNA polymerase (*Pf-540L; New England Biolabs*).

**Strain preparation**

The heterologous modules were integrated into the yeast strain W303. A euchromatic region on chromosome XVI (Flagfeldt et al. 2009) was selected as the site of integration (site 20 in [Flagfeldt et al. 2009]) for minimal interference in gene expression from neighboring genomic regions. The heterologous modules were PCR-amplified with primers GTAAGTTTAAAAATTTCAATCGGAAACACAA GAGCATAAGGGTTTTCGCCAAAAATTGGAGATCTGATTATCTCA and GAGTTCTGTATTTTGTGCTTCTCTCATATGTAAGCTGAC ATCCGACCTTCTTTACCGTGACGAGCCGAGT.

The italicized sequences were used for PCR-amplifying the heterologous modules and the bold sequences represent the genomic site of integration; they overlap with the genomic DNA for homologous recombination. As controls, GTAAGTTTTAAAAATTTCAATCGGAAACACAA GAGCATAAGGGTTTTCGCCAAAAATTGGAGATCTGATTATCTCA and GAGTTCTGTATTTTGTGCTTCTCTCATATGTAAGCTGAC ATCCGACCTTCTTTACCGTGACGAGCCGAGT were used to PCR-amplify the KIURA3 gene only. To create strains with the modules integrated in the reverse direction, the italicized sequences were switched between the two primers, whereas the bold sequences remained the same. The standard LiAc/SS carrier DNA/PEG method was used for high-efficiency yeast transformation (Gietz and Schiestl 2007), and transformed colonies were selected on synthetic defined (SD) complete plates with 2% glucose minus uracil (*#100217-544; Teknova*).
The genomic integration of heterologous modules was verified by PCR amplification. The promoter regions of our divergent promoter strains were sequenced and no mutation was detected over the pKIURA and pGAL1 promoter regions (Supporting Information, Table S6).

**Culture conditions and growth assay**

Before induction, cells were grown in liquid synthetic raffinose (2%) medium with additional 0.05% glucose (minimal amount to ensure even growth between different cell strains) (Acar et al. 2010) overnight for approximately 16 hr. The next day, these cells were inoculated into fresh media for exponential growth. Cells in exponential growth phase were then re-inoculated into synthetic raffinose (2%) medium with additional 0.05% glucose lacking uracil in the absence or presence of 2% galactose. After 24 hr of growth, cells were then transferred onto a 96-well plate for growth assays (Synergy H1; Biotek). The optical density (OD$_{600}$) was measured every 30 min for 6 hr, with 4 min of linear shaking immediately before measurements.

**Quantification of KIURA3 transcript**

The total mRNA was obtained from divergent and control strains after 24 hr of galactose induction or no induction. Cells were grown in 5 ml media [synthetic raffinose (2%) medium with 0.05% glucose lacking uracil] to logarithmic growth phase. At OD$_{600}$~0.6, these cells were harvested and homogenized with TRizol (#15596-026; Ambion). Cells were vortexed with acid beads (#12621-152; VWR) for 1 hr at 4°C followed by mRNA precipitation. The precipitated mRNA was treated with DNase I (#AM2222; Ambion) and was converted to cDNA with a kit from Invitrogen (#18080-400, SuperScript III First-Strand Synthesis SuperMix). The amount of cDNA was measured by standard quantitative real-time PCR (qPCR) with a PCR master mix (#4309155; Applied Biosystem). For KIURA3 quantification, primers CCGTGGGCGTGGATATCG and GAGGTTACTGT CGTTCGATTT were used. As control, the ACT1 gene was targeted with primers TGTCAACACTGGGACGATA and AACCA GCCGAAAATTGGAACG (Kessler et al. 2003).

**Nucleosome mapping**

The standard nucleosome scanning assay was performed as described (Infante et al. 2012). Briefly, divergent and control yeast cultures (100–200 ml) were grown for 24 hr in Gal$^-$ and Gal$^+$ conditions. At OD$_{600}$~0.6, they were fixed with formaldehyde, treated with zymolase for conversion of cells to osmotically fragile spheroplasts, and digested with varying concentrations of micrococcal nuclease (catalog #M0247S; New England BioLabs). The resulting mononucleosomal DNA was isolated and purified, and then was used to map nucleosome positions in chromatin by qPCR (#4309155; Applied Biosystem). The primer sets used in this study are listed in Table S7. For each primer set, a standard curve was obtained by serial dilution of the genomic DNA for both control and divergent-promoter strains. Using the standard curves, the relative concentration of mononucleosomal DNA (normalized to the reference genomic position) was calculated.

