Interallelic interaction and gene regulation in budding yeast

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In *Drosophila*, homologous chromosome pairing leads to "transvection," in which the enhancer of a gene can regulate the allelic transcription in *trans*. Interallelic interactions were also observed in vegetative diploid budding yeast, but their functional significance is unknown. Here, we show that a *GAL1* reporter can interact with its homologous allele and affect its expression. By ectopically inserting two allelic reporters, one driven by wild-type *GAL1* promoter (WT *GAL1pr*) and the other by a mutant promoter with delayed response to galactose induction, we found that the two reporters physically associate, and the WT *GAL1pr* triggers synchronized firing of the defective promoter and accelerates its activation without affecting its steady-state expression level. This interaction and the transregulatory effect disappear when the same reporters are located at nonallelic sites. We further demonstrated that the activator Gal4 is essential for the interallelic interaction, and the transregulation requires fully activated WT *GAL1pr* transcription. The mechanism of this phenomenon was further discussed. Taken together, our data revealed the existence of interallelic gene regulation in yeast, which serves as a starting point for understanding long-distance gene regulation in this genetically tractable system.

homologous pairing | interallelic interaction | interallelic regulation | transvection | single-cell gene expression

Cell proliferation and differentiation depend on rigorously controlled gene activities. The mechanism of gene regulation is best understood at the level of linear organization of the genome, including the primary DNA sequences and arrays of closely associated regulatory proteins. Three-dimensional (3D) organization of chromosomes also plays an important role in gene regulation. For example, distant *cis*-regulatory elements can be looped to the target promoters and regulate the corresponding genes, affecting both the average expression level and the cell-to-cell variability (1–4). In yeast, promoters and terminators can form loops to allow rapid reactivation of the gene following a period of repression (5, 6). Highly transcribed genes tend to associate with nuclear pore complex, and this association helps to maintain the transcriptional memory (7–9). Elucidating the regulatory roles of high-order chromosome organization is thus a key step toward the understanding of eukaryotic gene regulation.

One type of high-order interaction involves pairing between homologous chromosomes in somatic diploid or polyploid cells. Homologous pairing is most prominent in the salivary gland cells in *Drosophila*, where many copies of homologous chromosomes bundle along their entire length to form a polytene chromosome. Such pairing underlies a phenomenon called "transvection," in which the regulatory region of a gene influences the transcription of the paired gene in "*trans*" (10, 11). For a pair of essential genes, one with a defective open reading frame (ORF) and the other with a defective enhancer, the functional enhancer can activate the WT ORF in *trans* and lead to phenotypic rescue (10, 11). These observations demonstrate the physiological importance of transvection.

Homologous pairing was also observed in diploid budding yeast during vegetative growth, although the extent of the pairing is controversial among literature (12–15). Unlike the *Drosophila* homologous chromosomes that stably associate with each other, the yeast chromosomes tend to interact more dynamic and sporadic at interstitial locations (12, 14). Similarly, in interphase mammalian cells, homologous chromosomes occupy different territories (16), but discrete regions on these chromosomes may loop out of the territories and interact with each other dynamically (17–20). Whether such dynamic interactions play a role in gene regulation is not clear.

In this study, we picked the promoter of a galactose metabolism gene *GAL1* (*GAL1pr*) as a model to investigate the potential interallelic *trans*-regulatory effect in budding yeast. According to a statistical analysis of the Hi-C data, the Gal4 binding sites (*GAL1* activator) cluster in the nucleus (21). β-estradiol–induced *GAL1pr* on two different chromosomes could form *trans* interactions under the activating condition in haploid yeast (22). The upstream activation sequence (UAS) of *GAL1pr* was able to activate transcription in *trans* when introduced into the *Drosophila* genome (23). Based on this evidence, we reasoned that *GAL1pr* is a good candidate for long-distance gene regulation. Indeed, our findings below support the notion that *GAL1* promoter can interact with its homologous allele and influence its activity during galactose induction.

Results

The Activation of a Defective *GAL1* Promoter Is Accelerated by a Wild-Type Allele. To mimic the classic transvection experiment, we generated two reporter genes, one with a truncated *GAL1pr* (*Δ1pr*) driving WT *GFP*, and the other with WT *GAL1pr* driving inactive *gfp* (*GFP* with frame-shift mutation) (Fig. 1*A* and *Methods*). The WT *GAL1pr* contains the entire *GAL1-10* intergenic region and is flanked by a 170-bp segment of the *GAL10* ORF from the 5′ end (but not the rest of the *GAL10*). The *Δ1pr* deletes one of the four Gal4 binding sites and a few putative Rsc3 binding sites and shows delayed activation during galactose induction (24). We inserted these reporters either as a single copy (WT-*GFP*/- and *Δ1pr-GFP*/-) or as alleles (WT-*gfp*/*Δ1pr-GFP*) into a ChrII location in diploid yeast (*Methods*). This location was chosen to be far away from the

