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The VP16 Activation Domain Interacts with Multiple Transcriptional Components as Determined by Protein-Protein Cross-linking *in Vivo**

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Transcriptional activator proteins recruit the RNA polymerase II machinery and chromatin-modifying activities to promoters. Biochemical experiments indicate that activator proteins can associate with a large number of proteins, and many such proteins have been proposed to be direct targets of activators. However, there is great uncertainty about which biochemical interactions are physiologically relevant. Here, we develop a formaldehyde-based cross-linking procedure to identify protein-protein interactions that occur under physiological conditions. We show that the VP16 activation domain directly interacts with TATA-binding protein (TBP), TFIIB, and the SAGA histone acetylase complex *in vivo*.

Transcriptional activator proteins regulate the expression of eukaryotic genes in response to developmental and environmental cues. Such activator proteins contain a DNA-binding domain that recognizes specific promoter DNA sequences and a physically separate transcriptional activation region that stimulates mRNA initiation by RNA polymerase II (1–4). Activation domains are functionally autonomous; they function when fused to heterologous DNA-binding domains tethered at different positions in the promoter region. The best characterized activation domains are defined by short acidic regions that show little primary sequence homology (5, 6). Mutational analysis indicates that acidic and hydrophobic residues within these domains contribute to functional activity, although individual residues make only a minor contribution (4, 7–10). Acidic activation domains do not have a defined tertiary structure but become structured only upon specific interaction with another protein (11, 12). Taken together, these observations indicate that acidic activation domains are surfaces used to mediate protein-protein interactions. It is presumed that other types of activation domains, such as those rich in glutamine (13) or proline (14) residues, function in a similar manner.

Chromatin immunoprecipitation experiments indicate that, *in vivo*, activator proteins mediate the recruitment of the Pol II machinery and chromatin-modifying activities (*e.g.* the Swi/Snf nucleosome remodeling complex and the SAGA and NuA4 histone acetylase complexes) to promoters (15–20). However, such experiments do not define the direct targets of activator proteins. In yeast cells, individual components of the Pol II machinery associate with promoters in a mutually interdependent manner (15, 16), and direct connection of a DNA-binding domain to virtually any component of the Pol II machinery suffices for transcription (21, 22). Thus, activator-dependent recruitment of the Pol II machinery to promoters *in vivo* could be due to a direct contact to any component of the Pol II machinery. In addition, activator-dependent recruitment of the Pol II machinery could be an indirect consequence of activator-dependent changes in chromatin structure. Activator-dependent recruitment of Swi/Snf and SAGA can occur even when the Pol II machinery is not associated with promoters (17, 23, 24), consistent with the idea that activators directly interact with these chromatin-modifying complexes. However, chromatin immunoprecipitations experiments are inherently unable to determine which components of the Pol II machinery or which chromatin-modifying activities directly interact with activator proteins *in vivo*.

In vitro, transcriptional activators can interact with TATA-binding protein (TBP)[1] (25–27), TBP-associated factors (TAFs) (28, 29), TFIIA (30), TFIIB (31), TFIIH (32), components of the mediator subcomplex of RNA polymerase II holoenzyme (33–36), Swi/Snf (34, 37, 38), SAGA (39, 40), and NuA4 (40). However, it is generally not understood which of these interactions occur under physiological conditions and are relevant for transcriptional activation *in vivo*. In many cases, activator-target interaction experiments are performed under very artificial conditions. For example, standard GST pulldown experiments involve very high concentrations of activation domains and potential targets, and the potential targets are often assayed as isolated proteins rather than multiprotein complexes that occur in cells. GST pulldowns, and other techniques such as co-immunoprecipitation and far-Western blotting, are prone to binding artifacts, and this is particularly likely for acidic activation domains, which are largely unstructured and have an abundance of negative and hydrophobic residues. For example, while the strength of biochemical interactions between activation domains and several potential targets strongly correlates with the transcriptional potency of the activation domain, this correlation is equally strong for activator binding to lysozyme, a protein that is clearly not a physiologically relevant target (41).

Biochemical interactions between activators and potential targets have also been identified by photo-cross-linking. This approach has identified the Tra1 subunit of the SAGA complex (42), several subunits of the Swi/Snf complex (43), and the Srb4 subunit of the mediator complex (44) as direct targets of activation domains *in vitro*. In the case of Tra1, mutations that

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[1] The abbreviations used are: TBP, TATA-binding protein; TAFs, TBP-associated factors; HA, hemagglutinin.

reduce the interaction with the activation domain without compromising the integrity of the SAGA complex show transcriptional activation defects *in vivo*. These experiments provide strong evidence that the Tra1 subunit of SAGA is a physiologically relevant target. However, there is no direct physical evidence for these activator-target interactions *in vivo*.

