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Analysis of Transcriptional Activation at a Distance in *Saccharomyces cerevisiae*▽

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Most fundamental aspects of transcription are conserved among eukaryotes. One striking difference between yeast *Saccharomyces cerevisiae* and metazoans, however, is the distance over which transcriptional activation occurs. In *S. cerevisiae*, upstream activation sequences (UASs) are generally located within a few hundred base pairs of a target gene, while in *Drosophila* and mammals, enhancers are often several kilobases away. To study the potential for long-distance activation in *S. cerevisiae*, we constructed and analyzed reporters in which the UAS-TATA distance varied. Our results show that UASs lose the ability to activate normal transcription as the UAS-TATA distance increases. Surprisingly, transcription does initiate, but proximally to the UAS, regardless of its location. To identify factors affecting long-distance activation, we screened for mutants allowing activation of a reporter when the UAS-TATA distance is 799 bp. These screens identified four loci, *SIN4*, *SPT2*, *SPT10*, and *HTA1-HTB1*, with *sin4* mutations being the strongest. Our results strongly suggest that long-distance activation in *S. cerevisiae* is normally limited by Sin4 and other factors and that this constraint plays a role in ensuring UAS-core promoter specificity in the compact *S. cerevisiae* genome.

Many aspects of transcription initiation are conserved between *Saccharomyces cerevisiae* and other eukaryotes. This includes a high degree of conservation for many fundamental classes of transcription factors, such as RNA polymerase II, general transcription factors, particular coactivators, chromatin remodeling complexes, and histone modification enzymes (6, 30). Other features are less well conserved, particularly the DNA regulatory sites that control transcription. While TATA elements are used at a significant number of promoters in both *S. cerevisiae* and metazoans, the TATA-start site distance is fixed in metazoans but not in *S. cerevisiae*, and the start site itself is more conserved in metazoans (68). In addition, downstream promoter elements, widely used in metazoans, are not found in *S. cerevisiae* (40).

Another significant difference between yeast and metazoans concerns yeast upstream activation sequences (UASs) and their metazoan counterparts, enhancers. Both serve as binding sites for gene-specific activators, yet while UASs are usually positioned within a few hundred base pairs 5′ of the TATA box or core promoter, enhancers are often located several kilobases away from or even 3′ of the promoter (5, 30). While there is some evidence that yeast UASs cannot function when moved too far from their promoters (31, 71), this area has not been extensively studied. As the *S. cerevisiae* genome is compact, and the majority of yeast promoters range from approximately 150 to 400 bp (28, 59), it seems logical that there would be restrictions on activation distance in order to maintain specificity between a UAS and its target gene. However, there is no clear understanding of the effect of distance on activation in *S. cerevisiae* in vivo and no knowledge concerning factors that might play a role in controlling this effect.

Different mechanisms have been proposed for the ability of enhancers to act over long distances (5, 11, 25, 62). One mechanism, looping, stipulates that proteins bound at an enhancer associate directly with proteins bound at a core promoter with a looping out of the intervening chromatin. For example, loops have been demonstrated between the locus control region of the murine β-globin locus and activated genes located 40 to 60 kb away (72). Another proposed mechanism, tracking, requires the movement or binding of molecules along the DNA between the enhancer and core promoter. The tracking model postulates that RNA polymerase is recruited at the enhancer and moves along the DNA until it reaches the target promoter. The strongest evidence for this mechanism comes from analysis of a bacterial enhancer-like element (32). In *S. cerevisiae*, evidence consistent with tracking came from experiments in which LexA binding or a transcription terminator blocked transcriptional activation (8). Current evidence from metazoans supports several of these mechanisms, including combinations of mechanisms at particular enhancers (for an example, see reference 76).

The looping mechanism has been demonstrated to occur in *S. cerevisiae* in some circumstances. Looping has been detected in yeast between the promoters and terminators of long genes and this event has been correlated with active transcription (1, 58). In addition, looping has also been demonstrated within yeast telomeres, bringing the UAS and TATA box of a reporter gene located within that region into close proximity (22). Finally, genetic evidence for looping facilitated by the *Drosophila melanogaster* GAGA protein has been described previously (60). No evidence for looping as part of transcriptional activation, however, has been found, most likely because most *S. cerevisiae* UAS elements are close enough to the core promoter not to require looping as part of gene activation.

To study activation distance in *S. cerevisiae*, we established a

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype
FY3	*MAT***a** *ura3-52*
FY2549	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::HIS3*
FY2550	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$280-HIS3*
FY2551	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$380-HIS3*
FY2552	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$493-HIS3*
FY2553	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$574-HIS3*
FY2554	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$690-HIS3*
FY2555	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3*
FY2556	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-LexA642-HIS3*
FY2557	*MAT*α *his3*Δ*200 leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 ybr281c*Δ*::TRP1-UAS$_{GAL}$305-HIS3-natMX*
FY2558	*MAT*α *his3*Δ*200 leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 ybr281c*Δ*::TRP1-UAS$_{GAL}$606-HIS3-natMX*
FY2559	*MAT*α *his3*Δ*200 leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 ybr281c*Δ*::TRP1-UAS$_{GAL}$806-HIS3-natMX*
FY2560	*MAT*α *his3*Δ*200 leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 ybr281c*Δ*::TRP1-UAS$_{GAL}$806-HIS3-natMX sin4*Δ*0::LEU2*
FY2562	*MAT***a** *ade8-104 his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 sin4*Δ*0::LEU2*
FY2570	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 spt2*Δ*0::kanMX*
FY2571	*MAT***a** *ade8-104 his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 spt10*Δ*0::LEU2*
FY2572	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*1 ura3*Δ*0 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 (hta1-htb1)*Δ*::LEU2*
FY2573	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$1397-HIS3*
FY2574	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$1397-HIS3 sin4*Δ*0::LEU2*
FY2575	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$1995-HIS3*
FY2576	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$1995-HIS3 sin4*Δ*0::LEU2*
FY2577	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*1 ura3*Δ*0 bph1*Δ*::kanMX-UAS$_{GAL}$1397-HIS3 spt2*Δ*0::kanMX*
FY2578	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$1995-HIS3 spt2*Δ*0::kanMX*
FY2579	*MAT*α *his3*Δ*200 leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 ybr281c*Δ*::HIS3-natMX*
FY2580	*MAT*α *his3*Δ*200 leu2*Δ*0 trp1*Δ*63 ybr281c*Δ*::TRP1-UAS$_{GAL}$806-HIS3-natMX spt2*Δ*0::kanMX*
FY2581	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-LexA123-HIS3*
FY2582	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-LexA417-HIS3*
FY2583	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*1 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-LexA642-HIS3 spt2*Δ*0::natMX*
FY2584	*MAT*α *his3*Δ*200 leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::natMX-UAS$_{GAL}$799-HIS3 can1*Δ*::STE2-prLEU2 lyp1*Δ
FY2585	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$839-TERM$_{ADH1}$-HIS3*
FY2586	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 arg4-12 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$839-TERM$_{ADH1}$-HIS3 sin4*Δ*0::LEU2*
FY2587	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$839-TERM$_{ADH1}$-HIS3 spt2*Δ*0::kanMX*
FY2588	*MAT*α *his3*Δ*200 lys2-128*δ *ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 gal11*Δ*::TRP1*
FY2589	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 pgd1*Δ*0::LEU2*
FY2590	*MAT*α *ade8-104 his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 med2*Δ*0::URA3*
FY2591	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 nut1*Δ*0::URA3*
FY2592	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 med1*Δ*0::URA3*
FY2593	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 srb10*Δ*0::URA3*
FY2594	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 srb2*Δ*0::URA3*
FY2597	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 spt8*Δ*0::LEU2*
FY2600	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 arg4-12 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 spt6-140*
FY2601	*MAT***a** *his3*Δ*200 lys2-128*δ *leu2*Δ*1 ura3-52 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 spt21*Δ*0::kanMX*
FY2602	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 srb9*Δ*0::URA3*
FY2603	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 mot1-1*
FY2604	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 set2*Δ*::URA3*
FY2605	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-UAS$_{GAL}$799-HIS3 soh1*Δ*0::URA3*
FY2606	*MAT*α *his3*Δ*200 lys2-128*δ *leu2*Δ*0 ura3*Δ*0 trp1*Δ*63 bph1*Δ*::kanMX-LexA642-HIS3 sin4*Δ*0::URA3*