Significance

Homologous pairing occurs in budding yeast during vegetative growth, but its function in gene regulation is unknown. In this work, we revealed that a *GAL1* reporter gene can interact with its homologous allele and influence its activity. These results are significant because (*i*) it shows that the 3D organization of yeast chromosomes can contribute to gene regulation, (*ii*) it goes against the dogma that gene regulation in budding yeast only occurs over short distance via *cis* regulatory elements, and (*iii*) it contributes to the mechanistic understanding of long-distance gene regulation in yeast, as well as related phenomena in higher eukaryotes.

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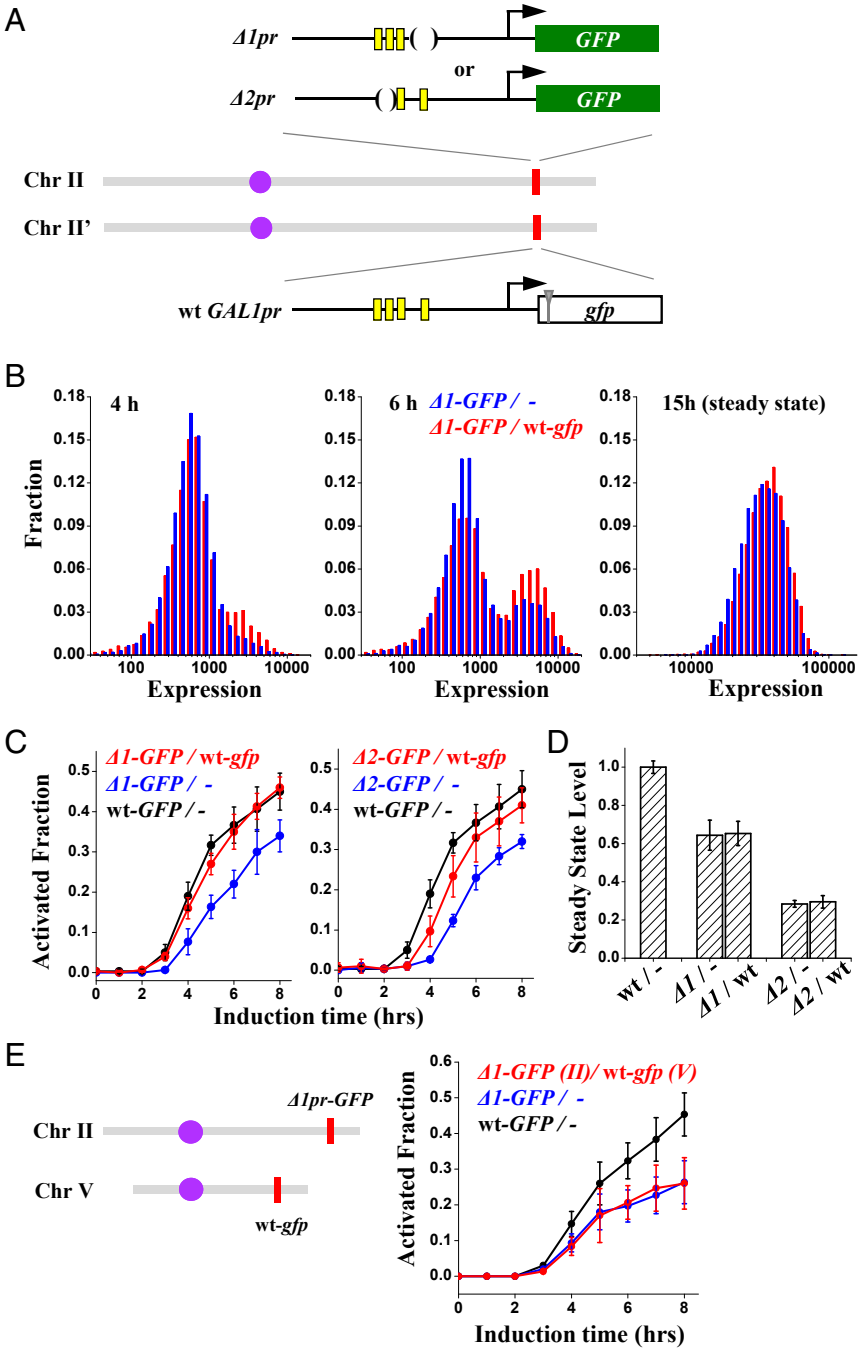