We wished to develop a new procedure that can detect protein-protein interactions inside living cells under physiological conditions. To that end, we utilized formaldehyde, which rapidly permeates the cell and generates protein-protein and protein-DNA cross-links. Proteins that are cross-linked to transcriptional activators are co-immunoprecipitated under stringent conditions and then identified by Western blotting after reversal of the formaldehyde cross-links. Kinetic experiments using formaldehyde cross-linking to measure protein-DNA association *in vivo* strongly suggest that formaldehyde inactivates cellular enzymes almost immediately upon addition to the growing cells and that the 20-min incubation time merely increases the cross-linking in fixed and metabolically inert cells (45–47). As such, formaldehyde cross-linking is likely to provide a snapshot of protein-protein interactions at the particular time point. Here we use this technique to address whether TFIID, TFIIA, and TFIIB and the SAGA histone acetylase complex interact directly with activation domains in yeast cells.

EXPERIMENTAL PROCEDURES

Construction of Plasmids and Yeast Strains—The plasmids listed in Fig. 1 were constructed by PCR amplification of the various segments and insertion into the indicated restriction sites of the *LEU2* vector YCplac111 (48). In addition, *Apa*I and *Sal*I sites and a His_6 tag were introduced between the *Nco*I site and the *CYC1* termination domain. The activation domains described in Fig. 7 were cloned into this construct between the *Bam*HI and *Nco*I sites (Hap4, Ace1, Adr1) or between the *Nco*I and *Sal*I sites (Gcn4, Put3). The *GAL-LacZ* reporter plasmid pRY131 contains a 2μ origin of replication and a *URA3* marker (49). All yeast strains were derived from a Research Genetics strain (record number 11044) with a *gal4* deletion (*MAT*α *his3*−Δ1 *leu2*-Δ0 *lys2*-Δ0 *ura3*-Δ0 *gal4*::*KAN*. Yeast strains expressing 3-Myc-tagged proteins from the normal genomic locus were obtained by using gene-specific PCR primers to amplify derivatives of pMPY-3xMYC DNA, introducing the resultant PCR fragment into yeast cells by one-step integration, followed by looping out of the *URA3* marker (50). pMPY-3xMYC DNA was modified by inserting the *CYC1* termination region (246 bp) into the *Bam*HI site (pDH035 for C-terminal tagging) or insertion of the *ADH1* (1200 bp) or *TEF1* (400 bp) promoter into the *Eco*RI site (pDH036 and pDH037 for N-terminal tagging); these modifications maintain stable expression of the target yeast protein before looping out of the *URA3* marker. All proteins were tagged at the C terminus except for TBP, TAF8, Spt7, Spt20, and Tra1. Yeast strains bearing SAGA deletions (*ada2*, no. 4282; *spt3*, no. 4228; *spt20*, no. 7390) were obtained from Research Genetics and were derived from BY4741 (*MAT***a** *his3*-Δ1 *leu2*-Δ0 *met15*-Δ0 *ura3*-Δ0). Strains containing the desired Myc-tagged protein, SAGA deletion, and *gal4* deletion were generated by mating and tetrad dissection.

Cross-linking—Cells were grown in 200 ml of synthetic complete medium lacking uracil and leucine to OD = 0.4, and then $CuSO_4$ (1 mM) was added for 1.5 h. A 37% solution of formaldehyde (5.4 ml) was added directly to the culture to bring the final concentration to 1%. After 20 min, cross-linking was quenched by addition of 2 M glycine (60 ml). The cells were harvested, washed with 400 ml of cold Tris-buffered saline followed by 40 ml of cold lysis buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 10 mM EDTA, 5 mM EGTA, and 1% Triton X-100). Cells were resuspended in 1 ml of lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 1× Complete™ protease inhibitor mixture (Roche Molecular Biochemicals). To this suspension, 1.5 ml of zirconia/silica beads (0.5 mm, BioSpec Products) was added, and then the cells were disrupted on a Mini-Beadbeater (BioSpec Products) with 6 pulses (1 min each) at full power with icing between cycles. The mixture was transferred to a Falcon tube and separated from the beads by centrifugation through a needle hole into a 30-ml tube. The beads were further washed with 3 ml of lysis buffer plus protease inhibitors. The lysate was then sonicated with 4 × 30-s pulses (Branson Ultrasonics Sonifier Model 450, 50% pulses, power = 7) and centrifuged for 20 min at 32,000 × *g*. The supernatant was transferred to a 15-ml tube taking care that no solid material from the pellet was dislodged. α-HA monoclonal antibody (12CA5) was coupled to protein A-Sepharose beads (Amersham Biosciences) with dimethyl pimelimidate. In the case of Western blots involving proteins larger than 100 kDa, the antibody was not covalently coupled to the resin due to high molecular mass background. For each reaction, 30 μl of ascites fluid and 60 μl of beads were incubated with the supernatant for 1 h at 4 °C. The beads were transferred to a 2-ml column and washed 4 × 3 ml with lysis buffer with 0.1% sodium deoxycholate and 0.1% SDS, 2 × 3 ml of the same buffer but with 500 mM NaCl, 2 × 3 ml of 10 mM Tris-HCl, pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 2 × 3 ml of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The beads were then transferred to a 1.5-ml microcentrifuge tube with TE and pelleted. The TE was removed to dryness with a 25-gauge needle. The immunoprecipitated material was removed by incubating the beads in 60 μl of 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% SDS at 65 °C for 10 min. The beads were removed by filtration, and 5× SDS-PAGE buffer was added to the eluted material. After reversing the cross-links by boiling for 20 min, SDS-PAGE gels were run using 3 μl of immunoprecipitated material for α-HA Western blots, 15 μl for α-TAF61, and 30 μl for α-Myc. The α-TAF61 and α-Myc Western blots were detected with SuperSignal® West Femto Substrate (Pierce).