system using the well-characterized activator, Gal4, to examine the ability of the *GAL1* UAS to function at different distances from a TATA box in *S. cerevisiae*. These experiments clearly show that activation diminishes with distance, confirming the suggestions from previous studies (31, 71) and indicating that long-distance activation is normally repressed. Unexpectedly, we have also found that when a UAS is far from a TATA, the UAS directs expression of nonfunctional transcription from nearby start sites regardless of the location of the UAS, suggesting that promiscuous activation may be a property of UAS elements. To gain insight into factors that control activation distance in *S. cerevisiae*, we screened for mutants that permit activation from the normally nonpermissive distance of 800 bp. These screens have identified a small set of factors known to play roles in chromatin structure: Sin4, Spt2, Spt10, and histone H2A/H2B. The mutations that allow the strongest level of long-distance activation, in *SIN4*, expand the range of transcription start sites, thereby allowing long-distance activation. Finally, experiments to test the mechanism of long-distance activation in *sin4*Δ mutants have provided strong evidence against a tracking model.

MATERIALS AND METHODS

***S. cerevisiae* strains and plasmids.** All *S. cerevisiae* strains used (Table 1) are isogenic with a *GAL2*$^+$ derivative of S288C (79). Rich (yeast extract-peptone-dextrose [YPD]), minimal (SD), and synthetic complete drop-out media (for example, medium lacking histidine [SC-His]) were prepared as described previously (65). YPGal (1% yeast extract, 2% peptone, and 2% galactose) and SC-His Gal media contained 2% galactose as the carbon source. Where indicated, specified concentrations of 3-aminotriazole were added to SC-His Gal medium. Strains were constructed by standard methods, either by crosses or by transformation (4). The *spt2*Δ*::kanMX* (57), (*hta1-htb1*)Δ*::LEU2* (35), *mot1-1* (21), *spt6-140* (18), and *gal11*Δ*::TRP1* (63) alleles have been described previously. The

sin4Δ0::*LEU2*, *sin4Δ0*::*URA3*, *spt10Δ0*::*LEU2*, *pgd1Δ0*::*LEU2*, *spt8Δ0*::*LEU2*, *med1Δ0*::*URA3*, *med2Δ0*::*URA3*, *nut1Δ0*::*URA3*, *srb2Δ0*::*URA3*, *srb5Δ0*::*URA3*, *srb9Δ0*::*URA3*, *srb10Δ0*::*URA3*, *soh1Δ0*::*URA3* and *set2Δ0*::*URA3* deletion mutations were constructed by replacing their open reading frames (ORFs) with the auxotrophic marker *LEU2* or *URA3* (17). The *spt2Δ0*::*natMX* deletion mutation was constructed by replacing its ORF with the *natMX* marker, which confers resistance to nourseothricin (29). The *spt21Δ0*::*kanMX* deletion mutation was constructed by replacing its ORF with the *kanMX* marker which confers resistance to G418 (7, 75).

Long-distance activation reporters were made by a series of integrative transformations, resulting in either Gal4 or LexA binding sites integrated at different distances from a *HIS3* gene with its adjacent TATA elements. Each reporter was made within a long coding region, chosen to avoid consensus TATA or other binding sites for transcription factors. The oligonucleotides used in the construction of all reporter strains are listed in Table S1 at http://genetics.med.harvard.edu/~winston/Winston%20Lab%20Links.html. For the reporter in the *BPH1* gene, construction began with the integration of *URA3* at the 3′ end of the *BPH1* ORF. Next, the *HIS3* TATA and coding and terminator sequences were amplified by PCR from plasmid YIP55-Sc^{HIS3}A5 (36) with flanking homologies added so that the *URA3* marker would be replaced entirely by the *HIS3* sequences after transformation. Ura$^-$ transformants were selected by resistance to 5-fluoroorotic acid. To prevent transcription through *BPH1*, sequences from −500 to +49 (where +1 is the ATG) were deleted and replaced with copies of the sequence encoding the HA1 epitope, using a method described previously (66). This strain, FY2549, was used as the parent strain for all other *BPH1* reporter strains. To insert Gal4 binding sites at specified distances 5′ of the *HIS3* TATA in the *BPH1* reporter, a cassette containing four Gal4 binding sites and the *kanMX* marker was amplified by PCR from the *pFA6a-kanMX6-PGAL1* plasmid (47) using primers designed for recombination at specific distances 5′ of *HIS3*. To make strain FY2584 for use in synthetic genetic array analysis, integrative transformation was used to replace the *kanMX* marker with the *natMX4* marker, which confers resistance to the drug nourseothricin (29). To insert the *ADH1* terminator between the Gal4 binding sites and *HIS3* TATA in FY2555, the *ADH1* terminator was amplified from *pFA6a-GFP*(*S56T*)*-kanMX6* (47) and this PCR product was used in an integrative transformation to replace a *URA3* marker within *BPH1* using the same method described above. To place LexA binding sites 5′ of the *HIS3* TATA in FY2549, complementary oligonucleotides containing three overlapping LexA binding sites flanked by restriction sites were synthesized, annealed, and digested by BamHI and BglII. This LexA fragment was cloned into the BamHI and BglII sites of *pFA6a-kanMX6-PGAL1*, replacing the Gal4 binding sites and creating plasmid *pFA6a-kanMX6-LexA*. This plasmid was used as a template for PCR to generate DNA for integrative transformation into the *BPH1* locus, and G418^R transformants were selected. The *LexA-GAL4* (8) and *LexA-GCN4* plasmids were generously provided by Roger Brent and Mark Ptashne.

To construct the *YBR281C* reporter, we first marked *HIS3* so that it could be integrated at the *YBR281C* locus in one step. To do this, the *natMX4* cassette was amplified by PCR from *pAG25* (29) and inserted 3′ of the *HIS3* terminator at its genomic locus in a wild-type strain. Then the entire *HIS3-natMX4* region was amplified by PCR and inserted by integrative transformation at the 3′ end of the *YBR281C* ORF, selecting for nourseothricin resistance. Subsequently, a cassette containing four Gal4 binding sites and the *TRP1* auxotrophic marker was amplified from the *pFA6a-TRP1-PGAL1* plasmid (47), with flanking homology to specific sites within *YBR281C*. These PCR products were used to insert Gal4 binding sites by integrative transformation at the specified distances 5′ of the *HIS3* TATA within *YBR281C*. Transformants were selected by growth on SC-Trp. The design of the flanking homologous sequences was such that integration of the Gal4 binding sites within *YBR281C* simultaneously deleted all *YBR281C* sequences 5′ of that integration to −140 (where +1 is the ATG) in order to prevent transcription through *YBR281C*. Verification of all correct integration and recombination events was performed by PCR.