**Statistical analysis**

Paired $t$ tests were performed with the Matlab statistics toolbox to compare the growth patterns or the nucleosome occupancy between two different strains. Each paired $t$ test was based on at least three independent experiments. The growth rates were obtained by estimating the slope of a line fit to a semi-log plot of the growth curves (Table S1). Then, these growth rates between different strains were compared for statistical significance. For nucleosome occupancy, the control strain was compared with the uninduced or induced case of the divergent strain for statistical significance. The $t$ tests with $p$-value < 0.05 are considered to show a statistically significant difference between the compared strains.

**RESULTS**

**Construction of all pairwise arrangements of elements in a heterologous module**

We first constructed heterologous modules of all possible arrangements between a selection marker and a gene expression cassette as shown in Figure 1. The selection marker consists of the URA3 promoter (pKIURA) from *Kluyveromyces lactis* and its downstream protein-coding region KIURA3. The KIURA3 gene encodes the enzyme orotidine 5'-phosphate decarboxylase responsible for the synthesis of uracil, which is one of the nucleobases for mRNA. Thus, active pKIURA is essential for cell growth in media lacking uracil (URA$^-$ condition). The gene expression cassette consists of the galactose-inducible GAL1 promoter (pGAL1) driving the expression of GFP (pGAL1-GFP). This pGAL1 promoter is derived from the divergent GAL1-10 promoter by removing a significant portion of the cis-regulatory elements of pGAL10 (126 bp upstream of the coding region of the GAL10 gene). Such removal has been shown to disable bidirectional transcription and to allow gene expression only in the 5' to 3' direction in the derived GAL1 promoter (Johnston and Davis 1984). The pKIURA and pGAL1 promoters are widely used in yeast experiments and do not share common cis-regulatory elements or trans-factors. Whereas pKIURA is expected to be always active in the Ura$^-$ condition, pGAL1 is known to exhibit a wide dynamic range, up to 1000-fold change, in gene expression level (Johnston and Davis 1984) depending on galactose induction. Thus, the effect of pGAL1 activity on pKIURA can be analyzed for a wide range of galactose concentrations. These heterologous modules were inserted into the yeast genome. The resulting yeast strains are named after the heterologous modules they carry (i.e., a divergent strain is defined as a yeast strain that carries the divergent module). As controls, we constructed strains carrying only KIURA3. All our modules were integrated at a transcriptionally “neutral” site in the yeast genome (Flagfeldt et al. 2009), where the gene expression of the inserted construct was the highest compared to other genomic sites investigated. We expected that, at this site, neighboring genomic elements would not significantly affect the expression of heterologous modules. To experimentally confirm this, we genomically integrated our heterologous modules in both 5’ to 3’ and 3’ to 5’ directions in *Saccharomyces cerevisiae*.

**Divergent arrangement leads to significantly smaller colony size**

We first grew our strains on SD agar plates lacking uracil with glucose as the carbon source. In this condition, the growth of cells depends on their ability to synthesize uracil (via pKIURA activity). Interestingly, we observed a striking difference in colony size between our strains (Figure S1A). The colony size of divergent promoter strains was significantly smaller than that of serial or convergent promoter strains. This was also shown in our serial dilution spotting assays in Figure S1B. We note that these strains are genetically identical except for the relative arrangement of KIURA3 and pGAL1-GFP. Therefore, we reasoned that the difference in colony size was because of arrangement-dependent pKIURA activity. In support of this reasoning, when our strains were grown on SD agar plates supplemented with uracil (Ura$^+$), the difference in colony size was nearly eliminated (Figure S1A). These results suggest that the amount of uracil synthesized by...
cells in the Ura− condition (or pKIURA activity) is the limiting factor for cell growth and, thus, growth rates can be used as a proxy for pKIURA activity.

For more quantitative comparison of pKIURA activity between different strains, we measured their growth rates in liquid synthetic raffinose media lacking uracil. Using the growth rate as a proxy for pKIURA activity, we first tested whether the genomic site of integration was neutral to neighboring genomic regions. Validation of “neutrality” was important to ensure that the neighboring genomic elements do not significantly affect the transcriptional activity of pGAL1 and pKIURA. Our growth assays showed that the two control strains, which differ only in the directionality of integration, exhibited similar growth curves. We quantitatively compared these strains by performing paired t tests of their growth rates (extracted from their growth curves) (Table S1). Our results showed no statistically significant difference between the two control strains (Figure S2 and Table S2). These results suggest that gene expression at this genomic site is not considerably biased by either the upstream or the downstream genomic elements, and that the neighboring genomic regions do not significantly influence the behavior of pGAL1 and pKIURA.