RESULTS

In Vivo Cross-linking of VP16 Activation Domain to TAF12—The yeast strains in these experiments express the chimeric activator Gal4-VP16, which consists of the Gal4 DNA-binding domain fused to the VP16 transcriptional activation domain, or related proteins that contain mutated forms of either domain (Fig. 1*A*). To facilitate immunopurification, each protein also contains three copies of the HA epitope at the N terminus (51). As high levels of Gal-VP16 are toxic to yeast cells (52, 53), expression is controlled by a copper-inducible promoter that is essentially inactive in the absence of copper (54). The yeast strains also contain a multicopy *LacZ* reporter plasmid to monitor the transcriptional activity as well as to provide additional DNA binding sites from which to activate transcription. Within the range of copper tolerated by the cells, the level of transcription continuously increased, and no toxicity was observed (Fig. 1*B*).

To generate protein-protein cross-linking *in vivo*, copper-induced cells were treated with 1% formaldehyde for 20 min. Gal4-VP16 derivatives and cross-linked proteins were immunopurified from cell-free extracts with antibodies against the HA epitope under stringent conditions. A control Western blot with the antibody against the HA epitope verifies that equal amounts of each fusion protein were immunoprecipitated (Fig. 1*C*).

Western blots of the immunoprecipitated samples using an antibody against TAF12 (previously known as TAF68 or TAF61) (55), a component of both TFIID and SAGA, reveals that TAF12 cross-links to Gal4-VP16 but not to the Gal4 DNA-binding domain alone (Fig. 1*C*). VP16-TAF12 cross-linking depends on formaldehyde treatment (see Fig. 6), and the level of cross-linking is proportional to the amount of activator expressed (data not shown). DNase I treatment of the cell-free extract destroys the DNA but does not affect the level of the observed VP16-TAF12 cross-link (data not shown), indicating that observed interactions *in vivo* reflect protein-protein cross-linking that are not mediated through the DNA. Under the conditions shown here, we estimate that ~1% of the total TAF12 is cross-linked to the VP16 activation domain.

The level of cross-linking is correlated with the functional quality of the VP16 activation domain, because the Gal4-VP16-F442P derivative shows reduced levels of activation and TAF12 cross-linking. However, VP16-dependent cross-linking occurs at the same level even upon deletion of the N-terminal 31 residues of Gal4, which disrupts the zinc-finger domain essential for DNA binding and hence prevents Gal4-dependent tran-

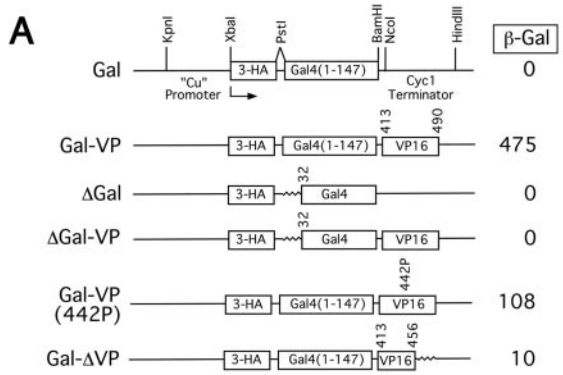
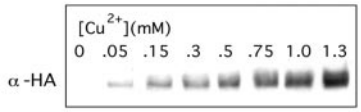
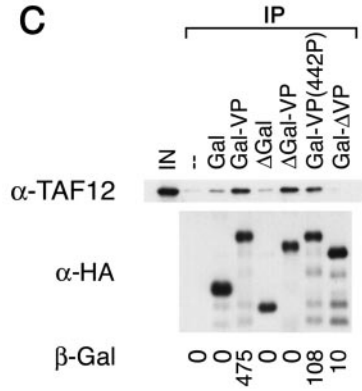