Isolation of long-distance activation mutants. Long-distance activation mutants were identified by four methods, a transposon insertion mutagenesis screen (12), spontaneous mutant selection, systematic genetic array analysis using the nonessential deletion set and the doxycycline-repressible essential allele set (53, 73), and testing of candidate genes. For insertional mutagenesis, a mutagenized yeast genomic library containing *LEU2*-marked transposon insertions (12) was digested with NotI and transformed into FY2555. Integrative transformants were selected by growth on medium lacking leucine (SC-Leu), and these colonies were replica plated onto SC-His-Leu Glu and onto SC-His-Leu Gal to screen for transformants that were His$^+$ only when galactose was the carbon source. Of 11 His$^+$ transformants, one was retested after purification. Vectorette PCR (2) was used to amplify the region where the transposon had integrated, and sequencing determined this location to be within the coding region of *SIN4*. Two methods of spontaneous selection were carried out using strains FY2554 and FY2555. For the first method, patches of FY2554 and FY2555 were grown on YPD and replica plated to SC-His Gal medium. Papillae that grew after 2 weeks at room temperature were purified and retested to check that the His$^+$ phenotype was galactose dependent. Of the four mutants that were rechecked, two FY2555-derived mutants were complemented by a wild-type *SIN4* plasmid and two FY2554-derived mutants remain unidentified. In the second method, four cultures of either FY2554 or FY2555 were grown overnight and 1×10^7 cells from each culture were plated on SC-His Gal and grown at 30°C. Three colonies were picked from each plate, making a total of 24 putative mutants, 19 of which were retested upon purification. Of the 10 FY2554 mutants, 6 failed to complement a deletion of *SIN4*, 1 failed to complement a deletion of *SPT10*, 1 failed to complement a deletion of *HTA1-HTB1*, and 2 others remain unidentified. Of the nine FY2555 mutants, five failed to complement a deletion of *SIN4*, two failed to complement a deletion of *HTA1-HTB1*, and two remain unidentified but are in the same complementation group. For the systematic genetic array analysis, we screened both the yeast nonessential deletion set (73) and a doxycycline-repressible set of essential genes (53) by crossing each set to strain FY2584 and screening for growth on SC-His Gal. Deletion mutants of *SIN4*, *SPT2*, and *HTA1* were identified as having a His$^+$ phenotype in these screens.

Northern analysis. RNA isolation and Northern hybridization experiments were performed as previously described (4). As indicated, strains were grown to mid-log phase in YPGal. Northern hybridization analysis was conducted with probes to the coding regions of *HIS3* (−27 to +376, where +1 is the ATG) and *ACT1* (+533 to +722) (see Table S1 at http://genetics.med.harvard.edu/~winston/Winston%20Lab%20Links.html).

5′ mapping of transcript start sites. 5′ end mapping of RNAs was carried out using the rapid amplification of cDNA ends (RACE) method. Total RNA was prepared as described above (4) and treated with DNase using the RNeasy Mini kit (QIAGEN). cDNA synthesis and PCR were performed using the SMART-RACE kit (Clontech) as described previously (80). The primer used for mapping the 5′ ends of transcripts annealed to sequences from +263 to +282 of the *HIS3* ORF (see Table S1 at http://genetics.med.harvard.edu/~winston/Winston%20Lab%20Links.html). The PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced.

3C. To assay possible loop formation between the Gal4 binding sites and *HIS3* TATA in the reporter, the chromosome conformation capture (3C) method was used (1, 23). We followed the method of Ansari and Hampsey (1) with some modifications. Fifty milliliters of cells was grown in YPD or YPGal to a density of 3×10^7 cells/ml and cross-linked with 1% formaldehyde for 30 min at room temperature. The reaction was quenched with the addition of glycine to 250 mM and 0.1% sodium dodecyl sulfate (SDS) and incubated for 5 min at room temperature. The cell pellet was washed with 10 ml of 1× Tris-buffered saline buffer plus 1% Triton X-100, resuspended in 1 ml 1× Tris-buffered saline, transferred to a 2-ml screw-cap tube, and pelleted. This pellet was resuspended in 750 μl of FA lysis buffer, approximately 500 μl of glass beads was added, and cells were lysed with vigorous shaking for 40 min at 4°C. Cell lysates were collected by puncturing the bottom of the tube with a 22-gauge needle and collecting the filtrate in a second 2-ml screw-cap tube set inside a 15-ml conical tube. The filtrate was then pelleted at top speed in a microfuge for 5 min at 4°C. The pellet was washed with 500 μl of FA lysis buffer and resuspended in 500 μl of 10 mM Tris, pH 7.5. Eighty microliters of this chromatin preparation was digested for 16 h, with shaking in a 125-μl digestion mixture with 10 μl each of enzymes BfaI (5,000 U/ml) and MseI (4,000 U/ml). Samples were pelleted at top speed in a microfuge for 5 min at room temperature and then resuspended in 90 μl of 10 mM Tris. Digestion was stopped by adding 10 μl of 10% SDS and incubating at 65°C for 20 min. To sequester the SDS, 75 μl of 10% Triton X-100 was added, and the reaction mixture was diluted to a volume of 750 μl. Ligations were carried out for 1 h at room temperature using 5 μl of Quick ligase (New England Biolabs). Two microliters of 10 mg/ml RNase was added, and the reaction mixture was incubated for 10 min at 37°C. Five microliters of 20 mg/ml proteinase K was then added, and the reaction mixture was incubated overnight at 65°C. The samples were extracted two times with phenol-chloroform and once with chloroform and ethanol precipitated in the presence of glycogen. The DNA concentration was determined, and 500 ng of DNA was used as the template for each PCR. PCRs (30 cycles) used same-strand oligonucleotides that anneal to the indicated regions of the reporter and require a cut between the two oligonucleotides to form a product (see Fig. 6A and Table S1 at http://genetics.med.harvard.edu/~winston/Winston%20Lab%20Links.html) (23). A PCR to control for the amount of template DNA was performed using convergent oligonucleotides that amplify a region of chromosome V lacking BfaI and MseI restriction

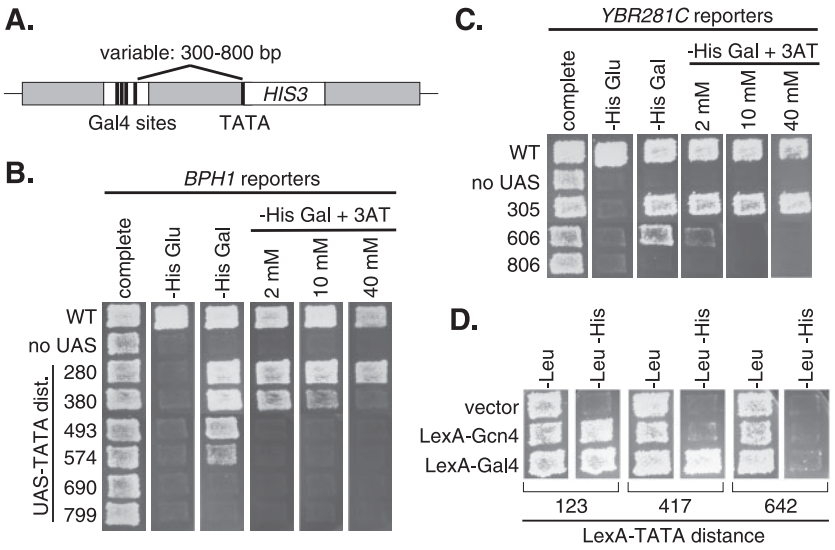

FIG. 1. The UAS-TATA distance affects *HIS3* expression. (A) A schematic of the reporters used in this study. Four Gal4 binding sites from the *GAL1* UAS were integrated at various distances 5′ of the *HIS3* gene (see Materials and Methods). (B) Growth of strains with the *HIS3* reporters integrated within the *BPH1* ORF. UAS elements were positioned at the indicated distances 5′ of the *HIS3* TATA. The strains shown are as follows: FY3 (the wild type [WT]), FY2549 (no UAS), FY2550 (UAS 280), FY2551 (UAS 380), FY2552 (UAS 493), FY2553 (UAS 574), FY2554 (UAS 690), and FY2555 (UAS 799). Patches of cells were grown on YPD, replica plated to the specified media, and grown for 2 days at 30°C. In this and all subsequent figures, patches for a particular growth medium were grown on the same plates, but have been arranged for ease of phenotype comparison between the different types of media. (C) Growth of strains with the *HIS3* reporters integrated within the *YBR281C* ORF. The strains shown are as follows: FY3 (WT), FY2579 (no UAS), FY2557 (UAS 305), FY2558 (UAS 606), and FY2559 (UAS 806). Strains were grown and incubated as described for panel B. (D) Growth of reporter strains with LexA binding sites serving as the UAS. Strains with LexA sites at 123 (FY2581), 417 (FY2582), or 624 (FY2583) bp 5′ of *HIS3* were transformed with *LEU2*-marked plasmids expressing LexA-Gcn4 or LexA-Gal4 fusion proteins or a vector-only control. Patches of cells were grown on SC-Leu, replica plated to the specified media, and grown for 3 days at 30°C. 3AT, 3-aminotriazole.

sites (see Table S1 at the URL mentioned above). PCR products were visualized on a 1.6% agarose gel using ethidium bromide.

