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Analysis of Gal4-directed transcription activation using Tra1 mutants selectively defective for interaction with Gal4

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Promoter-specific transcriptional activators (activators) stimulate transcription through direct interactions with one or more components of the transcription machinery, termed the “target.” The identification of direct in vivo targets of activators has been a major challenge. Previous studies have provided evidence that the Tra1 subunit of the yeast SAGA (Spt-Ada-Gcn5-acetyltransferase) complex is the target of the yeast activator Gal4. However, several other general transcription factors, in particular the mediator complex, have also been implicated as Gal4 targets. Here we perform a large-scale genetic screen to derive and characterize *tra1* alleles that are selectively defective for interaction with Gal4 in vivo [Gal4 interaction defective (GID) mutants]. In contrast to WT Tra1, Tra1 GID mutants are not recruited by Gal4 to the promoter and cannot support Gal4-directed transcription, demonstrating the essentiality of the Gal4–Tra1 interaction. In yeast strains expressing a Tra1 GID mutant, binding of Gal4 to the promoter is unexpectedly also diminished, indicating that Gal4 and Tra1 bind cooperatively. Consistent with cooperative binding, we demonstrate that the Gal4–Tra1 interaction occurs predominantly on the promoter and not off DNA. Finally, we show that although Tra1 is targeted by other activators, these interactions are unaffected by GID mutations, revealing an unanticipated specificity of the Gal4–Tra1 interaction.

bimolecular fluorescence complementation | acidic activation domain | protein–protein interaction

Transcription initiation by RNA polymerase II involves the assembly of general transcription factors on the core promoter to form a preinitiation complex (PIC). Promoter-specific activator proteins (activators) work, at least in part, by increasing PIC formation (1–4). Activators are modular proteins that contain a DNA-binding domain (DBD) and an activation domain (AD). Activator-mediated stimulation of PIC assembly is believed to result from a direct interaction between the AD and one or more components of the transcription machinery, termed the “target.” The unambiguous identification of the direct in vivo targets of activators has been a major challenge in the field.

Transcriptional induction of yeast genes involved in galactose utilization (*GAL* genes) has been a model experimental system for studying transcriptional activation mechanisms. The well-characterized acidic activator Gal4 is responsible for the transcriptional stimulation of *GAL* genes, such as *GALI*, which contain Gal4-binding sites in their promoters (5–7). In the absence of galactose, the negative regulator Gal80 binds to the Gal4 AD and inhibits its activity (5, 6). Galactose triggers an association between Gal80 and Gal3, relieving Gal80-mediated inhibition of Gal4.

We have been studying the yeast histone acetyltransferase (HAT) Spt-Ada-Gcn5-acetyltransferase (SAGA) complex and its potential role as an activator target (8–10). SAGA regulates the transcription of $\approx 10\%$ of yeast genes, including *GAL* genes (11). Previous studies from our laboratory and others have provided evidence that the Tra1 subunit of SAGA is a target of Gal4. Tra1 has also been proposed as a target of several other activators, in particular Gcn4, which like Gal4 has an acidic AD (12–15).

A number of factors in addition to Tra1 have also been implicated as Gal4 AD targets, including TATA-box binding protein

(TBP) (16, 17), TFIIB (16), the Swi/Snf complex (18), TBP-associated factors (19) and, in particular, one or more components of the mediator complex (19–23). In general, mutations in the Gal4 AD compromise the interaction with all of these proposed targets, confounding the question of which interaction(s) is essential for transcriptional activation in vivo. Here we derive Tra1 mutants that are selectively defective for interaction with Gal4 and use them to study how Gal4 stimulates transcription in vivo and the basis by which Tra1 is recognized by Gal4 and other activators.

Results

Isolation of *tra1* Mutants That Cannot Support Growth on Galactose.

The strategy we used to derive Tra1 mutants that fail to interact with the Gal4 AD is summarized in Fig. S1A and described below. We generated a library of random *tra1* mutants by in vitro hydroxylamine mutagenesis of a low-copy plasmid expressing *TRAI*. The library was transformed into a haploid yeast *tra1*- Δ strain that was complemented by WT *TRAI* expressed on a low-copy URA3-containing plasmid. After eviction of the WT *TRAI* plasmid on media containing 5-fluoroorotic acid (5-FOA), strains harboring the *tra1* mutants were analyzed for growth on media containing glucose (YPD) but not galactose (YPG). This approach yielded 13 *tra1* mutants that were unable to support growth on YPG (Fig. 1A).

Sequence analysis revealed that the 13 *tra1* mutants represented eight distinct alleles (Fig. S1B). Mutants 2–7 were found to contain the same mutations, and therefore, of these mutants only *tra1*-*mut2* was further analyzed. Immunoblot analysis showed that all of the Tra1 mutants were expressed at levels comparable to that of WT Tra1 (Fig. S1C).