FIG. 1. **The VP16 activation domain cross-links to TAF12.** *A*, expression of Gal-VP16 fusion proteins containing the indicated regions of the Gal4 DNA-binding domain and VP16 activation domain. All proteins contain three copies of the HA epitope at the N terminus and are expressed from a copper-inducible promoter (54). Levels of transcriptional activity on the pRY131 *GAL-LacZ* reporter gene are indicated in units of β-galactosidase activity after induction for 1.5 h with 1 mM $CuSO_4$. *B*, β-galactosidase activity (measured in strains expressing Gal and Gal-VP proteins) and Gal4-VP16 protein levels (Western blot probed with α-HA antibody) as a function of copper concentration. *C*, TAF12 cross-links to the VP16 activation domain. Formaldehyde-cross-linked proteins were immunoprecipitated with HA antibodies, and the resulting material analyzed by Western blotting using HA and TAF12 antibodies. Transcriptional activities conferred by the fusion proteins are indicated.

B

	β-Galactosidase	
$[Cu^{2+}]$ (mM)	Gal	Gal-VP
0.00	10	7
0.10	3	108
0.25	7	197
0.40	4	251
0.50	4	308
0.60	2	337
0.75	3	375
1.00	4	463
1.25	4	504





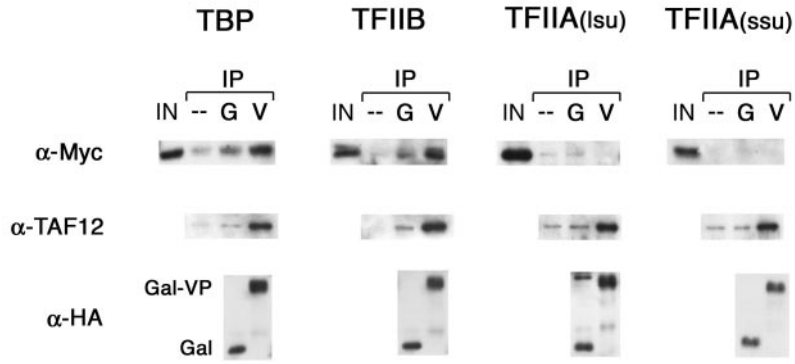


FIG. 2. **The VP16 activation domain cross-links to TBP and TFIIB but not to TFIIA.** Immunoprecipitations were performed with the α-HA antibody on cross-linked samples from strains expressing the Gal (*G*), the Gal-VP plasmid (*V*), or no plasmid (–) as well as Myc-tagged versions of TBP, TFIIB, and either the large (*lsu*) or small (*ssu*) subunit of TFIIA. The resulting materials were analyzed by Western blotting with antibodies against the Myc epitope, HA epitope (to monitor immunoprecipitation efficiency), and TAF12 (which serves as a positive control for each sample).

scription *in vivo*. Thus, although cross-linking depends on the presence of the VP16 activation domain, it does not depend on a functional DNA-binding domain.

VP16 Cross-linking to Other Transcriptional Components—To determine whether the VP16 activation domain cross-links to other potential target proteins *in vivo*, we generated an isogenic set of yeast strains in which individual proteins were tagged with the Myc epitope. In each case, following cross-linking, the immunoprecipitated material was analyzed with antibodies against the Myc epitope as well as the antibody against TAF12, which serves as an internal control to verify the cross-linking and co-immunoprecipitation procedure. As shown in Fig. 2, we observed a VP16-dependent cross-link to TBP and TFIIB, but to neither subunit of TFIIA. Next, we examined all 14 TAF components of TFIID (56) for their ability to cross-link with the VP16 activation domain *in vivo* (Fig. 3). VP16-dependent cross-linking is observed with a number of TAFs, but only TAFs that are also present in the SAGA complex are cross-linked (TAF5, TAF6, TAF9, and TAF12; weak cross-linking to TAF10 is also observed).

These results suggest that the VP16 activation domain interacts directly with TBP, TFIIB, and SAGA *in vivo*. Further support for the VP16 interaction with SAGA comes from the observation that many non-TAF components of SAGA (Ada1, Spt3, Spt7, Spt20, Tra1) also co-purify with VP16 following cross-linking. Some SAGA subunits (Ada2, Ada3, and Gcn5, which is responsible for the histone acetylase activity of the SAGA complex), are present in the related ADA complex (57). However, we did not detect VP16-dependent cross-linking to Ahc1, a subunit that is specific to the ADA complex (Fig. 4), suggesting that VP16 does not interact with ADA *in vivo*.