RESULTS

Construction and analysis of long-distance reporters. A small number of studies of *S. cerevisiae* have suggested that a UAS-TATA distance greater than 300 bp can impair transcriptional activation at some promoters (31, 71). To examine the effect of the UAS-TATA distance on transcriptional activation in a systematic fashion, we constructed reporter strains in which the UAS-TATA distance was increased in a stepwise fashion (Fig. 1A and Table 2). These reporters were constructed at two different locations within the genome, within the coding regions of the long, nonessential genes *BPH1* and *YBR281C*. At each location, we integrated the *HIS3* TATA, coding, and terminator sequences into regions of *BPH1* and *YBR281C* that were devoid of consensus TATA or Gal4 binding sites. Then, the *GAL1* UAS, with its four Gal4 binding sites, was integrated 5′ of *HIS3* at distances that ranged from approximately 300 to 800 bp (Table 2).

TABLE 2. Long-distance activation reporters

Genomic location	UAS element	UAS-TATA distances (bp)[a]
BPH1	*GAL1* (four Gal4 binding sites)	280, 380, 493, 574, 690, 799, 1397, 1995, 839*
YBR281C	*GAL1*	305, 606, 806
BPH1	Three LexA binding sites	123, 417, 642

[a] The asterisk indicates that the 839 reporter includes the *ADH1* terminator element between the UAS and TATA.

The level of Gal4-activated expression of *HIS3* in these reporter strains was initially assayed by testing growth on SC-His Gal. We also included tests on a range of concentrations of 3-aminotriazole, a competitive inhibitor of His3, as a further test of expression strength. Our results show that at *BPH1*, UAS-TATA distances less than or equal to 493 bp were strongly His^+, a distance of 574 bp was weakly His^+, and distances of 690 bp and greater were His^- (Fig. 1B). The *YBR281C* location gave similar results, although this site was somewhat more permissive, with a UAS-TATA distance of 606 bp conferring a His^+ phenotype and a distance of 806 bp being His^- (Fig. 1C). The addition of different 3-aminotriazole concentrations revealed a progressive change of expression even at closer distances, with the greatest expression occurring at the smallest UAS-TATA distance. The expression of *HIS3* was Gal4 dependent, as all reporters were His^- when glucose was the carbon source instead of galactose.

To test whether other activators would function similarly to Gal4 as the UAS-TATA distance was increased, we constructed three additional reporter strains in which three overlapping LexA binding sites were integrated 123, 417, and 642 bp 5′ of the *HIS3* TATA (Table 2). Using these reporters, we compared activation via two fusion proteins, LexA-Gal4 and LexA-Gcn4 (Fig. 1D). While the LexA-Gal4 fusion protein was able to activate transcription from distances of 123 and 417, similar to Gal4 alone, LexA-Gcn4 could activate transcription from only the shortest distance. None of the reporters were

TABLE 3. Genes identified in long-distance activation screens

Gene	Function	Identification method[a]
SIN4	Subunit of Mediator coactivator complex; positive and negative regulator of transcription	Transposon insertion, spontaneous, SGA
SPT2	HMG box protein; involved in transcriptional elongation	Candidate, SGA
SPT10	Putative histone acetyltransferase; positively regulates a subset of histone genes	Spontaneous
HTA1-HTB1	Histones; integral component of chromatin	Spontaneous, SGA*

[a] The asterisk indicates that only *hta1*Δ was identified by synthetic genetic array (SGA); the deletion set does not contain *htb1*Δ. Spontaneous, spontaneous mutagenesis; candidate, candidate gene approach.

activated when transformed with a control plasmid that did not express an activator.

Isolation and characterization of mutants that allow long-distance activation. In order to discover factors that might determine the distance at which a UAS can activate in *S. cerevisiae*, *BPH1* reporter strains with the UAS located either 690 or 799 bp 5′ of *HIS3* were screened for factors which, when mutated, enable the expression of *HIS3* in a galactose-dependent manner. Four methods were used: transposon insertion mutagenesis, spontaneous mutagenesis, a candidate gene approach, and synthetic genetic array analysis with both the yeast nonessential deletion set as well as the essential repressible allele set (described in Materials and Methods) (12, 53, 73).

We found a small number of factors affecting long-distance activation of gene expression, all of which have been previously implicated in chromatin structure (Table 3 and Fig. 2). Mutations in *SIN4* were recovered in all our screens, and *sin4* mutations conferred the strongest His^+ phenotype (Fig. 2A). *SIN4* encodes a subunit of the tail domain of the Srb/Mediator coactivator complex and plays both positive and negative roles in the regulation of transcription (15, 16, 19, 20, 37–39, 45, 48, 69, 77). *SPT2* encodes an HMG-like protein, binds DNA in a sequence-independent manner, and has been implicated in both transcription elongation and the recruitment of mRNA cleavage/polyadenylation factors (33, 43, 44, 57). Both *SIN4* and *SPT2* were previously isolated in a screen for suppressors of a transcriptional defect at an *HO-lacZ* fusion in strains mutated for members of the Swi/Snf chromatin remodeling complex (69). *SPT10* encodes a site-specific DNA binding protein that regulates histone gene transcription and is also a putative histone acetyltransferase (24, 26, 34, 56, 67). Finally, *HTA1-HTB1* encodes the core histones H2A and H2B. Mutations in *SIN4*, *SPT10*, and *SPT2* share other phenotypes with histone gene mutants, such as the suppression of Ty and δ insertions (Spt^- phenotype), reinforcing their roles in controlling chromatin structure (18, 27, 38, 43, 55, 64, 69, 78). In all four cases, complete deletion of the gene allowed long-distance activation, showing that the phenotype is caused by a loss of function. In addition, for each mutant, long-distance activation occurred only in the presence of galactose, showing that the mutations did not merely bypass the requirement for a UAS. The rest of our analysis is focused primarily on long-distance activation in *sin4*Δ mutants, as they have the strongest phenotypes, with some analysis of *spt2*Δ for comparison.

To determine whether the long-distance activation observed in *sin4*Δ and *spt2*Δ mutants is specific for either location or activator, additional reporters were tested. First, we tested the effects of *sin4*Δ and *spt2*Δ mutations at *YBR281C*, testing the