**Relative position of pGAL1 determines pKIURA activity**

Next, we compared basal growth patterns of all our strains in liquid synthetic raffinose media lacking uracil without galactose induction (Gal−). If pGAL1-GFP and KIURA3 were transcriptionally independent, we would not expect differences in growth patterns between strains. Consistent with this notion, our convergent and serial promoter strains showed similar growth rates as control strains (blue in Figure 2, A and B and Figure S3). Interestingly, however, we observed a significant decrease in growth rate in divergent promoter strains compared to other strains (see Table S3 for statistical analyses). We note that the observed decrease in growth rate (or repressed pKIURA activity) is unlikely to be explained by transcriptional interference via RNAP, given that pGAL1 is known to be tightly repressed when not induced (Johnston and Davis 1984). This is also evidenced by our measurement of basal GFP level without galactose induction (Figure S4). When our strains were supplemented with uracil (Ura+), they showed similar growth patterns (Figure S5), consistent with our growth assay on agar plates in Figure S1. We concluded that the relative arrangement of pKIURA and pGAL1 can account for the variable colony size and growth rate, given that the relative arrangement is the only difference between our strains.

**Activity of pGAL1 determines pKIURA activity**

We also measured changes in growth rate on pGAL1 induction by adding galactose (Gal+) (red in Figure 2, A and B and Figure S3).

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**Figure 1** Construction of all pairwise arrangements of heterologous gene expression modules. Our heterologous modules consist of two functionally unrelated genes: the KIURA3 gene from _K. lactis_ with its own promoter (pKIURA) driving the downstream protein coding region (represented by the red box) with the native flanking region (represented by the red line) and the GAL1 promoter (pGAL1) driving the expression of GFP (represented by the green box) with the ADH1 terminator signal (represented by the green line). The length of each element (in bp) is indicated. These genes are adjacently placed in various arrangements: bidirectional (transcribing away from each other), serial (transcribing in the same direction), and convergent (transcribing in the opposite direction). The control strains consist of only the KIURA3 gene. These modules were integrated in both 5’ to 3’ (top row) and 3’ to 5’ directions (bottom row) into the budding yeast genome.

**Figure 2** Modulation of pKIURA activity by galactose induction. After 24 hr of growth without uracil in the Gal− or Gal+ condition, a small portion of cells were moved to fresh media on a 96-well plate and their growth at steady state was measured for 6 hr at 30-min intervals with a plate reader. (A) Growth curves, as measured by OD600, of the designated strains. Control strains grown in either condition are plotted as black lines with square boxes for comparison. The growth curves in the Gal− or Gal+ conditions are denoted by blue or red lines with square boxes, respectively. A representative set of experiments is shown here. Additional set of growth experiments can be found in Figure S3. The top and bottom panels differ in the direction of genome integration. The growth curves reflect “raw” OD600 measurements on a plate reader and are not corrected by the baseline OD600. (B) Growth rates extracted from the growth curves. The error bars represent at least three independently measured growth rates. The growth rates in the Gal− or Gal+ condition are denoted by blue or red squares, respectively.
this condition, we observed three distinct growth patterns. First, we observed a minor increase in growth rate on galactose induction (in convergent and control strains). This was expected for all cell strains because galactose, being a carbon source, can improve overall cell growth. However, our serial promoter strains did not show a significant change in growth rate between Gal- and Gal+ conditions, reminiscent of transcriptional interference in the serial arrangement of promoters (Shearwin et al. 2005; Sneppen et al. 2005). Finally, we observed an increase in growth rate in divergent promoter strains, and this increase was significantly larger than the minor increase attributable to galactose addition (Table S3). We discuss potential explanations in the Discussion section. These results together suggest that pGAL1 activity can control pKIURA activity when divergently arranged.

Thus far, we have assumed that growth rate can be used as a proxy for pKIURA activity, and that pKIURA activity can be extrapolated from the growth rate. To validate this assumption, we quantified the KIURA3 transcript level in the same experimental conditions as growth assays, in the absence of galactose and in the presence of 2% galactose (Table S4). Consistent with our growth assays, our results showed that pKIURA activity in divergent promoter strains is nearly halved in comparison to the control strains without galactose and doubled with galactose induction. Our growth assay and KIURA3 transcript quantification results demonstrate that pKIURA transcription level is positively correlated to the growth rate, although the relationship may be nonlinear.