Fig. 1. Interallelic gene regulation measured by FACS. (*A*) Strain constructs. We constructed diploid strains containing WT *GAL1pr* driving *gfp* (*GFP* with frame-shift mutation) and mutant promoter (*Δ1* or *Δ2*) driving WT *GFP*. The two reporters were integrated at allelic loci on ChrII. Yellow bar, Gal4 binding site; parentheses, deleted sequence; arrow, TSS; red bar, integration site; purple circle, centromere. Same notations apply to the figures below. (*B*) Histograms of *Δ1pr-GFP* FACS data at three time points of induction. *Δ1pr-GFP/-*, diploid cells with one ChrII containing *Δ1pr-GFP* and the other intact; *Δ1pr-GFP*/wt-*gfp*, diploid cells with one ChrII containing *Δ1pr-GFP* and the other containing wt-*gfp* at the allelic locus. To enhance the difference between the WT and mutant promoter, the stains were induced with 3% galactose + 2% raffinose + 0.1% glucose (for the 4 h and 6 h data). For the steady-state measurement, the strains were induced with 3% galactose + 2% raffinose (no glucose) for 15 h. (*C*) Fraction of activated cells containing *Δ1pr-GFP* or *Δ2pr-GFP* in the presence or absence of the allelic WT *GAL1pr-gfp*. (*D*) The steady-state level of *GFP* expression in these strains (normalized by that of WT *GAL1pr-GFP*) (*D*). Note that the induction rate was increased, but not the steady-state level. (*E*) The same as in *C* except that the *Δ1pr-GFP* and WT *GAL1pr-gfp* are at nonallelic location.

endogenous *GAL1-10* locus and other galactose-related genes so that the insertion would not perturb the galactose metabolism. Indeed, these strains have the same growth rate as wild type in both glucose and galactose media.

We induced the *GAL1pr* by switching the media from glucose to galactose [3% (wt/wt)] + raffinose [2% (wt/wt)] + glucose (0.1%) and measured the *GFP* intensity using flow cytometry (FACS) at different induction times. The small amount of glucose was used to slow down the induction and enhance the difference in the activation rates between the WT and mutated *GAL1pr*. Consistent with previous reports that *GAL1pr* turns on stochastically in individual cells (25, 26), *GFP* intensity showed an "on or off" bimodal