To confirm that the *tra1* mutants selectively abolished *GAL* gene expression, we monitored expression of two *GAL* genes, *GALI* and *GAL3*, by quantitative RT-PCR (qRT-PCR). In strains harboring the eight *tra1* mutants, transcription of *GALI* and *GAL3* in galactose-containing media was severely compromised relative to that observed in a WT *TRAI* strain (Fig. 1B). By contrast, expression of *RPS5* and *RPS0B*, which are SAGA-independent genes, were unaffected by the *tra1* mutants (Fig. S1D).

Development of a Bimolecular Fluorescence Complementation Assay for Detecting Interactions Between Activators and Tra1 in Vivo.

To detect direct interactions between the mutant Tra1 proteins and Gal4 in vivo, we developed a bimolecular fluorescence complementation (BiFC) assay, which has been used for the in vivo

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo/ (accession no. GSE31391).

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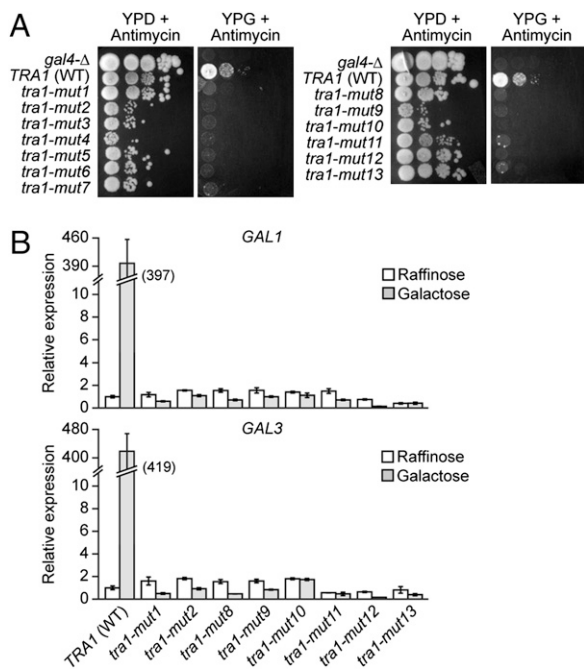


Fig. 1. Isolation of *tra1* mutants that cannot support growth on galactose. (A) Growth of *tra1* mutants 1–13 on YPD and YPG media supplemented with antimycin. Growth of WT *TRA1* and *gal4-Δ* strains are shown as controls. Cells were spotted as 10-fold serial dilutions. (B) qRT-PCR analysis monitoring expression of *GAL1* and *GAL3* in strains expressing WT *TRA1* or *tra1* mutant grown in raffinose or galactose. Expression of each gene was normalized to that observed in the WT *TRA1* strain grown in raffinose, which was set to 1. The fold induction in galactose in the WT *TRA1* strain is indicated. Error bars indicate SD.

detection of a wide variety of protein–protein interactions (24, 25). The BiFC assay is based on the formation of a fluorescent complex comprising two fragments of YFP, which are brought together by association of two interacting proteins fused to the fragments (reviewed in ref. 26).

To verify the feasibility of this approach we first performed a series of control experiments that monitored the interaction between Tra1 and two SAGA-dependent activators, Gal4 and Gcn4. The experimental strategy for detecting activator–Tra1 interactions using the BiFC assay is shown in Fig. 2A. We derived a pair of haploid yeast strains, one in which the endogenous Tra1 protein was tagged at the C terminus with the N-terminal fragment of a YFP variant known as Venus (27) (Tra1-VN), and a second strain of opposite mating type in which the endogenous activator was tagged at its C terminus with the C-terminal Venus fragment (Gal4-VC or Gcn4-VC). Tra1-VN was fully functional, as evidenced by its ability to substitute for WT Tra1 in a complementation assay (Fig. S2). The two haploid strains were then mated, and the resulting diploid cells were analyzed by fluorescence microscopy for a nuclear BiFC signal.

As described above, because of Gal80-mediated inhibition, the Gal4–Tra1 interaction is expected to occur in the presence but not the absence of galactose. Fig. 2B (Upper) shows that a BiFC signal was detected in cells expressing Tra1-VN and Gal4-VC and grown in YPG but not YPD. Identical results were obtained when the Venus fragment was fused to Tra1 at its N terminus (VN-Tra1) (Fig. 2B, Lower). Thus, the BiFC assay detected the Gal4–Tra1 interaction, which, as expected, was galactose dependent.