Modulation of pKIURA activity by pGAL1 may be mediated by repositioning of nucleosomes

To explain transcriptional suppression, we measured nucleosome occupancy over pKIURA in Gal- and Gal+ conditions. First, we mapped nucleosome occupancy over pKIURA in the control strain (Figure 3A). In the control strain, we did not expect galactose to have an effect on the nucleosome occupancy over pKIURA. As expected, our results did not show a significant difference between Gal- and Gal+ conditions (Figure S6). The nucleosome occupancy pattern resembles that of its orthologous UR3A promoter (Jansen et al. 2012): strongly positioned -1 and +1 nucleosomes of ~147 bp surrounding the nucleosome-depleted region (NDR) of ~100 bp. The -1 nucleosome is positioned over the genomic region immediately next to the NDR region of pKIURA, and the +1 nucleosome is centered around the start codon of the KIURA3 gene. The putative TATA signal (Mizukami and Hishinuma 1988) resides immediately next to pGAL1 (~185 bp upstream of the start codon of KIURA3).

Then, we also mapped nucleosome occupancy over pKIURA in the divergent strain and performed statistical analysis to compare with the control strain in Gal- and Gal+ conditions (Figure 3B and Table S5). Without galactose, our results showed that the divergent placement of pGAL1 generally increases nucleosome occupancy over pKIURA (blue line in Figure 3B) relative to the control strain. In particular, we observed a significant increase in the nucleosome occupancy over the putative TATA signal. Such increase in nucleosome occupancy is generally associated with transcriptional repression (Shivaswamy et al. 2008) and may account for the repressed pKIURA activity in the Gal- condition. With galactose induction, the nucleosome occupancy over pGAL1 is substantially lowered (red line in Figure 3B), resulting in exposure of the TATA signal on pKIURA. Such exposure would “restore” pKIURA activity to the level observed in the control strain but is insufficient to account for the two-fold increase in the KIURA3 transcript level. Possible explanations are provided in the Discussion section.

pGAL1 activation patterns in divergent arrangements are significantly different from those in other cases

Finally, we measured pGAL1 activity in varying concentrations of galactose in the Uracil condition (top histograms in Figure 4). The galactose signaling pathway is known to switch on in an all-or-none manner (Acar et al. 2005). Consistent with this, pGAL1 activity remained low, with all cells expressing only the basal level of GFP without galactose induction. With increasing galactose concentration, pGAL1 activity in all our strains switched from the low mode to the high mode of GFP (ON cells) in a bimodal manner. We separated the
low and high modes of GFP using a Gaussian Mixture Model and calculated the fraction of ON cells (Figure S7A) and their mean GFP level (Figure S7B). Interestingly, we observed that \textit{pGAL1} activation patterns in divergent promoter strains are significantly different from other strains. For example, only \(\sim25\%\) of divergent cells showed \textit{pGAL1} activation at 0.2% galactose, whereas \(\sim75\%\) of serial and convergent cells showed \textit{pGAL1} activation at the same galactose concentration. Furthermore, the mean GFP level was nearly twice as high in divergent promoter strains as in other strains.

We suspected that the observed difference in \textit{pGAL1} activation patterns may be attributable to different growth rates arising from arrangement-dependent \textit{pKIURA} activity. To test this, we measured \textit{pGAL1} activity of all our strains in the Ura\(^+\) condition (bottom panels in Figure 4). In this condition, we showed that the growth rate is nearly identical between strains (Figure S5). With uracil supplemented, the difference in the fraction of ON cells and their mean GFP levels was nearly eliminated (bottom histograms in Figure 4 and Figure S7, A and B). Interestingly, similar \textit{pGAL1} activation patterns were also observed without uracil supplemented in the growth media, as long as cells were grown at a similar rate with sufficiently strong galactose induction. Such growth rate-dependent \textit{pGAL1} activation patterns illustrate how the relative arrangement of \textit{pGAL1} and \textit{pKIURA} can fine-tune the gene expression dynamics of both promoters.

**DISCUSSION**

We experimentally demonstrated that the relative arrangement of \textit{pKIURA} and \textit{pGAL1} has a profound impact on gene expression dynamics. Most notably, we observed that the activity of \textit{pKIURA} can be altered when arranged with \textit{pGAL1} in the divergent configuration. Compared to its normal level without \textit{pGAL1}, the \textit{pKIURA} activity is reduced when \textit{pGAL1} is not induced. This reduction may be attributable to repositioning of nucleosomes in the \textit{KIURA3} promoter region as a result of \textit{pGAL1} arrangement. This idea is supported by previous studies performed by other groups. In one study, it was shown that \textit{pGAL1} contains nucleosome-phasing elements (Floer \textit{et al.} 2010) that may reposition neighboring nucleosomes. In another study, when \textit{pURA3} was placed in various genomic contexts, the “strongly” positioned nucleosomes were either repositioned or even removed (Jansen \textit{et al.} 2012). These results suggest that nucleosome positioning strongly depends on the nearby genomic elements. Consistent with these studies, our nucleosome mapping also showed that a nucleosome becomes repositioned closer to the putative TATA box of the \textit{KIURA3} promoter without galactose induction, likely accounting for reduced \textit{pKIURA} activity.