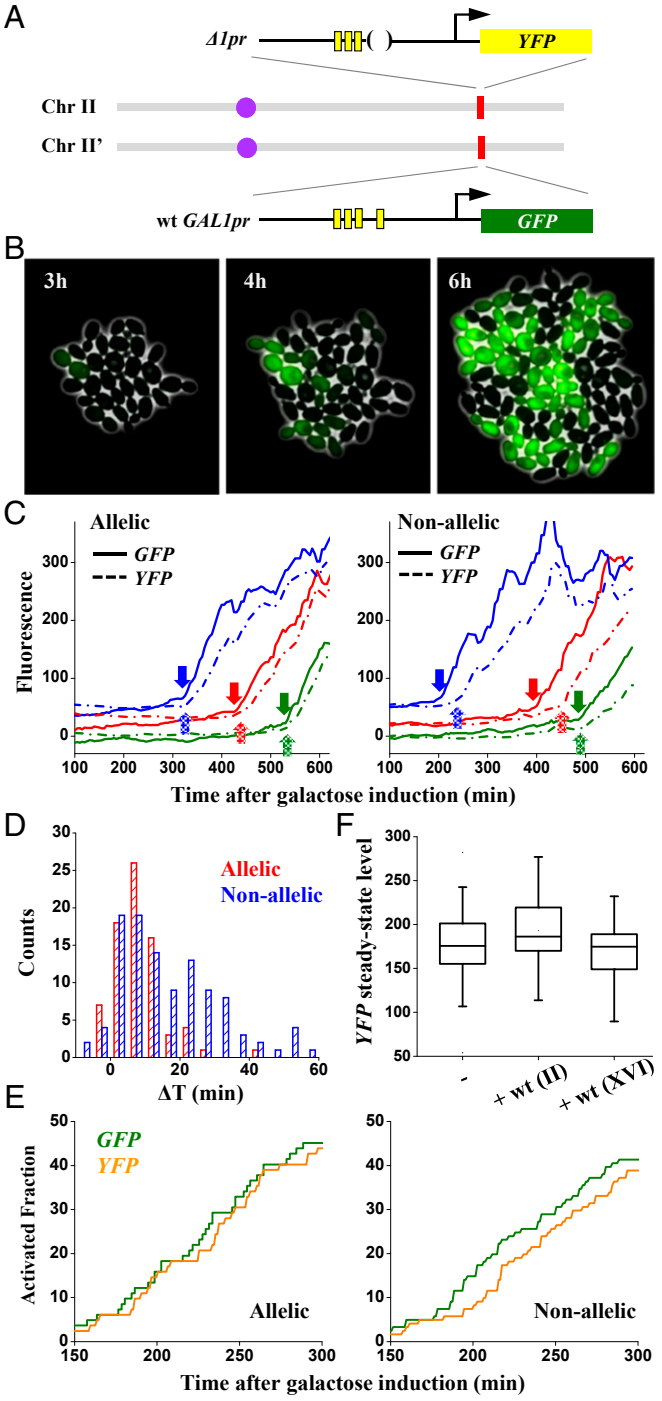

Fig. 2. Interallelic gene regulation measured by time-lapse fluorescence microscopy. (*A*) Strain constructs. The diploid strains contain *Δ1pr-YFP* and WT *GAL1pr-GFP* at allelic (II/II′) or nonallelic (II/XVI) locations. (*B*) Time-lapse live-cell imaging of the allelic strain during induction with 3% galactose + 2% raffinose + 0.05% glucose. (*C*) *GFP* and *YFP* intensity (after cross-talk elimination) as a function of time in three single cells, each with a different color. The solid/stippled arrows point to the activation time in the *GFP*/*YFP* traces, respectively. (*D*) Histogram of the difference in *GFP* and *YFP* activation time (ΔT) in the allelic vs. the nonallelic strain. The activation of *GFP* and *YFP* are more synchronized in the allelic case. Total trace number: allelic, 82; nonallelic, 128. (*E*) Activated fraction as a function of time. The plot shows the cumulative distribution of the activation time derived from the single-cell traces in the allelic (*Left*) and the nonallelic (*Right*) strain. The *GFP* activation follows similar rate in these two strains, but *YFP* activation is faster in the allelic case. (*F*) Steady-state *YFP* intensity in *Δ1pr-YFP(II)/-*, *Δ1pr-YFP(II)/wt-GFP(II)*, and *Δ1pr-YFP(II)/wt-GFP(XVI)* strains. The box plot shows the distribution of the steady-state level in single cells, which was quantified as the average *YFP* intensity >4 h after activation.

distribution before reaching the steady state (Fig. 1*B*, 4 h and 6 h). At each time point, we quantified the activated fraction by fitting the distribution with two skewed Gaussians.

As a single copy (*Δ1pr-GFP*/-), *Δ1pr* showed both slower activation and lower steady-state expression comparing with the WT (Fig. 1 *B*–*D*) (24). However, in the presence of allelic WT *GAL1pr-GFP* (*Δ1pr-GFP*/WT-*gfp*), the activation of *Δ1pr-GFP* was accelerated to a near-wild type level (Fig. 1 *B* and *C*). In contrast, there was no significant change in the steady-state level with or without the WT allele (Fig. 1*D*). Similar results were obtained with another mutant *GAL1* promoter where two of the four Gal4 binding sites were deleted (*Δ2pr*) (Fig. 1 *A*, *C*, and *D*). Importantly, when we inserted the two reporters into nonallelic locations by keeping *Δ1pr-GFP* on ChrII and moving WT-*gfp* to ChrV, this effect disappeared (Fig. 1*E* and *Methods*). Therefore, this *trans*-regulatory effect is sensitive to the relative location of the two reporters.

The Activation of *Δ1pr* Is Highly Synchronized with the WT Allele in Each Single Cell. A "snap-shot" method like FACS cannot follow gene activation dynamics in single cells. To visualize the acceleration effect in real time, we performed time-lapse live cell imaging in strains containing *Δ1pr-Venus* (a version of *YFP*) and/or WT *GAL1pr-GFP* at allelic or nonallelic locations (Fig. 2*A*). *GFP* and *Venus* have comparable brightness and maturation rate in yeast, allowing us to directly compare their intensity (27, 28). Using a flow cell device that allows rapid media exchange, we pregrew the cells in glucose, switched to galactose + raffinose + 0.05% glucose, and monitored the *GFP* and *YFP* intensity during the induction process (Fig. 2*B* and *Methods*). We then mathematically removed the cross-talk between the *GFP* and *YFP* fluorescence and analyzed their activation dynamics separately (*Methods*).

Consistent with previous reports (25, 26), we observed a large cell-to-cell variability in *GFP* and *YFP* activation: Some cells were turned on within 3 h after the media exchange, and others remained "off" for 8 h. However, when the two reporters are at allelic sites, the activation of *GFP* and *YFP* in the same cell are almost perfectly synchronized (Fig. 2 *C*–*E*). For nonallelic reporters, their activation timings are also correlated, but the *Δ1pr-YFP* activation tends to have a 20- to 60-min delay relative to WT *GAL1pr-GFP*, so the activation curve of *YFP* lags behind that of *GFP* (Fig. 2 *C*–*E*). We also analyzed the steady-state *Δ1pr-YFP* activity (*YFP* intensity > 4 h after activation), and they are essentially the same with or without the allelic WT copy (Fig. 2*F*). Overall, the data in Figs. 1 and 2 indicate that (*i*) the main rate-limiting step of the *GAL1pr* activation is the switch of the *GAL* network that affects Gal4-regulated genes globally, (*ii*) *Δ1pr* has an intrinsic slower response to the global *GAL* activation signal, and (*iii*) WT *GAL1pr* allele causes a faster response of *Δ1pr* by triggering synchronized transcription without affecting the steady-state *Δ1pr* activity.