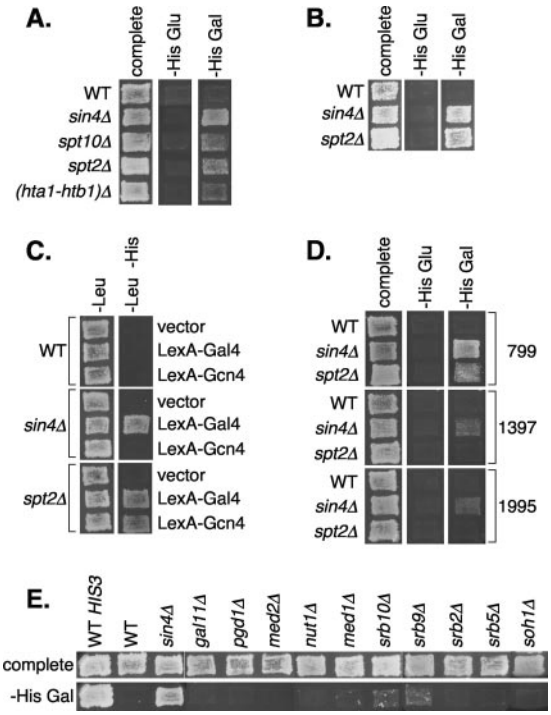

FIG. 2. Phenotypic analyses of mutants permissive for long-distance activation. (A) Growth of mutant strains with the *HIS3* UAS 799 reporter integrated into the *BPH1* ORF. Cells were patched on YPD, replica plated to the specified media, and grown for 3 days at 30°C. The strains shown are as follows: FY2555 (wild type [WT] with reporter), FY2562 (*sin4*Δ), FY2570 (*spt2*Δ), FY2571 (*spt10*Δ), and FY2572 [(*hta1-htb1*)Δ]. (B) Growth of mutant strains containing the UAS 806 reporter integrated into the *YBR281C* ORF. The strains shown are as follows: FY2559 (WT with reporter), FY2560 (*sin4*Δ), and FY2580 (*spt2*Δ). Strains were grown and incubated as described for panel A. (C) Growth of strains containing a reporter with LexA binding sites positioned 642 bp 5′ of *HIS3* in the *BPH1* ORF. Strains were transformed with *LEU2*-marked plasmids expressing LexA-Gal4 or LexA-Gcn4 fusion protein or a vector-only control. The strains shown are as follows: FY2556 (WT with LexA reporter), FY2563 (*sin4*Δ), and FY2583 (*spt2*Δ). Strains were patched on SC-Leu, replica plated to the specified media, and grown for 3 days at 30°C. (D) Growth of wild-type, *sin4*Δ, and *spt2*Δ strains containing reporters with UAS elements positioned at the specified distances 5′ of the *HIS3* TATA within *BPH1* (right side of panel). The strains shown are as follows: FY2555 (WT UAS 799), FY2562 (*sin4*Δ UAS 799), FY2570 (*spt2*Δ UAS 799), FY2573 (WT UAS 1397), FY2574 (*sin4*Δ UAS 1397), FY2577 (*spt2*Δ UAS 1397), FY2575 (WT UAS 1995), FY2576 (*sin4*Δ UAS 1995), and FY2578 (*spt2*Δ UAS 1995). Strains were grown and incubated as described for panel A. (E) Growth of strains containing the *BPH1 GAL* UAS 799 reporter in Mediator mutants. The strains shown are as follows: FY3 (WT), FY2555 (WT with UAS 799 reporter), FY2562 (*sin4*Δ), FY2588 (*gal11*Δ), FY2589 (*pgd1*Δ), FY2590 (*med2*Δ), FY2591 (*nut1*Δ), FY2592 (*med1*Δ), FY2593 (*srb10*Δ), FY2602 (*srb9*Δ), *srb2*Δ (FY2594), FY2595 (*srb5*Δ), and FY2605 (*soh1*Δ). Strains were patched and grown for 4 days at 30°C.

TABLE 4. Direct testing of mutants for a long-distance activation phenotype

Mutation	Identity	His phenotype
*sin4*Δ	Mediator tail	+
*gal11*Δ	Mediator tail	−
*pgd1*Δ	Mediator tail	−
*med2*Δ	Mediator tail	−
*nut1*Δ	Mediator middle	−
*med1*Δ	Mediator middle	−
*soh1*Δ	Mediator middle	−
*srb10*Δ	Mediator Srb 8-11	−/+
*srb9*Δ	Mediator Srb 8-11	−/+
*srb2*Δ	Mediator head	−
*srb5*Δ	Mediator head	−
*spt8*Δ	SAGA	−
spt6-140	Elongation factor	−
*set2*Δ	Histone methlytransferase	−
mot1-1	Transcription factor	−
*spt21*Δ	Transcription factor	−

[a] Strains in the table are FY2562 (*sin4*Δ), FY2588 (*gal11*Δ), FY2589 (*pgd1*Δ), FY2590 (*med2*Δ), FY2591 (*nut1*Δ), FY2592 (*med1*Δ), FY2605 (*soh1*Δ), FY2593 (*srb10*Δ), FY2602 (*srb9*Δ), FY2594 (*srb2*Δ), FY2595 (*srb5*Δ), FY2597 (*spt8*Δ), FY2600 (*spt6-140*), FY2604 (*set2*Δ), FY2603 (*mot1-1*), and FY2601 (*spt21*Δ*0*::*kanMX*).

reporter in which the UAS-TATA distance is 806 bp. As can be seen by growth on SC-His Gal medium, *sin4*Δ and *spt2*Δ mutations allow long-distance activation at this site as well, showing that their phenotype is not location dependent (Fig. 2B). To discover whether *HIS3* activation is activator specific, we tested our mutants with the reporter in which LexA binding sites are located 642 bases 5′ of the TATA. When testing *sin4*Δ and *spt2*Δ mutants, we found that both are His^+ in strains expressing a LexA-Gal4 fusion protein; however, only *spt2*Δ mutants are His^+ in strains expressing a LexA-Gcn4 fusion protein (Fig. 2C). This result suggests that long-distance activation in *sin4*Δ mutants may be more activator dependent than in *spt2*Δ mutants.

To determine whether activation at even longer distances could occur in *sin4*Δ or *spt2*Δ mutants, we constructed two additional reporters within *BPH1* with UAS-TATA distances of 1,397 and 1,995 bp. While both reporter strains were His^- in combination with *spt2*Δ, a low but visible level of growth was observed in *sin4*Δ mutants (Fig. 2D). This result confirms that *sin4*Δ mutants are quite permissive for long-distance activation. The ability to activate transcription from a distance of almost 2 kb makes the UASs in *sin4*Δ mutants behave similarly to *Drosophila* or mammalian enhancers.

Long-distance activation does not generally occur in Mediator mutants or other mutants that affect chromatin or transcription. Although we had screened both the yeast nonessential deletion set and the *tet*-repressible essential gene set for mutants that allow long-distance activation, our results prompted us to retest particular mutants. To do this, we directly examined additional genes for their role in long-distance activation by combining mutations in these genes with the *BPH1* reporter having a UAS-TATA distance of 799 bp and examining growth on SC-His Gal medium. First, as Sin4 is part of the large Mediator coactivator complex, it was of interest to test whether mutations in other Mediator genes might also allow long-distance activation. Therefore, we tested mutations in almost all of the other genes encoding nonessential subunits of Mediator. With the exception of *srb9*Δ and *srb10*Δ mutants, which showed an extremely weak His^+ phenotype, none of the other Mediator mutants tested were His^+ (Fig. 2E and Table 4).

Some of the mutations that we isolated have also been shown to cause other transcription defects. For example, to various degrees, all four cause an Spt^- phenotype (18, 27, 38, 78), some allow transcription from cryptic promoters within ORFs (41, 51, 57), and some suppress the requirement for a UAS (61). Therefore, to determine whether mutations that affect these phenotypes might also generally affect long-distance activation, we tested a set of mutations previously shown to cause these other phenotypes. Our results (Table 4) show that mutations in *SPT8*, *SPT6*, *SPT21*, *SET2*, and *MOT1* do not allow long-distance activation. Furthermore, we note that there are qualitative differences that suggest that long-distance activation and these other phenotypes are caused by different mechanisms. For example, *spt6* mutants are among the strongest cryptic initiation mutants (41); however, they fail to detectably allow long-distance activation. In contrast, *sin4* mutants have the strongest known long-distance activation phenotype, yet have only a weak cryptic initiation phenotype (V. Cheung, personal communication). These data strongly suggest that we have identified a new class of transcriptional mutants with a distinct phenotype.