In the same divergent configuration, we also observed that the \textit{pKIURA} activity is higher than normal when \textit{pGAL1} is induced. This increased \textit{pKIURA} activity with galactose induction can be accounted for by the recruitment of \textit{trans}-factors involved in \textit{pGAL1} activation in the vicinity of \textit{pKIURA}. This idea is supported by the fact that \textit{pGAL1} is taken from the divergent \textit{pGAL1-10} promoter that is known to be intrinsically bidirectional in its transcriptional activity. These \textit{trans}-factors can be general transcription factors, chromatin remodeling complexes, or histone-modifying complexes. Widespread bidirectional transcription among yeast promoters is also a possibility (Xu \textit{et al.} 2009; Churchman and Weissman 2011; Trinklein \textit{et al.} 2004; Chen \textit{et al.} 2010). Further experimental investigation is warranted to identify the \textit{trans}-factors that spread from \textit{pGAL1} to the \textit{pKIURA}.

The activity of \textit{pGAL1} was also different in divergent promoter strains compared to serial and convergent promoter strains. However, the change in \textit{pGAL1} activity only occurred in uracil starvation and was more complicated than would be expected from simple crosstalk between promoters; whereas the fraction of ON cells decreased, the mean expression level of the ON cells increased (Figure S7). Given nearly identical GFP activation patterns when cells grow at similar rates (either by uracil supplement or by strong galactose induction), we suggest that the unique GFP activation patterns are likely attributable to the global effects of growth rate on gene expression (Klumpp \textit{et al.} 2009). These results suggest that the relative arrangement between the selection marker and the gene expression cassette is an important parameter in the design of heterologous gene expression modules.

Regardless of the exact mechanism, we showed that two functionally unrelated promoters can be transcriptionally coupled in the divergent arrangement. It is worth noting that naturally occurring divergent promoters are distinguished from our divergent promoters in that they may belong to similar categories of gene function (Wakano \textit{et al.} 2012) and often share common \textit{cis}-regulatory or \textit{trans}-regulatory elements (Trinklein \textit{et al.} 2004). The mode of interaction in divergent promoters can be quite diverse. Coexpression can be explained by bidirectional transcription attributable to various factors, including common \textit{cis}-regulatory elements (in \textit{MAL6T-MAL6S}) (Bell \textit{et al.} 1995) or chromatin organization (in \textit{UGA3-GLT1}) (Ishida \textit{et al.} 2006). In contrast, some studies reported no dependence on gene orientation (Bae \textit{et al.} 2008) and others reported anticorrelated gene expression between divergent genes (Lin \textit{et al.} 2007). Multiple factors may account for the differences in how divergent promoters behave, including the structure and properties of promoters (i.e., noisy vs. robust or strong vs. weak) and how they are expressed in the cell.
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(i.e., genomically vs. from plasmids). In addition, the genomic site
of integration may be an important parameter that governs whether
the inserted promoters can affect or be affected by the neighboring
genes. Therefore, generalization of transcriptional coupling would
require rigorous and systematic characterization of promoters and
experimental conditions.

In summary, we propose that any inducible promoter with elements
that can affect its neighboring nucleosome positioning can be
divergently fused to modulate its adjacent promoter activity. In
fact, transcriptional coupling with an inducible promoter may be an
alternative design principle for efficient control of gene expression
with unique benefits. By externally controlling the chromatin state of
pGAL, we observed “overexpression” and “knockdown” of the target
gene (KU3A in our study) (Figure 2), which are approaches com-
monly used to study gene functions. The unique benefits of this
method are that the expression of the target gene can be driven by
its own native promoter, and its activity can be externally modulated
around its native activity level. In other words, a constitutive promoter
can be engineered to behave as an inducible promoter when
divergently fused with an inducible promoter. Moreover, two inducible
promoters that are divergently positioned may generate nontrivial
gene expression patterns. Consistent with this notion, recent theoret-
ical and bioinformatics studies suggested that divergent arrangement
of two genes in proximity may be a noise-reducing mechanism (Wang et al. 2011; Woo and Li 2011). Mathematical models will be necessary
to improve design and characterization of such promoter interactions.

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