Cross-linking in SAGA Mutant Strains—The above results

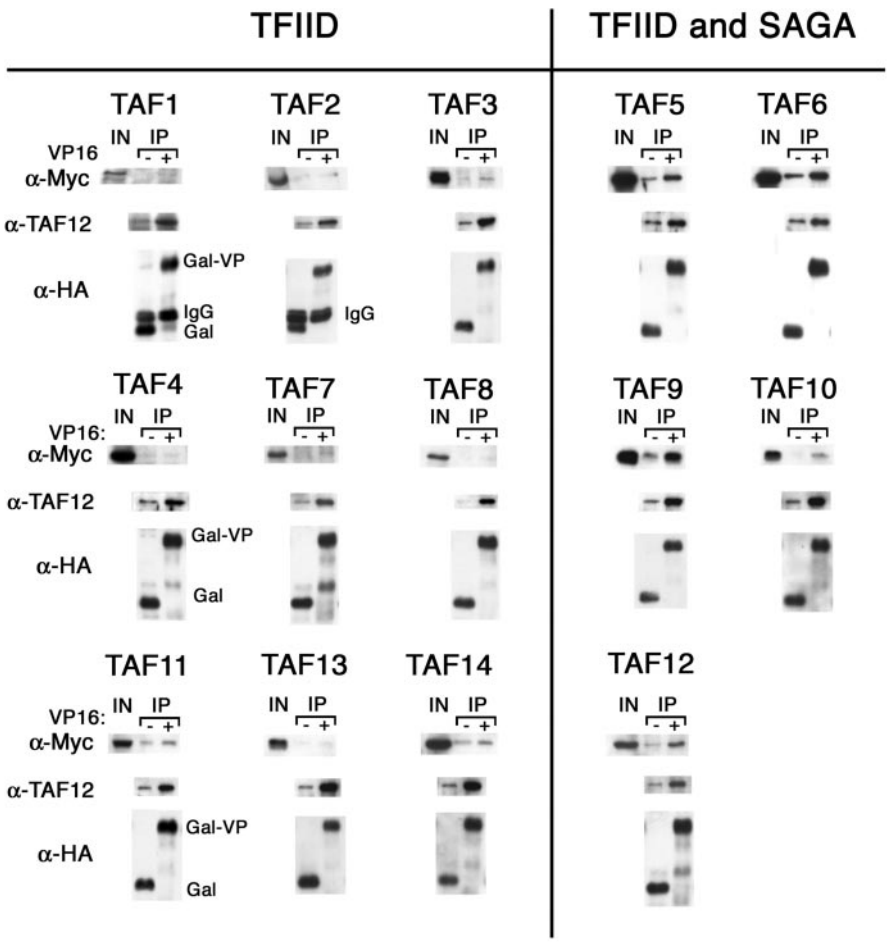


FIG. 3. **The VP16 activation domain cross-links to multiple TAFs in the SAGA complex but not to TFIID-specific TAFs.** Immunoprecipitations were performed with the α-HA antibody on cross-linked samples from strains expressing the Gal (–) or Gal-VP plasmid (+) as well as Myc-tagged versions of the indicated TAFs. The resulting samples were analyzed as described in Fig. 2.

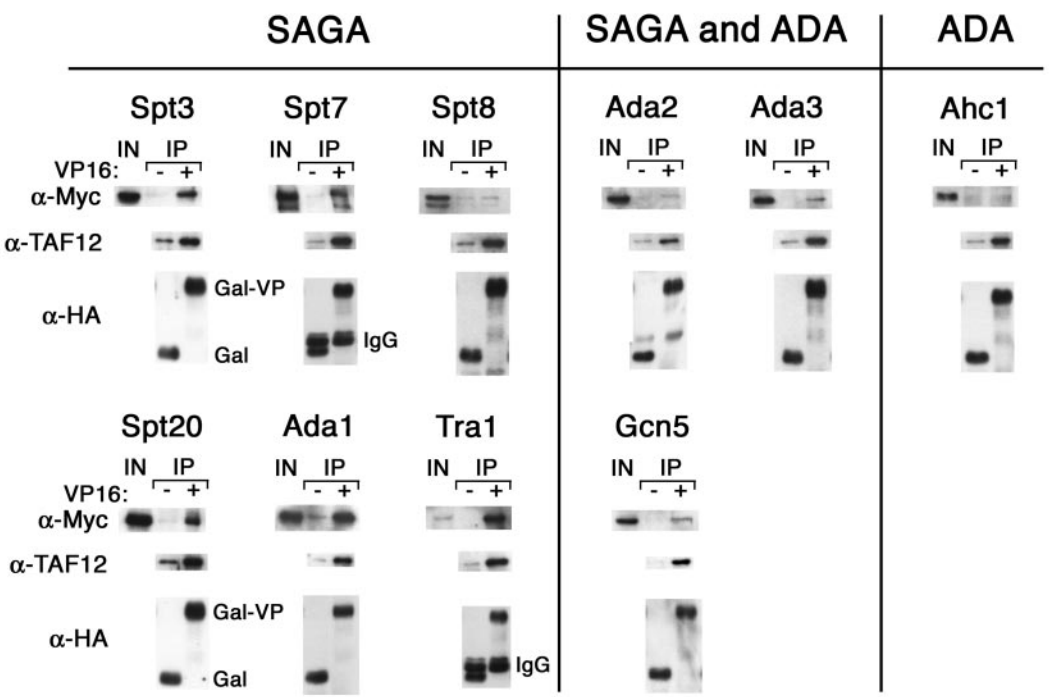


FIG. 4. **The VP16 activation domain cross-links to multiple subunits of SAGA but not to the ADA complex.** Immunoprecipitations were performed with the α-HA antibody on cross-linked samples from strains expressing the Gal (–) or Gal-VP plasmid (+) as well as Myc-tagged versions of the indicated SAGA and/or ADA subunits. The resulting samples were analyzed as described in Fig. 2.