A Gcn4–Tra1 interaction is expected to occur only under conditions of amino acid starvation. Fig. 2C shows that a nuclear BiFC signal was not detected in cells expressing Tra1-VN and Gcn4-VC grown in nutrient-rich media (YPD) but was readily detected in amino acid-starved cells grown on histidine (His)-lacking medium in the presence of the competitive inhibitor 3-aminotriazole (3-AT). Collectively, the results of Fig. 2

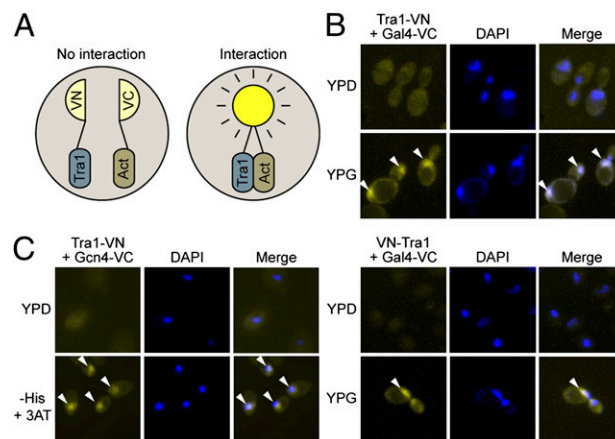


Fig. 2. Development of a BiFC assay for detecting interactions between activators and Tra1 in vivo. (A) Schematic diagram depicting the BiFC assay. Tra1 is tagged at the C terminus with the N-terminal Venus fragment (VN), and the activator (Act) is tagged at the C terminus with the C-terminal Venus fragment (VC). (B) BiFC assay monitoring interaction between Tra1 and Gal4 in vivo, as evidenced by intense YFP signal (arrowheads) in YPG. The Tra1–Gal4 interaction occurs in the nucleus, as evidenced by colocalization (arrowheads) with the DNA stain DAPI. Tra1 was tagged at either the C terminus (Upper) or N terminus (Lower). (C) BiFC assay monitoring interaction between Tra1 and Gcn4 in vivo, as evidenced by intense YFP signal in response to amino acid starvation (–His + 3-AT media).

demonstrate that the BiFC assay can be used to detect interactions between activators and Tra1.

Identification of Tra1 Mutants That Are Unable to Interact with Gal4.

We used the BiFC assay to analyze the interaction between Gal4 and the Tra1 mutants. In these and subsequent BiFC experiments described below, the endogenous activator was tagged at the C terminus with VC in a haploid *tra1-Δ* strain that was complemented by WT *TRA1* expressed on a URA3-containing plasmid. The strain was then transformed with a low-copy plasmid expressing a Tra1 mutant protein fused to VN at either the C terminus (Tra1-mut1, -mut2, -mut8, -mut9, -mut12, and -mut13) or N terminus (Tra1-mut10 and -mut11), and the BiFC signal was monitored after eviction of the WT *TRA1* plasmid. As expected, in cells expressing WT Tra1-VN, a nuclear BiFC signal could be detected upon growth on galactose (Fig. 3A). By contrast, a BiFC signal was not detected in cells expressing any of the Tra1 mutants.

We have previously proposed that Tra1 must be incorporated into an intact SAGA complex for interaction with Gal4 in vivo (10). Therefore, one explanation for the inability of the Tra1 mutants to interact with Gal4 is a failure to be incorporated into SAGA. To address this issue, we analyzed the stable association between Tra1 mutants and the SAGA subunit Spt20 in a coimmunoprecipitation assay. Fig. 3B shows that Tra1-mut1 and Tra1-mut8 coimmunoprecipitated with Spt20 at levels comparable to that of WT Tra1. Similar results were obtained with two other SAGA subunits, Spt3 and Spt7 (Fig. S3A). By contrast, the other mutants were completely (Tra1-mut2, -mut9, -mut10, -mut12, and -mut13) or partially (Tra1-mut11) defective for interaction with Spt20. Tra1 is also a subunit of another HAT complex known as NuA4 (28). Fig. S3B shows that all Tra1 mutants coimmunoprecipitated with the NuA4 subunit Eaf1 at WT or, in one instance (Tra1-mut2), modestly reduced levels, indicating that they were all incorporated into the NuA4 complex. Collectively, these results indicate that Tra1-mut1 and Tra1-mut8 are incorporated into the SAGA complex but are unable to interact with Gal4 and thus can be classified as Gal4 interaction defective (GID) mutants. Tra1-mut8 is of particular importance because it contains only a single amino acid substitution at position 400 (H400Y) (Fig. S1B). The inability of Tra1-mut1 and Tra1-mut8 to interact with Gal4 and support transcription of *GAL* genes