Northern and 5′ RACE analysis of long-distance activation. To assay long-distance activation at the transcriptional level and to correlate it with the His phenotype conferred by our reporters, we examined *HIS3* mRNA levels by Northern analysis. We first examined *HIS3* expression in the *BPH1* reporter strains. As expected, in reporter strains that have a His^+ phenotype, wild-type-length *HIS3* mRNA is produced, with the level corresponding to the UAS-TATA distance (Fig. 3A, lanes 3, 4, and 5). At UAS-TATA distances of 690 and 799 bp, which are His^-, extremely low levels of wild-type-length *HIS3* mRNA are detected. Therefore, wild-type-length *HIS3* mRNA levels correlate with the growth phenotypes conferred by the report-

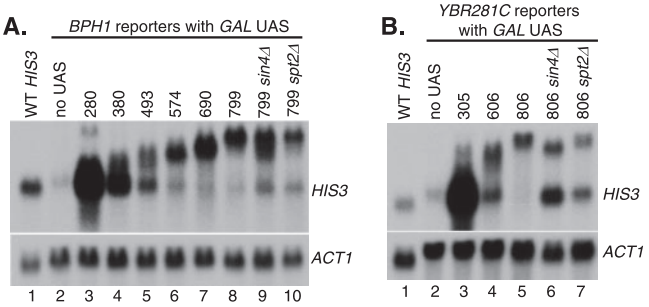

FIG. 3. Northern analysis of reporter strains. (A) Northern analysis of *BPH1 GAL* UAS reporter strains and mutants. A Northern blot was hybridized with a probe for *HIS3* (top panel) and *ACT1* (bottom panel). The strains shown are as follows: FY3 (lane 1), FY2549 (lane 2), FY2550 (lane 3), FY2551 (lane 4), FY2552 (lane 5), FY2553 (lane 6), FY2554 (lane 7), FY2555 (lane 8), FY2562 (lane 9), and FY2570 (lane 10). The numbers above lanes 3 to 10 indicate the UAS-TATA distance in base pairs. (B) Northern analysis of *YBR281C GAL* UAS reporter strains and mutants. The strains shown are as follows: FY3 (lane 1), FY2579 (lane 2), FY2557 (lane 3), FY2558 (lane 4), FY2559 (lane 5), FY2560 (lane 6), and FY2580 (lane 7). WT, wild type. Base pair distances are shown as in panel A.

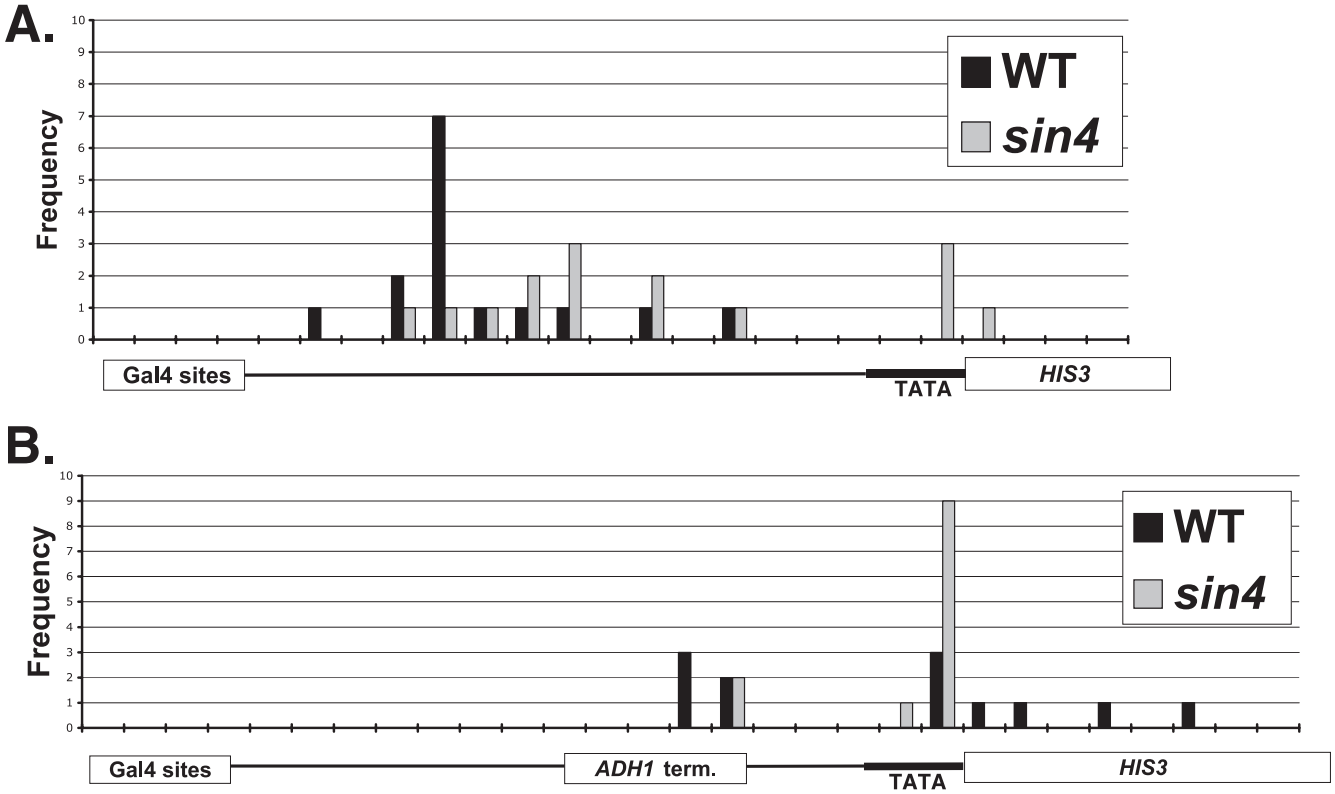

FIG. 4. 5′ RACE analysis of reporter strains, with and without the *ADH1* terminator. (A) Frequency of observed start sites among 5′ RACE clones (see Materials and Methods). The number of clones observed within each 50-bp region (represented by marks along the *x* axis) is plotted on the *y* axis, and the position along the *BPH1* UAS 799 reporter gene is plotted along the *x* axis. Positions of the Gal4 binding sites and *HIS3* promoter and coding regions are indicated. (B) Frequency of observed start sites among 5′ RACE clones of strains containing the terminator reporter (term.), the wild type (WT) (FY2585), and the *sin4*Δ mutant (FY2586). The number of clones observed is plotted on the *y* axis, and the position along the reporter is plotted on the *x* axis. Positions of the Gal4 binding sites, *ADH1* terminator sequences, and *HIS3* promoter and coding regions are indicated. A greater portion of the *HIS3* coding region is shown in panel B to enable the depiction of start sites within the *HIS3* ORF. Note that transcripts that terminate 5′ of the terminator will not be detected, as they will not anneal to the primer used.

ers, showing that greater distance decreases the level of activation. In *sin4*Δ and *spt2*Δ mutants, when we examined a UAS-TATA distance of 799 bp, we found that, as expected, wild-type-length *HIS3* mRNA is produced at a level significantly greater than that in a wild-type background (Fig. 3A, compare lane 8 to lanes 9 and 10). Similar results were observed with the second reporter at *YBR281C*, where we observed even greater levels of *HIS3* mRNA at the most proximal Gal4 binding site locations and in *sin4*Δ and *spt2*Δ mutants (Fig. 3B). Taken together, these results demonstrate that *sin4*Δ and *spt2*Δ mutations allow long-distance activation by Gal4.

In addition to the wild-type-length *HIS3* mRNA, we were surprised to detect long, galactose-induced RNAs expressed in almost every case examined. This pattern was observed for the *GAL1* UAS located at either *BPH1* or *YBR281C* or for LexA or Ace1 binding sites inserted at *BPH1* (Fig. 3; also data not shown). The sizes of these RNAs correspond to the location of the UAS and are consistent with transcription start sites just 3′ of the UAS, regardless of its position. As there are no consensus TATA sequences in any of these reporters except for the known *HIS3* TATA, this transcription is TATA independent. Furthermore, Northern analysis showed that in *sin4*Δ mutants, while the level of the long transcripts was approximately the same as in the wild type, the band corresponding to the long transcripts was reproducibly more diffuse, suggesting that a greater range of start sites are used in this mutant (Fig. 3A, compare lanes 8 and 9). The identification of these long transcripts suggests that UAS elements, in the absence of a nearby promoter, have the potential to initiate promiscuous transcription nearby. This property suggests that it is beneficial for UAS elements to be positioned close to their target promoters.