strongly suggest that the VP16 activation domain directly interacts with the SAGA complex and not TFIID *in vivo*. To demonstrate this directly, cross-linking was examined in strains deleted for individual genes encoding SAGA subunits. SAGA subunits have been categorized into three functional types based on genetic and biochemical observations (23, 24,

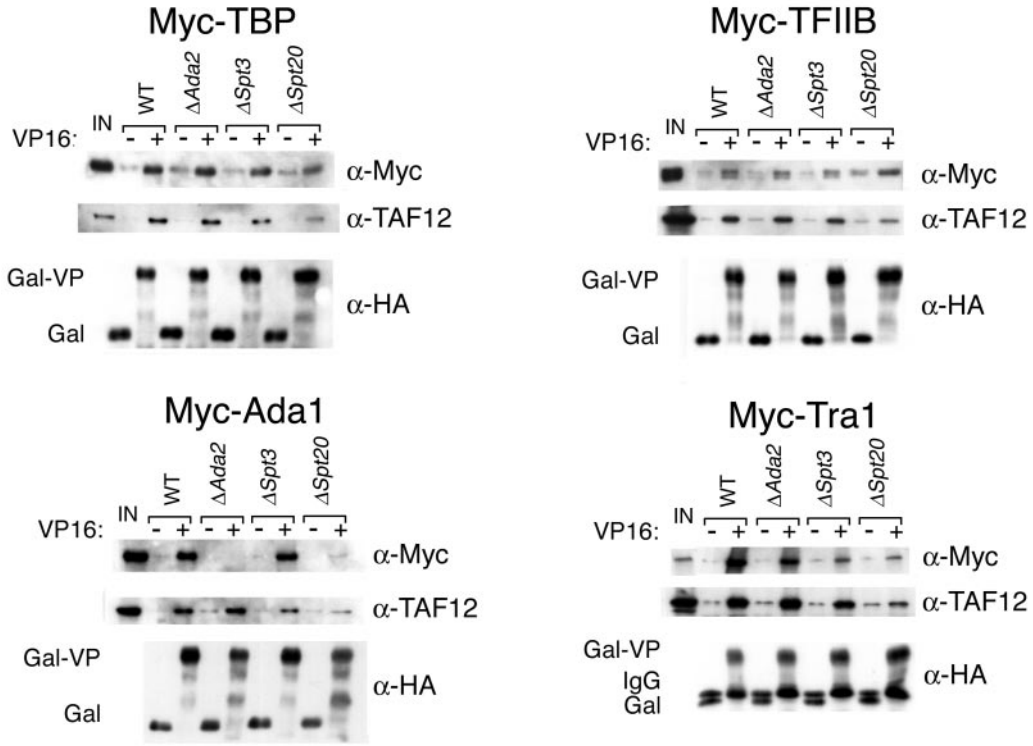


FIG. 5. **Cross-linking in strains lacking an intact SAGA complex.** Immunoprecipitations were performed with the α-HA antibody on cross-linked samples from wild-type and the indicated mutant strains expressing the Gal (–) or Gal-VP plasmid (+) as well as Myc-tagged versions of the indicated proteins. The resulting samples were analyzed as described in Fig. 2. Variable amounts of each immunoprecipitated sample were loaded so that each would contain approximately equal levels of Gal or Gal-VP as assayed by the α-HA Western blot.

58–60). Some subunits, such as Spt20, are required for the integrity of the SAGA complex, and hence all SAGA functions. Subunits such as Ada2 are required for histone acetylase activity and hence chromatin structure but are not required for certain transcriptional functions of SAGA. Conversely, subunits such as Spt3 are important for transcriptional functions that connect SAGA to the general transcription machinery, particularly TBP.

We examined cross-linking of TBP, TFIIB, Ada1, Tra1, and TAF12 in wild-type and mutant strains representing each class of SAGA subunit (Fig. 5). For this experiment, the Gal4 and Gal4-VP16 proteins were expressed from the *EFT2* promoter, which is less sensitive to SAGA mutations than the copper-inducible promoter. VP16-dependent cross-linking of TAF12, which is present in both TFIID and SAGA, is essentially eliminated in the *spt20* strain, in which the SAGA complex is completely disrupted. However, the amount of Gal4-VP16 cross-linking to TAF12 is only slightly reduced in the *ada2* and *spt3* strains. Similar results are observed for cross-linking to Ada1 and Tra1, although cross-linking to Ada1 is more sensitive to loss of Ada2 and Tra1 is more sensitive to deletion of Spt3. In contrast to these results with SAGA subunits, cross-linking to TBP and TFIIB is only slightly reduced in any of the mutant strains. The total cellular levels of these SAGA subunits as well as TBP and TFIIB are similar in wild-type and all three mutant strains (data not shown). These results strongly suggest that the VP16 activation interacts with SAGA but not TFIID *in vivo*.