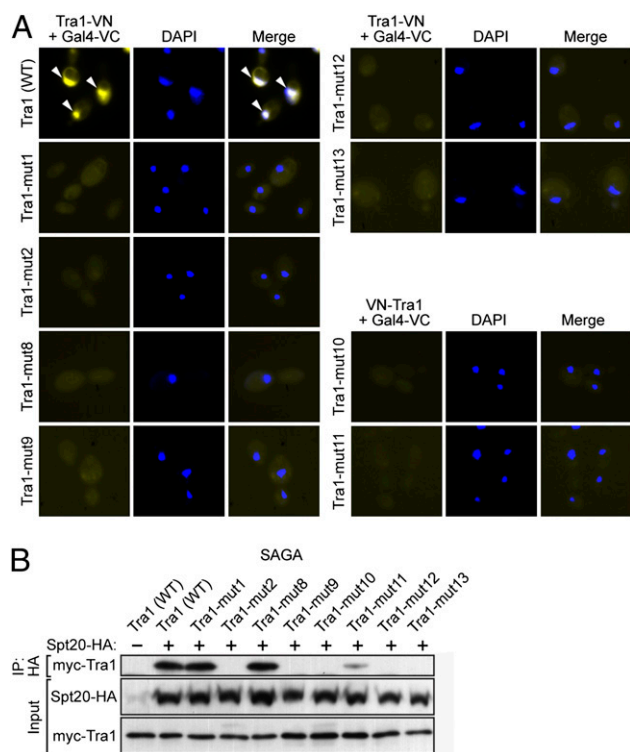


Fig. 3. Identification of Tra1 mutants that are unable to interact with Gal4. (A) BiFC assay monitoring the interaction between Gal4 and the mutant Tra1 proteins. (B) Coimmunoprecipitation assay. Spt20-HA was immunoprecipitated with an anti-HA antibody, and the immunoprecipitate analyzed for the presence of Tra1. The levels of Spt20 and Tra1 in the input extract are shown.

conclusively establishes that the Gal4–Tra1 interaction is essential for Gal4-directed transcription activation.

Gal4 and Tra1 Bind Cooperatively to the *GAL1* Promoter. The interaction between Gal4 and Tra1 is expected to result in recruitment of the SAGA complex to the promoters of *GAL* genes. Therefore, in a yeast strain expressing a Tra1 GID mutant, the SAGA complex should not be recruited to *GAL* genes. To confirm this prediction we performed a series of ChIP assays. As expected, in galactose association of Tra1-mut1 and Tra1-mut8 with the *GAL1* promoter was substantially reduced relative to WT Tra1 (Fig. 4A). Likewise, association of Spt20 with the *GAL1* promoter was comparably reduced in the two Tra1 GID mutant strains. By contrast, recruitment of Tra1-mut1 and Tra1-mut8 to the promoter of *RPS0B*, a NuA4-dependent gene, was comparable to that of WT Tra1.

Fig. 4A also shows that in raffinose Gal4 binding was roughly equivalent in the WT Tra1 and Tra1 GID mutant strains. Unexpectedly, in galactose binding of Gal4 to the *GAL1* promoter was substantially reduced in the Tra1 GID mutant strains, even though Gal4 levels were equivalent to that observed in the WT Tra1 strain (Fig. S4A). Similar results were observed at the *GAL7* promoter (Fig. S4B). Binding of Gal4 to the *GAL1* promoter was also reduced in an *spt20*- Δ strain (Fig. 4B), which does not support the Gal4–Tra1 interaction (10). Collectively, the results in Fig. 4A and B indicate that as a result of the Gal4–Tra1 interaction, Gal4 and Tra1/SAGA bind cooperatively to the promoter.

Gal4–Tra1 Interaction Occurs Predominantly on the Promoter and Not off DNA. An important, unresolved question is whether in vivo the interaction between an activator and its target occurs predominantly on the promoter or whether the activator–target interaction is sufficiently stable that it occurs off DNA. To address this issue, we used the BiFC assay to monitor the interaction between

Tra1 and a LexA–Gal4 AD fusion-protein that can bind to DNA only in yeast strains engineered to contain LexA-binding sites. A summary of the experimental design is shown in Fig. 4C and discussed below. We first constructed a haploid *tra1*- Δ strain harboring plasmids expressing Tra1-VN and a LexA DBD–Gal4 AD fusion-protein tagged with the C-terminal Venus fragment [LexA(DBD)–Gal4(AD)–VC]. This strain was transformed with a series of constructs to derive three strains, the first of which (strain 1) expressed a high-copy plasmid containing four LexA-binding sites located upstream of a *GAL1-lacZ* reporter gene (29). Strain 2 was identical to strain 1 except that the construct lacked upstream LexA-binding sites (29). In strain 3 the *GAL1* core promoter sequence, which is the site at which SAGA and other PIC components are recruited, was deleted from the *GAL1-lacZ* reporter gene. Immunoblot analysis confirmed that the LexA(DBD)–Gal4(AD)–VC fusion-protein was expressed equivalently in the three strains (Fig. S5).

We analyzed the interaction between the Gal4 AD and Tra1 in these three strains using the BiFC assay. Fig. 4D shows that a nuclear BiFC signal was detected in strain 1 but not in strain 2, indicating a requirement for LexA-binding sites. As expected, in strain 1 the nuclear BiFC signal was observed only in galactose. The nuclear BiFC signal was also absent from strain 3, which lacked the *GAL1* core promoter. Collectively, these results indicate that the Gal4 AD–Tra1 interaction occurs predominantly on DNA and is dependent upon both activator-binding sites and the core promoter. We note that in a previous study (8) a Gal4–Tra1 interaction could be detected on a minimal Gal4-binding site in a ChIP assay, perhaps because the cross-linking agent formaldehyde enabled “trapping” of the low-affinity, otherwise transient interaction between Gal4 and Tra1.