To precisely determine the 5′ ends of all of the RNAs produced from a long-distance reporter, we used 5′ RACE, comparing wild-type and *sin*Δ strains (Fig. 4A). Our results lead to two main conclusions. First, in strains carrying *sin4*Δ, but not in wild-type strains, initiation occurs at the wild-type *HIS3* start site (36, 70). Second, in both wild-type and *sin4*Δ strains, the long transcripts initiate over a range of positions, all 3′ of the *GAL1* UAS; however, in the *sin4*Δ mutant, the degree of specificity for any site is reduced and the range is significantly greater. These results suggest that Sin4 normally constrains the distance over which transcription start sites can be used. Furthermore, in *sin4*Δ mutants, the distance barrier that exists in wild-type strains is surmounted, allowing accurate long-distance transcriptional activation to occur.

Construction and analysis of a reporter containing a terminator. The discovery of the long transcripts raised the possibility that this transcription might play a role either in the

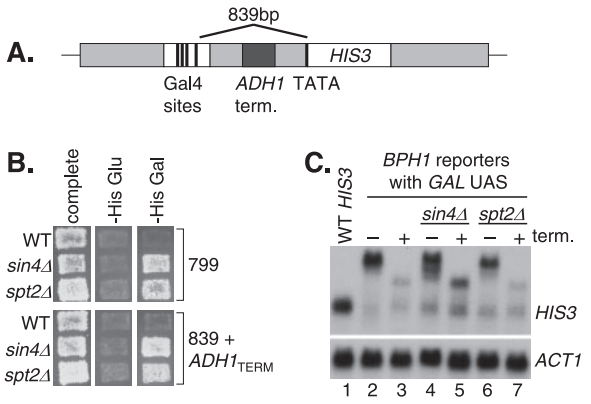

FIG. 5. Experiments with a reporter strain containing the *ADH1* terminator. (A) Schematic of a *BPH1 GAL* UAS reporter with the *ADH1* terminator (term.) sequences integrated between the UAS and *HIS3*. In this reporter strain, the UAS is 839 bp 5′ of the TATA. (B) Comparison of growth between strains containing the *BPH1 GAL* UAS 799 reporter and the *BPH1 GAL* UAS 839 reporter with the *ADH1* terminator. The strains shown are as follows: FY2555 (wild-type [WT] UAS 799), FY2562 (*sin4*Δ UAS 799), FY2570 (*spt2*Δ UAS 799), FY2585 (WT term), FY2586 (*sin4*Δ term), and FY2587 (*spt2*Δ term). Strains were patched on YPD, replica plated to indicated media and grown for 3 days at 30°C. (C) Northern analysis comparing the *BPH1 GAL* UAS 799 reporter and the *BPH1 GAL* UAS 839 *ADH1* terminator reporter. Blots were hybridized with a probe to *HIS3* (top panel) and *ACT1* (bottom panel). The strains shown are as follows: FY3 (lane 1), FY2555 (lane 2), FY2585 (lane 3), FY2562 (lane 4), FY2586 (lane 5), FY2570 (lane 6), and FY2587 (lane 7). −, absence of terminator; +, presence of terminator.

inhibition of long-distance activation in wild-type strains or in enabling long-distance activation in *sin4*Δ and *spt2*Δ mutants. For example, in wild-type strains, the long transcript might somehow prevent long-distance activation by transcriptional interference over the *HIS3* TATA region. Alternatively, in *sin4*Δ and *spt2*Δ mutants, the long transcript might be required for long-distance activation by allowing RNA polymerase to track from the UAS to the *HIS3* core promoter. To attempt to address these issues, we integrated the strong *ADH1* terminator between the *GAL1* UAS and the *HIS3* TATA in the *BPH1* reporter with a UAS-TATA distance of 839 bp (Fig. 5A). If transcriptional interference is the primary mechanism by which long-distance transcriptional activation is prevented, then integration of the terminator will result in the expression of the reporter and a His^+ phenotype. Such a result was obtained in the case of transcriptional interference at the *S. cerevisiae SER3* gene (50). We found, however, that a wild-type strain with the *ADH1* terminator integrated between the *GAL1* UAS and *HIS3* TATA is still His^-, supporting the hypothesis that distance, and not transcriptional interference, is the reason that activation does not occur at the *HIS3* start site (Fig. 5B). We also tested the *BPH1* reporter containing the *ADH1* terminator in *sin4*Δ and *spt2*Δ mutants to determine whether transcription across the length of the *BPH1* reporter was required for *HIS3* expression in these mutants. Both *sin4*Δ and *spt2*Δ mutants are His^+, even with the terminator between the UAS and TATA, suggesting that transcription through the *BPH1* reporter is not required for long-distance activation (Fig. 5B). This result suggests that RNA polymerase II does not need to track along the template to activate at a distance.

Northern analysis of these strains confirms that the long transcript is efficiently terminated (Fig. 5C; also data not shown), and as expected, wild-type-length *HIS3* mRNA is made in *sin4*Δ and *spt2*Δ mutants (Fig. 5C, lanes 4 to 7). An unexpected finding, however, was that in wild-type strains with this modified reporter, there is a transcript produced at a low level that appears to be the length of wild-type *HIS3* mRNA (Fig. 5C, lane 3). This possibility seemed unlikely, since the strain has a tight His^- phenotype. To analyze this class of RNAs more precisely, we performed 5′ RACE analysis to determine the 5′ ends, from both wild-type and *sin4*Δ backgrounds. Our results again showed there to be a range of transcription start sites, extending from just 3′ of the terminator to within the ORF of *HIS3* (Fig. 4B). Unlike what was observed for the reporter strains lacking the terminator, in wild-type strains, transcription could now start over a wider range of positions, including a low level of initiation from the normal *HIS3* start site. The insertion of the terminator between the UAS and TATA, then, does seem to help the UAS to communicate with the correct start site at a low level, suggesting that transcriptional interference may play some role in preventing *HIS3* transcription. However, given the His^- phenotype of this strain, there is not an adequate level of functional *HIS3* mRNA to alter the phenotype. In *sin4*Δ mutants, transcripts started at the correct location significantly more often than in the wild-type strain (Fig. 4B). These results support the view that Sin4 normally functions to select the proper sites for transcription initiation.

3C analysis. Since the long transcript is efficiently terminated by the *ADH1* sequences, we hypothesized that a loop might form between the UAS and the TATA for transcription to initiate 3′ of the terminator. To test for such a loop, we used 3C analysis (1, 23). In this technique, chromatin was cross-linked and digested with the restriction enzymes BfaI and MseI that cut on either side of, and at several sites between, the Gal4 binding sites and the *HIS3* TATA (Fig. 6A). Digested chromatin was then ligated at low concentration to maximize intramolecular ligations over intermolecular ones, and PCR was performed to detect the ligation products by using primers that anneal to specific regions in the reporter gene (Fig. 6A). If the Gal4 binding sites and *HIS3* TATA are held physically close to one another by a bridge of transcription factors, then these regions should remain physically associated after restriction enzyme digestion. Our results show that the growth of either wild-type or *sin4*Δ strains in galactose leads to an enrichment of a PCR product specific to a ligation between the restriction fragments containing the Gal4 binding sites and *HIS3* TATA (Fig. 6B). Importantly, these PCR products require formaldehyde cross-linking and ligation (Fig. 6C). We do not detect a difference between wild-type and *sin4*Δ mutants, indicating that *sin4*Δ mutants affect start site selection and long-distance activation at a step subsequent to loop formation.