Relative Cross-linking Efficiencies of SAGA Subunits Are Not Affected by Reducing the Overall Level of Cross-linking—As formaldehyde is a rather nonspecific cross-linking agent and as SAGA is a very stable complex, our experimental procedure is likely to generate significant (and perhaps extensive) cross-linking between SAGA subunits *in vivo*. Thus, it is difficult to determine whether an observed VP16-dependent interaction *in vivo* reflects a direct cross-link with the protein examined or is due to a network of protein-protein cross-links in which the protein examined is not a direct target. Because a majority of the SAGA subunits co-purify with Gal4-VP16 following cross-linking, we suspected that some of them might not be directly cross-linked to the VP16 activation domain. As an attempt to investigate this possibility, we carried out experiments at reduced concentrations of formaldehyde. We reasoned that reduced cross-linking efficiency would have less of an effect on direct targets of the VP16 activation domain (*i.e.* those requiring a single protein-protein cross-link) as opposed to SAGA components that do not contact the VP16 activation domain (*i.e.* those requiring multiple protein-protein cross-links). As expected, the level of cross-linking decreases as the formaldehyde concentration is reduced. However, as shown by a comparison to TAF12 in each case, a decrease in relative cross-linking efficiency was not observed for any of the six proteins tested (Fig. 6).

Cross-linking of Other Activation Domains to TBP and SAGA—We analyzed other activation domains for their ability to cross-link to TBP and TAF12 *in vivo* (Fig. 7). Specifically, we analyzed these activation domains in the context of a Gal4 fusion protein in the same manner described for Gal4-VP16. While the activator proteins are expressed at different levels (assayed by the Western blotting with the HA antibody), the relative cross-linking of TBP and TAF12 can be compared among the activators. In this regard, it is interesting that the VP16, Gcn4, Put3, and Adr1 activation domains cross-link to both TBP and TAF12, whereas the Hap4 and Ace1 activation domain cross-link preferentially to TAF12.

DISCUSSION

Analysis of Protein-Protein Interactions in Vivo—Protein-protein interactions are responsible for a great deal of biological specificity, but it has been difficult to identify such interac-