Two Allelic Reporters Interact in *trans* Under the Inducing Condition. For WT *GAL1pr* to influence the activation of *Δ1pr*, they presumably need to form physical interactions. To directly probe such *trans* interaction, we performed a chromosome configuration capture (3C) measurement between either allelic or nonallelic *Δ1pr* and WT *GAL1pr* in both glucose and galactose media (Fig. 3*A* and *Methods*). We also carried out the same measurement by using two *cis* segments 13 kb apart on ChrXV as a positive control (22).

As shown in Fig. 3 *B* and *C*, the 3C signal was only detected between the allelic *Δ1pr* and WT *GAL1pr* under the inducing condition, whereas the control signal remained constant. When

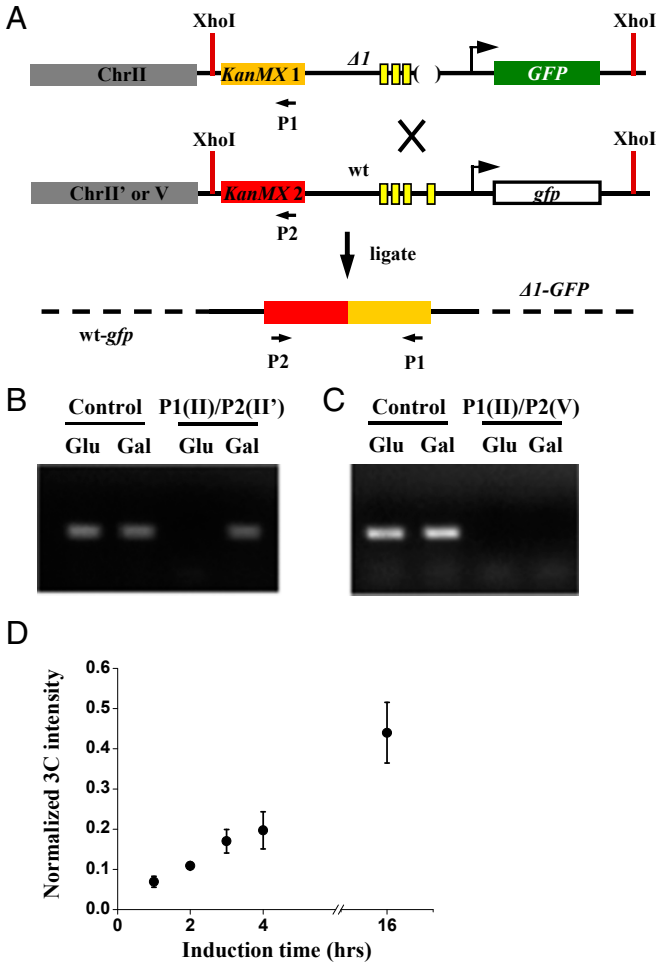

Fig. 3. Inducible *trans* interaction between *Δ1* and the WT *GAL1* promoter. (*A*) Design of the 3C assay. Two flanking *KanMx* segments and XhoI cutting sites were inserted for PCR purpose. Cross-linked chromosomes were digested with XhoI, ligated, and subjected to PCR between primers P1 and P2. The two alleles were either inserted at allelic (II/II′) or nonallelic loci (II/V). (*B* and *C*) Representative gel showing the result of the 3C assay. The P1/P2 PCR product was only visible between alleles after galactose induction. The interaction between two *cis* regions on ChrXV was detected as a positive control. (*D*) The intensity of the P1/P2 3C signal as a function of induction time. The intensity was quantified by qPCR and normalized by the 3C signal of the positive control.

we repeated the 3C measurements between the allelic *Δ1pr-GFP* and WT-*gfp* at different induction times and quantified the 3C signal with quantitative PCR (qPCR), we found that the interaction gradually increased over time and continued to be present after *GAL1pr* reached the steady state (Fig. 3*D* and *Methods*). These results show that, at least among the integration sites we selected, the chromosomal interaction can only be established between two allelic reporters. The interaction strength is well-correlated with transcription, indicating that the association may be mediated by proteins involved in *GAL1pr* activation.