As a control, we performed an analogous experiment to monitor the interaction between Gal4 and its negative regulator, Gal80. Fig. 4E shows that a nuclear BiFC signal was detected in all three yeast strains, indicating, as expected, that the Gal4–Gal80 interaction is not dependent upon either activator binding sites or the core promoter. Notably, unlike the Gal4–Tra1 interaction, the Gal4–Gal80 interaction occurred in both raffinose and galactose, which is in agreement with previous biochemical experiments showing that in galactose Gal80 remains physically associated with Gal4 at a second site (30).

Gal4-Interaction Site on Tra1 Is Highly Selective. We next performed experiments to determine whether the Tra1 GID mutants were selectively defective for interaction with the Gal4 AD or were unable to interact with other activators that also targeted Tra1. Fig. 5A shows that both Tra1 GID mutant strains grew on His-lacking medium containing 3-AT, indicating that the mutants could support Gcn4-directed transcription. Furthermore, a nuclear BiFC signal could be detected in strains expressing Gcn4-VC and either WT Tra1 or a Tra1 GID mutant (Fig. 5B). Thus, unlike Gal4, Gcn4 functionally interacts with Tra1 GID mutants.

To further investigate the selectivity of the Gal4 interaction site on Tra1, we sought to identify other activators that interact with Tra1 in the SAGA complex and determine their sensitivity to the Tra1 GID mutations. Toward this end, we first identified Tra1-dependent genes by comparing the mRNA population of a WT *TRAI* strain to that of a strain bearing a temperature-sensitive *tra1* allele (*tra1-2^{ts}*) (31) under nonpermissive conditions. After inactivation of Tra1, $\approx 3\%$ of yeast genes were down-regulated greater than twofold (Dataset S1), consistent with a previous study that analyzed other *tra1* alleles (32).

To identify a set of Tra1-dependent genes that were also SAGA-dependent, we analyzed the 20 genes most affected by Tra1 inactivation (Table S1) for dependence on Spt20. We first mined a published expression profiling study for genes whose transcription is compromised in an *spt20*- Δ strain (11) and then confirmed these in silico results by qRT-PCR. This combined analysis identified 11 genes whose transcription was compromised, relative to WT Tra1, by inactivation of Tra1 or loss of Spt20 (Fig. 6A). In addition, ChIP analysis showed, as expected, that Tra1 was bound to the promoters of all 11 genes (Fig. S6A).