DISCUSSION

In this study, we designed a system using *S. cerevisiae* to examine long-distance transcriptional activation in vivo and have used it to demonstrate that activation by Gal4 diminishes with increasing UAS-TATA distance. Our results confirm those of previous studies that showed that increasing the dis-

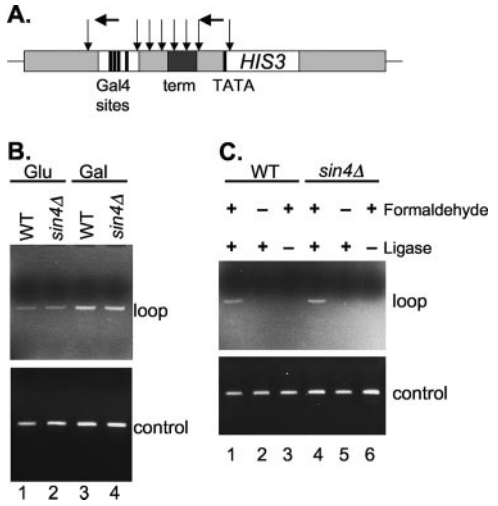

FIG. 6. Molecular evidence for galactose-induced looping between the UAS and TATA. (A) Schematic of *BPH1 GAL* UAS 839 *ADH1* terminator reporter (term.). Horizontal arrows denote the positions of same-strand oligonucleotide sequences that were used in 3C analysis (see Materials and Methods). Vertical arrows show the positions of some of the BfaI and MseI restriction enzyme cut sites along the reporter; there are 13 total sites between the TATA and Gal4 binding sites. (B) 3C analysis of looping between the UAS and TATA in the wild-type (WT) (FY2585) and the *sin4*Δ mutant (FY2586) strains (see Materials and Methods). Products of PCR using same-strand oligonucleotides correspond to the ligation of fragments containing the Gal4 binding sites and *HIS3* TATA. Control PCR to normalize the amounts of DNA in the samples was performed using convergent oligonucleotides that amplify a region of chromosome V with no restriction enzyme cut sites. (C) Gal-induced looping between the UAS and TATA is dependent upon cross-linking and ligation. 3C analysis was performed as described in Materials and Methods, except without cross-linking (lanes 2 and 5) or addition of ligase (lanes 3 and 6). −, absence of formaldehyde and ligase; +, presence of formaldehyde and ligase.

tance between a UAS and TATA impedes transcriptional activation (31, 71). In addition, our studies yielded two new sets of information regarding *S. cerevisiae* UAS function and the potential for long-distance activation. First, we were surprised to discover that when the *GAL1* UAS is located distant from a core promoter, it directs transcription initiation events just 3′ of its position, independently of both genomic location and activator and apparently in the absence of core promoter elements. This result suggests that UAS-proximal transcription is an inherent property of UAS elements. Such events would not normally be detected because of the compact nature of the *S. cerevisiae* genome. Second, our isolation of loss-of-function mutations that allow long-distance activation suggests that long-distance activation is normally repressed in *S. cerevisiae.* Our genetic evidence suggests that this repression depends upon a small number of factors, as our screens identified mutations in only four loci that cause this phenotype. Interestingly, other previously studied mutations known to suppress the requirement for a UAS or to allow initiation from cryptic promoters do not allow long-distance activation, indicating that the repression of long-distance activation that occurs in wild-type cells does not require several factors known to be required for a repressive chromatin state. Thus, long-distance activation may be normally repressed by an aspect of chromatin structure outside those that are currently known to regulate transcription initiation and elongation.

Our results have shown that the *GAL1* UAS directs the expression of a long RNA whose transcription is independent of genomic location or activator. This finding suggests that promiscuous transcription initiation at proximal sites may be an inherent property of UAS elements. This conclusion is consistent with recent studies of transcription start sites in *Drosophila*, mouse, and human. Whole-genome analysis of *Drosophila* has discovered a large number of intergenic transcripts that correspond to newly identified 5′ exons of known genes (49). Additionally, an analysis of mammalian genomes has uncovered a number of "megatranscripts" that span megabases (13). Strikingly similar to what we have observed with our reporter genes, a transcript originates from the locus control region of the mammalian β-globin locus as those genes are actively being transcribed (3, 42). In these larger eukaryotes, it has been proposed that such intergenic transcription may keep chromatin structure in the region of the enhancer open and accessible for binding by transcription factors (11). Thus, the ability to direct nearby transcription initiation events may be a property conserved between UASs and enhancers.

The importance of strictly limiting long-distance activation in *S. cerevisiae* is evident from its genome organization. In contrast to what is seen for larger eukaryotes, the *S. cerevisiae* genome is very compact, with the distance between ORFs generally within a range of 150 to 400 bp, depending upon the gene configuration, with many coding regions under 1 kb (28, 59). The mutations we have identified enable communication between UAS and TATA regions that are at least 800 bp apart, and in the case of *sin4*Δ mutants, almost 2 kb apart. If the entire yeast genome were able to make efficient UAS-TATA interactions at this distance, there would be a significant disruption to normal regulation and transcription. Therefore, it is clearly critical for *S. cerevisiae* and other organisms with compact genomes to constrain activation distance.

Given the importance of constraining long-distance activation, one might expect that mutations that are permissive for this type of event would cause poor growth or inviability due to widespread aberrant gene expression. However, of the loci we identified, only *HTA1-HTB1* is essential for growth (46). Furthermore, there is little correlation between the growth rate and the strength of long-distance activation in our mutants, as the strongest long-distance activation mutant, *sin4*Δ, has only a mild growth defect. Consistent with this observation, recent microarray analysis of *sin4*Δ suggests that it does not cause dramatic changes in transcript levels genome-wide (74). The fitness of these strains is most likely explained by the model that a UAS will preferentially act at a nearby core promoter even in a *sin4*Δ mutant, where it has the modest ability to activate at a distance. This preference for activation from proximal sites is supported by our observation that the *GAL1* UAS in a wild-type strain will activate primarily from proximal sites, even in the absence of core promoter elements. Thus, in a *sin4*Δ mutant, nearby core promoters may serve as barriers or insulators for long-distance activation. It seems possible that mutations that allow a stronger level of long-distance activation might be able to overcome such barriers.

While our results have clearly shown that long-distance activation in *sin4*Δ and other mutants can occur, we do not yet

understand the mutant alteration that allows this type of activation. Initial genetic tests suggest that a *sin4*Δ *spt2*Δ double mutant is similar to a *sin4*Δ single mutant with respect to long-distance activation strength, suggesting that they may work by similar means. In addition, the presence of the *ADH1* transcription terminator does not inhibit long-distance activation in either *sin4*Δ or *spt2*Δ mutants, suggesting that tracking of RNA polymerase II along the template is an unlikely mechanism. A previous study of UAS-TATA interactions found that in a wild-type strain with a UAS-TATA distance of less than 300 bp, the same *ADH1* terminator did block transcriptional activation (8). The difference between those results and ours seems likely to be caused by the *sin4*Δ and *spt2*Δ mutant backgrounds in which our experiment was performed. Given the nature of the genes identified, chromatin structure also seems likely to play a role in long-distance activation, at the level of either nucleosome positioning or histone modification. In addition, other mechanisms, such as localization of the gene within the nucleus (10, 14, 52), might play a role. As the level of transcription in our long-distance activation mutants is considerably lower than that in genes usually studied for chromatin effects, such as *GAL1* or *PMA1*, it is conceivable that the events that allow long-distance activation may be below the level of detection. The isolation of stronger long-distance activation mutants, or enhancers of those already found, will facilitate molecular analysis.

Our studies also suggest that both local differences in chromatin structure as well as activator strength affect the potential for long-distance activation. Between the two reporters that we have studied, the one located at *YBR281C* on chromosome II is detectably more permissive for long-distance activation than the one at *BPH1* on chromosome III, and LexA-Gal4 is a better activator than LexA-Gcn4, consistent with their relative strengths at short distances (9). Previous studies of *PHO5* showed that activation of that gene occurs even after the insertion of several hundred base pairs between the UAS and TATA (54), indicating that the location of *PHO5* or activation by Pho4 is better suited for long-distance activation than the cases we have examined. These results suggest that the threshold at which UAS-TATA distance is too great for activation is not static but varies depending upon local chromatin structure and activator strength.

Taken together, our data have contributed to a clearer understanding of the constraints on long-distance transcriptional activation in yeast. In addition, our results have provided evidence that proximal transcription is a common property of UAS elements, similar to metazoan enhancers. Our reporters, especially our LexA reporters, provide a system for the study of specific transcription factors in long-distance transcriptional activation. Further studies of our mutants should reveal the mechanisms by which wild-type cells are able to repress long-distance interactions between UAS and TATA elements.

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