The Gal4 Binding Is Required for the Interaction Between the Two Alleles, but Not Sufficient for the Transactivation Effect. *GAL1pr* activation starts with the binding of transcription activator Gal4. To determine whether Gal4 binding is required for the interallelic interaction and *trans* regulation, we constructed a *GAL1pr* with all four Gal4 binding sites deleted (*Δ4pr*) and integrated the *Δ4pr-gfp* and *Δ1pr-GFP* into allelic locations on ChrII (same location as in Fig. 1*A*). The *Δ4pr* does not bind to Gal4 and has no galactose-induced activity (29). The 3C signal was completely abolished between these two alleles under the activating condition (Fig. 4*A*), demonstrating that Gal4 is indispensable for the interallelic interaction. No acceleration effect was observed for *Δ1pr-GFP* in the presence of allelic *Δ4pr-gfp* (Fig. 4*A*).

The absence of the interallelic interaction and regulation between *Δ1* and *Δ4* promoters may be due to the loss of Gal4 binding per se or the loss of transcription. To differentiate between these two possibilities, we mutated the TATA box of the WT *GAL1pr* (*ΔTATApr*) to selectively abolish the transcription without affecting the Gal4 binding. This particular TATA mutation was shown to severely decrease the *GAL1pr* activity (29), and our FACS measurement of the *ΔTATApr-GFP*/- strain confirmed that the steady-state *GFP* level driven by the *ΔTATApr* is less than 10% of that by the WT *GAL1pr*. Interestingly, allelic *ΔTATApr-gfp* and *Δ1pr-GFP* can still form physical contacts, but *Δ1pr-GFP* activation is no longer accelerated (Fig. 4*B*). These findings suggest that the WT level of *GAL1pr* transcription is not required for the allelic interaction, but is essential for the *trans*-regulatory effect on gene expression.

Discussion

Genes in budding yeast are traditionally thought to be regulated over short distances through *cis* regulatory elements immediately upstream the core promoters. However, numerous long-distance intrachromosomal and interchromosomal interactions have been observed in the yeast genome (30), raising the question as to whether these interactions play a role in gene regulation. In this study, we focused on the interaction and *trans* regulation between allelic/nonallelic reporter genes in diploid budding yeast. We found that a defective *GAL1* promoter can make contacts with an allelic WT *GAL1pr* during galactose induction and acquires a faster activation rate.

Our findings have implications for the 3D organization of the genome. First, the interaction between the *GAL1* reporters was only detected under the activating condition. This observation is consistent with previous statistical analysis, indicating that coregulated genes tend to cluster, and genes that are physically proximal tend to coexpress (21, 31, 32). Coactivated genes can also form "transcription factories" or "multigene complexes" in higher eukaryotic species (33, 34), so our findings here are likely to be one example of a more general phenomenon. This result can also explain why there have been conflicting observations on homologous pairing in yeast (12–15): The experimental outcome likely depends on the inspected regions and their transcription status. The molecular nature of the interallelic interaction requires further elucidation. Because the interaction requires the presence of Gal4 on both alleles (Fig. 4*A*), but not the WT *GAL1pr* transcription (Fig. 4*D*), the simplest possibility is that the Gal4 dimers can interact and bring the two alleles together, but to our knowledge there is no evidence for Gal4 oligomerization. Alternatively, the interaction may be mediated by some downstream factors recruited by Gal4 before transcription initiation.

Second, among the integration sites we have chosen, the interaction was only detected between the allelic reporters. We can think of two explanations. The first possibility is that the *GAL1* promoter does not explore the whole nuclear space for binding partners, and the contacts it can make are restricted to a nuclear subvolume. Similar observations have been made in mammalian cells (4), and it was proposed that this constraint is imposed by the limited motion of the chromosomal context. The second possibility is that when the two reporters are at nonallelic sites, one or both of them can find better binding partners, preventing them from interacting with each other. In either case, our results indicate that the allelic reporters occupy similar 3D space before galactose induction and, therefore, have higher probability to form contact.

Third, in all our experimental conditions where the interaction between the two reporters was lost, the acceleration effect also disappeared (e.g., in Figs. 1*E* and 4*A*). In contrast, there is a case