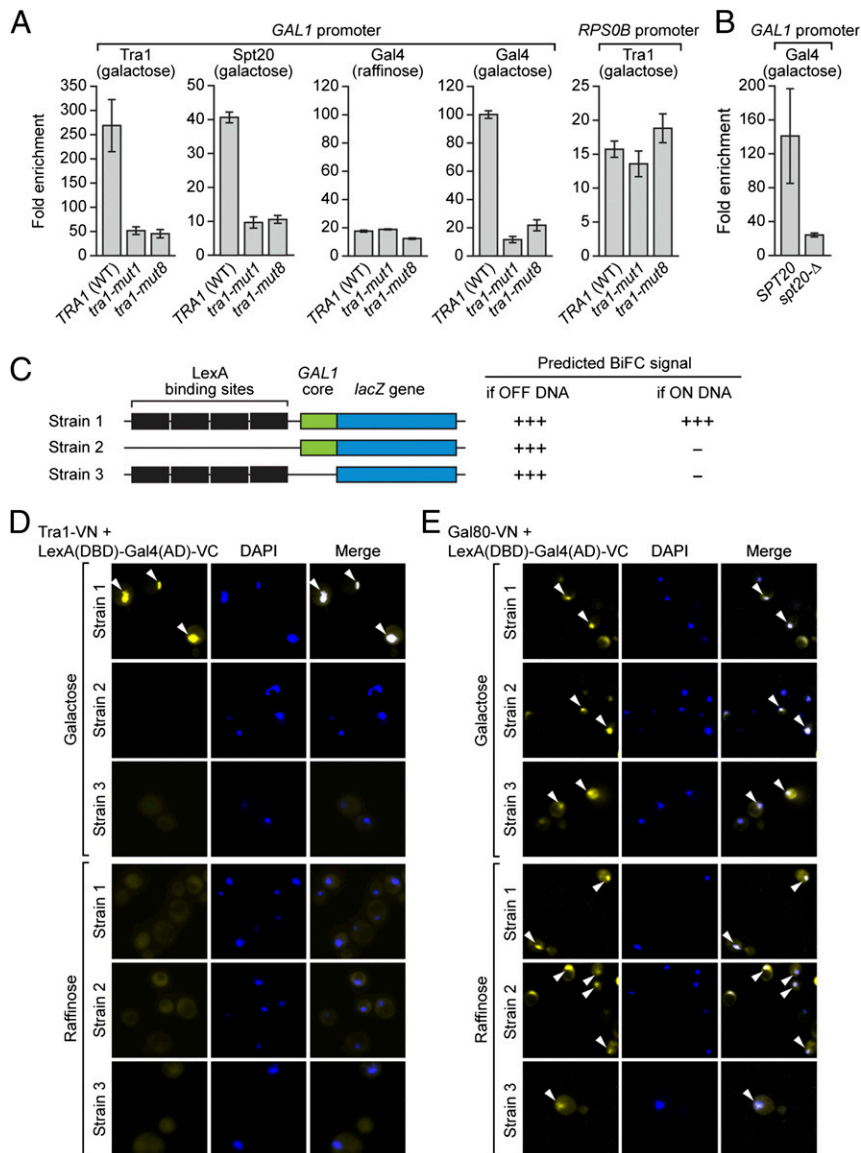


Fig. 4. Gal4 and Tra1 bind cooperatively to the *GAL1* promoter. (A) ChIP assay monitoring recruitment of Tra1, Spt20, and Gal4 to the *GAL1* and *RPS0B* promoters in strains expressing WT *TRAI1*, *tra1-mut1*, or *tra1-mut8* and grown in media containing galactose or raffinose. Error bars indicate SD. (B) ChIP assay monitoring recruitment of Gal4 to the *GAL1* promoter in a WT *SPT20* or *spt20-Δ* strain. (C) Schematic diagram of the BiFC-based strategy to detect whether the Gal4–Tra1 interaction occurs predominantly on or off DNA. (D) BiFC assay monitoring the interaction between Tra1-VN and LexA(DBD)-Gal4(AD)-VC in the three yeast strains grown in galactose or raffinose. (E) BiFC assay monitoring the interaction between Gal80-VN and LexA(DBD)-Gal4(AD)-VC in the three yeast strains grown in galactose or raffinose.

Significantly, the two Tra1 GID mutants did not significantly affect transcription of any of these 11 Tra1- and SAGA-dependent genes (Fig. 6A).

Next, we tested the ability of the activators that mediate expression of the 11 Tra1- and SAGA-dependent genes to interact with WT Tra1 and the Tra1 GID mutants. To identify activators involved in regulating expression of the Tra1- and SAGA-dependent genes, we searched published genome-wide ChIP-microarray (ChIP-chip) studies (33–37). Using this approach, predicted activators could be identified for 10 of the 11 Tra1- and SAGA-dependent genes analyzed above (Table S1).

We selected five activators for further analysis: Cbf1, Fkh2, Mcm1, Reb1, and Zap1. Fig. 6B shows that a nuclear BiFC signal was detected in yeast strains expressing WT Tra1-VN and either Cbf1-VC, Fkh2-VC, Mcm1-VC, Reb1-VC, or Zap1-VC (see also Fig. S6B). Thus, as predicted, all five of these activators directly interact with Tra1. Notably, a nuclear BiFC signal was also observed with all five activators in strains expressing either Tra1 GID mutant. Thus, consistent with the transcription results of Fig. 6A, these activators directly interact with Tra1, but this interaction is insensitive to the GID mutations.

Finally, in an independent approach, we attempted to identify genes whose transcription was affected by the Tra1 GID mutants

by comparing genome-wide expression profiles of yeast strains harboring either WT *TRAI1*, *tra1-mut1*, or *tra1-mut8* grown in YPD. Remarkably, we found only two genes (*GSC2* and *HSP30*) whose expression was affected more than twofold by the *tra1-mut1* mutation and no genes that were affected more than twofold by the *tra1-mut8* mutation (Fig. 6C and Dataset S1). Moreover, even for *GSC2* and *HSP30*, qRT-PCR did not confirm the difference in expression levels observed by microarray analysis and instead revealed that expression of these two genes was comparable in *tra1-mut1*, *tra1-mut8*, and the WT *TRAI1* strains (Fig. S6C). Collectively, these results indicate that remarkably few, and possibly no other, yeast activators target the same Tra1 region at which Gal4 interacts.

Discussion

The results presented here definitively establish Tra1 as an essential *in vivo* target of Gal4 by showing that Tra1 mutants selectively defective for interaction with Gal4 cannot support Gal4-directed transcription. It seems most likely that the sites of the GID mutations correspond to the region(s) of Tra1 at which Gal4 interacts. However, we cannot rule out the alternative possibility that the mutations induce a conformational change that affects Gal4 interaction at a putative distinct site on Tra1. In either case,

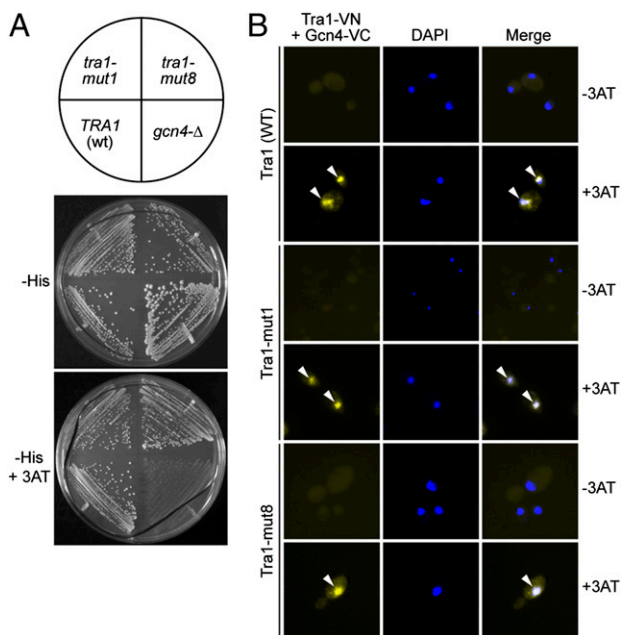


Fig. 5. Gcn4 functionally interacts with the Tra1 GID mutants. (A) Growth of *tra1-mut1* and *tra1-mut8* on His-lacking media containing or lacking 3-AT. Growth of WT *TRA1* and *gcn4-Δ* strains are shown as controls. (B) BiFC assay monitoring the interaction between Tra1-VN and Gcn4-VC in His-lacking media containing or lacking 3-AT.

the Tra1 mutants are defective for interaction with Gal4 and therefore do not affect our major conclusions. For Tra1 to interact with Gal4 it first must be incorporated into an intact SAGA complex. The amino acids in Tra1 that we find compromise assembly into the SAGA complex are consistent with the results of a recent study analyzing Tra1 functional domains (38). Our results indicate that Tra1 does not have an intrinsic ability to interact with the Gal4 AD but rather requires proper presentation within the SAGA complex. It seems likely that this finding is relevant to the selective interaction of Gal4 with SAGA and not the NuA4 complex, which also contains Tra1.

Several experimental observations have led to the suggestion that an activator will have multiple, functionally redundant targets. For example, *in vitro* protein–protein interaction experiments have shown that a single activator such as Gal4 (16–23) or Gcn4 (39, 40) can interact with multiple components of the transcription machinery. Likewise, in artificial recruitment experiments, a wide variety of transcription components can stimulate transcription and thus could potentially function as targets (3). However, in contrast to this view, we demonstrate that interaction of Gal4 with a single site on Tra1 is required for Gal4-directed transcription.

The fact that many yeast activators contain acidic ADs, with apparently similar sequence features, has suggested that activators have common targets and recognition sites. Surprisingly, however, we find that the Gal4-interaction region on Tra1 is remarkably specific. Our collective results suggest that at most very few, and likely no other, yeast activators functionally interact with the same region of Tra1 that is recognized by the Gal4 AD.

In this study, we have performed a series of BiFC experiments whose results show that the interaction between the Gal4 AD and Tra1 occurs predominantly on the promoter and not off DNA. These new results explain our previous finding that a Gal4 mutant lacking its DBD failed to interact with Tra1 *in vivo* (10). Our results help explain how cellular activators avoid a transcription inhibitory process referred to as “squenching,” which occurs after overexpression of a strong AD, such as the herpes simplex virus VP16 AD (41, 42). Squenching results from the sequestration of the target by the activator off the promoter; the target is thus unavailable for promoter-bound activators,