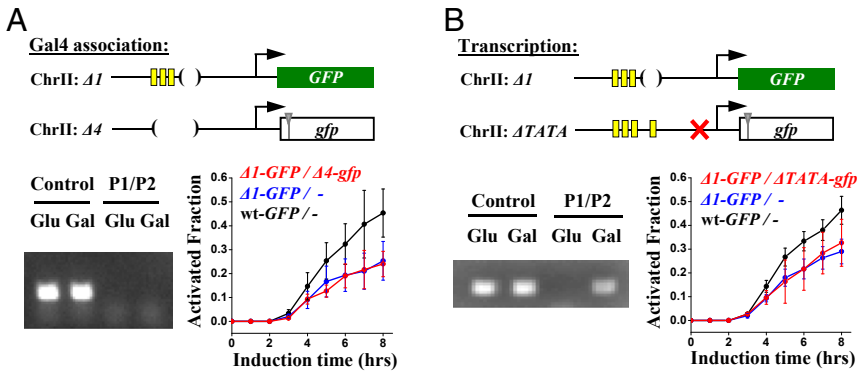

Fig. 4. Interallelic interaction and regulation between varied alleles. (*A*) Interallelic interaction and regulation between *Δ1pr-GFP* and *Δ4pr-gfp*. In *Δ4pr*, the entire UAS from the WT *GAL1pr* was deleted. (*Lower Left*) Gel image of the 3C measurement between the two alleles. (*Lower Right*) Activated fraction of *Δ1pr-GFP* as a function of time with or without allelic *Δ4pr-gfp*. (*B*) The same as *A* except that the *Δ4pr* was replaced by *ΔTATApr*, where the TATA element of the WT *GAL1pr* was mutated (*Methods*).

where the interaction remained, but we could not detect any acceleration (Fig. 4*B*). These data suggest that the interallelic interaction is necessary but not sufficient for *trans* regulation.

How does the interallelic interaction lead to change in the *Δ1/Δ2pr* expression? One possibility is that the UAS on the WT *GAL1pr* can directly recruit and deposit transcription machinery onto the paired allele. However, this model could not explain why the WT *GAL1pr* selectively enhances the activation rate but not the steady-state level of the *Δ1/Δ2pr* expression. In addition, this model predicts a competitive relation between the *cis* and *trans* core promoters, and we would expect the *trans*-regulatory effect to be enhanced after the removal of the *cis* TATA element (an observation made for transvection in *Drosophila*; refs. 23 and 35). Instead, we found that the deletion of TATA in the WT *GAL1pr* eliminated the *trans* regulation. We therefore suspect that the acceleration of the *Δ1/Δ2pr* expression is not due to direct activation in *trans*. This result also argues that the mechanism used here is different from that of transvection (or at least some classes of transvection).

Another component in the *GAL* regulatory pathway is long noncoding RNA (lncRNA). Two lncRNAs from the *GAL1-10* and *GAL7* locus can regulate endogenous *GAL1* expression both in *cis* and in *trans* (36–38). These lncRNAs are not expressed from our reporter constructs because they lack the 3′ end *GAL10* ORF sequence that is required for the initiation of these lncRNAs. Our reporters also do not contain the 5′ end of the *GAL1* ORF that is critical for the *trans* regulation of the lncRNA (38). The lncRNAs disappear after *GAL* activation, but the allelic *Δ1pr-GFP* and *wt-gfp* remain in contact (Fig. 3*D*). *Δ1pr-GFP/-* and *Δ1pr-GFP/wt-gfp* strains have the same amount of *GAL10* lncRNAs from the native site, but *Δ1pr-GFP* has faster activation in the latter strain. Overall, the evidence argues against the idea that the allelic interaction and *trans* regulation is mediated by the *GAL10* lncRNAs.

We propose an indirect mechanism where the WT *GAL1pr* stimulates the *Δ1/Δ2pr* expression by generating a more transcriptionally engaging environment. Recent studies indicate that pol II tends to form concentrated foci in the nucleus, and such pol II "clustering" may present a rate-limiting step for transcription activation (39). Because the *Δ1/Δ2pr* has less Gal4 binding capacity, we speculate that they are not as effective as the WT promoter in the initial recruitment of general transcription factors and pol II, causing the delayed activation. By physical association, actively transcribed WT *GAL1pr* may provide higher local pol II concentration, allowing *Δ1/Δ2pr* to have higher transcription rate at the early stage of induction when pol II is still limiting. In contrast, the steady-state expression level of the *Δ1/Δ2pr* may be limited by the maximum rate of the mediator/pol II recruitment at saturating concentration. Therefore, further increase in the pol II concentration will not result in an increase in the expression level.

Methods

Strain and Plasmids Construction. All plasmids used in this study were derived from pRS yeast shuttle vectors, and the *GAL1pr* reporters were inserted into the multiple cloning sites. The *Δ1*, *Δ2*, and *Δ4* promoters were constructed by deletion of the −395 to −335 bp, −312 to −277 bp, and −395 to −277 bp fragments from the WT *GAL1pr*, respectively (relative to the start of the ORF). For the *ΔTATA* promoter, the original TATA box "ATATAA" was mutated to "gcgTAA." *GFP* frame shift mutation was generated by inserting a 4-bp sequence "ATCC" between 104 and 105 bp of *GFP* ORF. This mutated *GFP* shows no green fluorescence signal. All plasmids were confirmed by Sanger sequencing.