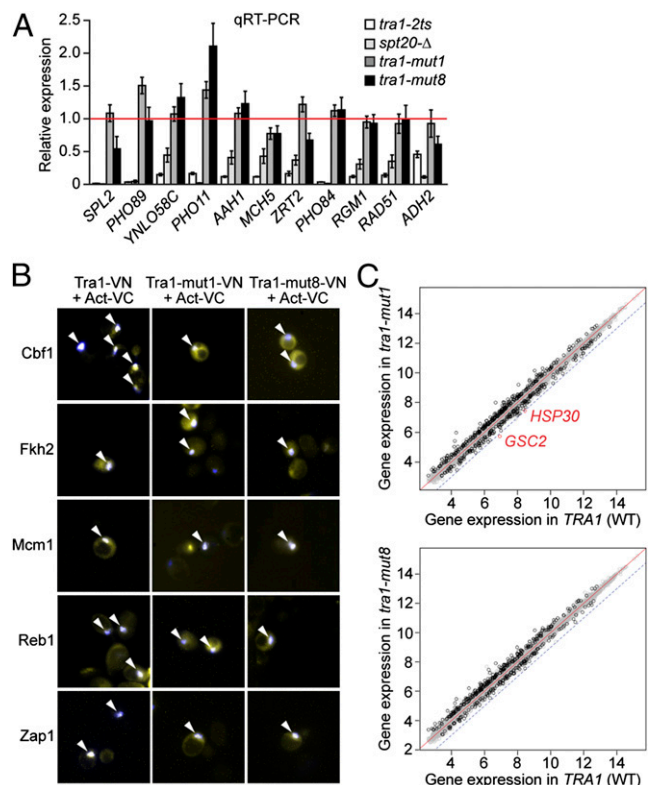


Fig. 6. The Gal4-interaction site on Tra1 is highly selective. (A) qRT-PCR analysis monitoring expression of 11 Tra1- and SAGA-dependent genes in *tra1-ts*, *spt20-Δ*, *tra1-mut1*, and *tra1-mut8* strains. Gene expression is presented relative to that observed in a WT strain, which was set to 1 (indicated by the red line). Error bars indicate SD. (B) BiFC analysis monitoring the ability of WT Tra1, Tra1-mut1, or Tra1-mut8 to interact with various activators. Only merged images are shown (see also Fig. S6B). (C) Scatter plot analyses comparing gene expression in a WT *TRA1* strain (Upper) or *tra1-mut8* strain (Lower). The red line represents no change in gene expression; the blue dotted line represents twofold down-regulation. Gray circles represent all of the genes on the array; black circles represent genes with a P value < 0.05 ; red circles represent genes with a P value < 0.05 and down-regulated more than twofold.

resulting in transcriptional inhibition. By interacting with Tra1 predominantly on the promoter and not off DNA, Gal4 avoids squenching. Squenching is dependent upon both the strength and concentration of the overexpressed AD (41, 42). On the basis of these considerations, we speculate that cellular activator–target interactions are in general weak, thus ensuring that they occur only on the promoter where they are stabilized by the many other protein–protein and protein–DNA interactions in the PIC.

Methods

TRA1 Mutagenesis Screen. Plasmid pRS414-pDED1-myc-*TRA1* (SI Methods) was mutagenized by treatment with 1 M hydroxylamine (Sigma), 50 mM sodium pyrophosphate (pH 7.0), 100 mM NaCl, and 2 mM EDTA at 75 °C for 30 min (43). The mutagenized library was amplified in bacteria and transformed into a haploid *tra1-Δ* strain LLY154 (Table S2). Cells were plated on –Trp-5-FOA media, and $\approx 1,200$ 5-FOA-resistant colonies were patched and replica plated onto YPD and YPG media containing 20 μ g/mL antimycin A (Sigma). Colonies able to grow on YPD but not YPG were selected, and the plasmid was isolated and sequenced. Strains carrying *tra1-mut1* and *tra1-mut8* were also analyzed for growth on –His media containing 50 mM 3-AT.

qRT-PCR. Total RNA was extracted (44), and reverse transcription was performed using SuperScript II Reverse Transcriptase (Invitrogen) followed by qPCR using Fast SYBR Green Master Mix (Applied Biosystems) using the primers listed in Table S3. For the experiments shown in Fig. 1B and Fig. S1D, cells were grown in 2% raffinose followed by 2% galactose for 5 min.

BiFC Assay. The BiFC signal in cells was examined by fluorescence microscopy using a Zeiss AXIO Imager Z2 microscope. A total of 100 cells from at least seven different fields were counted; representative examples are shown.

Immunoblotting and Coimmunoprecipitation Assays. For Fig. S1C, whole-cell extracts were prepared as previously described (45), and blots were probed with a myc (Santa Cruz) or actin (Abcam) antibody. For Fig. S5, extracts were prepared from strains grown in galactose or raffinose medium, and blots were probed with a LexA (Santa Cruz) or actin (Abcam) antibody. For the coimmunoprecipitation experiments of Fig. 3B and Fig. S3B, Spt20-HA or Eaf1-HA was immunoprecipitated with an HA antibody, and blots were probed with an HA (Abcam) or myc (Santa Cruz) antibody. For the coimmunoprecipitation experiments of Fig. S3A, myc-Tra1 was immunoprecipitated with a myc antibody (Abcam), and endogenous Spt proteins were detected using an Spt3 (Abcam) or Spt7 (Santa Cruz) antibody.

ChIP. ChIP was performed as described previously (33) using a myc (Abcam), HA (Abcam), or Gal4 (Abcam) antibody. After reversal of the cross-links, the DNA was PCR-amplified using gene-specific primers (listed in Table S3).

Microarray Analyses. Strains MDC1 and MDC3 were grown at 30 °C and shifted to 37 °C for 60, 90, and 120 min. Haploid *TRA1*, *tra1-mut1*, and *tra1-mut8* strains were generated as described in *SI Methods*. RNA was extracted according to standard protocols (44) and hybridized to an Affymetrix YG-598 array. The *tra1-mut1/TRA1* and *tra1-mut8/TRA1* experiments were done in duplicate. Statistical analyses were performed using R (46). The RMA method (47) in the Affy package from Bioconductor (48) was used to summarize the probe level data and normalize the dataset to remove across array variation. The Limma package (49) with randomized block design was used to determine whether a gene's expression level differs between mutant and WT regardless of time point. Genes with adjusted *P* value using the B-H method (50) <0.05 were considered significant. The microarray data from this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE31391.

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