All of the yeast strains are W303-congenic (see Table S1 for strain list). We first generated two background haploid strains, MMY116 (W303a, *ADE2*) and W303-TRP (W303α, *TRP1*), and integrated the reporters into these strains (in most cases with the *URA3* marker). To direct the reporters into ChrII, we inserted a homologous region (ChrII 664401–664682) into the plasmid, linearized by KpnI, and transformed into the haploid yeast following the standard protocol. Similarly, we used the segments ChrV 394711–394317 and ChrXVI 67026–67427 for integration into ChrV and ChrXVI.

After each transformation, we picked six to eight yeast colonies from the selection plate and performed three PCR tests. (*i*) To confirm the location of integration, we carried out PCRs between primer pairs flanking the intended integration site, one in the genomic DNA and the other inside the plasmid. The presence of the PCR product confirms that the reporter is inserted into the intended locus. (*ii*) To make sure that the reporter is inserted as a single copy, we performed PCRs between two primers facing toward each other in the plasmid sequence flanking the homologous region. Because we cut the homologous region in the middle for transformation, this PCR should not produce any product; the presence of the product indicates multiintegration. For some strains, we also used the *GFP* fluorescence intensity to detect multiintegration. (*iii*) To confirm that the integrated *Δ1*, *Δ2*, and *Δ4* promoters carry the intended deletions, we performed PCRs flanking the deleted sequence and we expected to see two products with different lengths, one from the endogenous *GAL1pr*, and the other from the reporter. After validation of the haploid strains, we mated them into diploids (in most cases selected with SCD-Ade-Trp). All of the experiments were repeated with at least two different diploid colonies.

FACS. Overnight culture in glucose (OD 0.5–0.7) was washed and diluted to OD 0.1 in synthetic media containing 3% (wt/wt) galactose, 2% (wt/wt) raffinose, and 0.1% glucose. We diluted the culture by using fresh media after each hour of induction to replenish glucose and maintain the OD between 0.1 and 0.2. The samples were taken at different time points between 0–8 h of induction, kept on ice, sonicated, and subject to FACS measurement by using The BD LSRFortessa cell analyzer in a 96-plate format.

Time-Lapse Fluorescence Microscopy. The time-lapse assay was performed as described (28). Briefly, live yeast cells were placed into CellAsic flow-cells,

pregrown in 2% glucose for 4 h, and induced in 3% galactose, 2% raffinose, and 0.05% glucose for 8 h. In the meantime, we took *GFP* and *YFP* fluorescent images every 5 min. The movies were analyzed by using semiautomated data analysis software written in Matlab (27) to obtain the fluorescent intensity of individual cells at each movie frame. We then eliminated the cross-talk between the *GFP* and *YFP* signals through linear subtraction (28), which resulted in the single cell activation curves shown in Fig. 2*C*. The curves were then smoothed, and the activation time was quantified as the point where the second derivative reached the maximum.

3C Assay. We cloned two distinct 100-bp fragments from KanMX ORF, 462–561 and 362–461 (KanMX1 and KanMX2 in Fig. 3*A*), and inserted them upstream of the *GAL1* reporters. We introduced two XhoI sites into the plasmids, one upstream of the KanMX fragments and the other downstream of *GFP* transcript. After integrating the plasmids into yeast, we followed standard 3C protocol described (40). Cells were incubated overnight from a freshly growing colony and induced by switching from 2% glucose to 3% galactose and 2% raffinose for 8 h to an OD ∼0.65–0.8. Cells were cross-linked by formaldehyde (final concentration 1%) for 20 min and quenched by glycine for 5 min followed by centrifugation. Cell pellets were washed with TBS twice and frozen by liquid nitrogen. Five hundred microliters of FA-lysis buffer was added to resuspend the cells, and an equal volume of glass beads was added to disrupt the cells. The cell extracts were collected by centrifugation, washed with FA-lysis buffer, and resuspended in 500 μL of 10 mM Tris·HCl (pH 7.5). We digested 50 μL of the cross-linked chromatin sample with XhoI overnight before heating the sample at 65 °C to inactivate XhoI. After resuspension of the digested chromatin in 100 μL of 10 mM Tris·HCl, we added 400 U of T4 ligase and ligated overnight. We then digested RNA with RNase, and reversed cross-linking with SDS and proteinase K at 65 °C overnight. The DNA was pheno-extracted, resuspended in 50 μL of Tris-EDTA (pH 8.0), and subject to PCR/qPCR analysis. The two primers used here are "ATTCGTGATTGCGCCTGAGC" and "CGCCTGAGAATGGCAAAAGC". The interaction between two *cis* regions on the ChrXV (near 878307 and 891189) was measured as a positive control and for normalization of the qPCR signal. The two primers used for the control are "CGGTAACCGAATTCTTCTCTCATG" and "TTCTTTGTCACTGTCGTGCGTG".

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