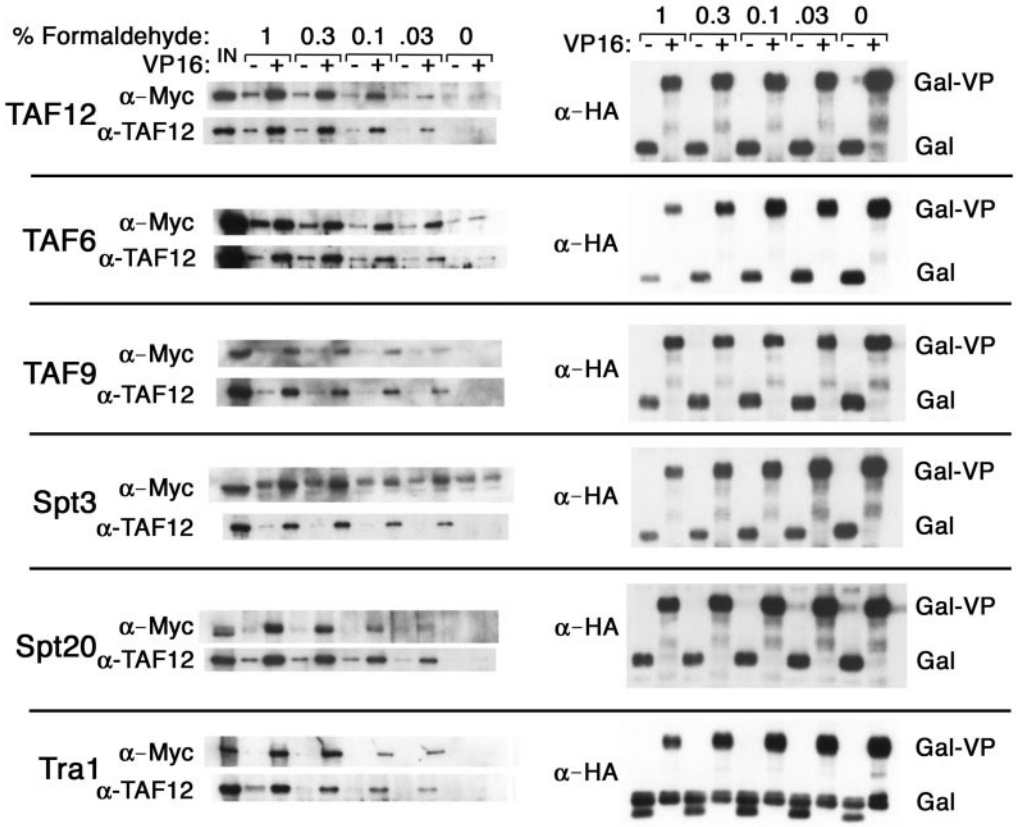


FIG. 6. **Cross-linking as a function of formaldehyde concentration.** Proteins were cross-linked at the indicated concentrations of formaldehyde, and immunoprecipitations were performed with the α-HA antibody on samples from strains expressing the Gal (–) or Gal-VP plasmid (+) as well as Myc-tagged versions of the indicated proteins. The resulting samples were analyzed as described in Fig. 2. The efficiency of the immunoprecipitation increases as the concentration of formaldehyde is lowered, probably due to modification of the HA epitope by formaldehyde.

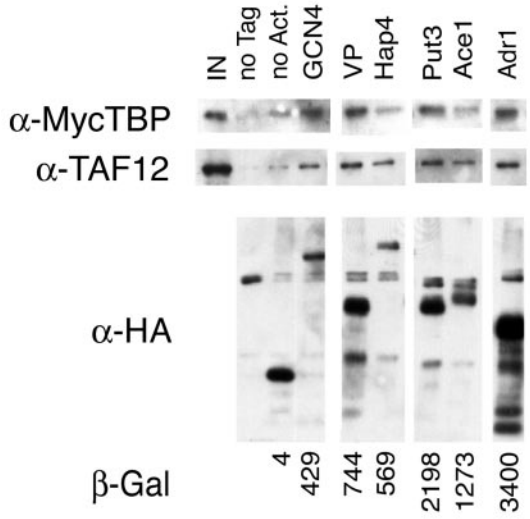


FIG. 7. **Cross-linking of various activation domains to TAF12 and TBP.** Immunoprecipitations were performed with the α-HA antibody on cross-linked samples from strains expressing Gal4 derivatives containing the indicated activation domains (and control proteins lacking the epitope tag or activation domain) as well as Myc-tagged TBP. The resulting samples were analyzed as described in Fig. 2. The levels of transcriptional activation (β-galactosidase units) for each derivative are indicated.

tions under physiological conditions. Biochemical assays, by definition, are not performed under physiological conditions, and indeed many such assays employ extremely high protein concentrations and/or isolated proteins out of their natural context within multiprotein complexes. Co-immunoprecipitation experiments from cell-free extracts are performed under arbitrarily defined conditions, and extract preparation results in the indiscriminate mixing of components that were physically separate when inside cells. Two-hybrid experiments are performed *in vivo*, but the components of interest are presented in an artificial manner. The combination of genetic analysis and biochemical analysis involving wild-type and mutant protein provides the best evidence for identifying protein-protein interactions that are physiological significant. However, it is important to stress that the existence of such protein-protein interactions *in vivo* is inferred, rather than directly demonstrated by physical means.

Here, we describe a general method for detecting protein-protein interactions *in vivo* under physiological conditions. This method, which is based on formaldehyde cross-linking, has several advantages. First, formaldehyde is a small molecule such that cross-linking requires that the two proteins be in close physical proximity. Second, as formaldehyde is a rather nonspecific cross-linking agent that rapidly permeates intact cells, it should be generally useful for detecting a wide range of protein-protein interactions. Third, kinetic experiments strongly suggest that formaldehyde inactivates cellular enzymes almost immediately upon addition to the growing cells (45–47). This consideration suggests that the method should provide a snapshot of protein-protein interactions at the time of formaldehyde addition and that the contribution of artifactual interactions that occur during the cross-linking period should be minimized. Fourth, the use of a common epitope tag on putative target proteins makes it possible to examine every subunit of a given complex and to approximate the relative

molar amounts of these proteins that immunopurify with a given protein of interest (Gal4-VP16 in the case here).

Although the method is capable of detecting protein-protein interactions *in vivo*, it has limitations that are worth noting. An important limitation is that an apparent protein-protein interaction *in vivo* could either represent a direct cross-link between the two proteins or it might be due to multiple cross-links that indirectly connect the two proteins. In this regard, Gal4-VP16 appears to cross-link with numerous SAGA subunits, and it is unclear which of these directly contact the activation domain (see below). Another limitation is that the amount of formaldehyde cross-linking depends on the number and physical location of lysines (and perhaps other residues) within the interacting surfaces, parameters that vary among protein-protein interactions. For this reason, the failure to observe a cross-link between two proteins does not necessarily mean that the proteins are not in contact. Lastly, although the observed protein-protein interactions occur under physiological conditions, the experiments do not address the intracellular location where the interaction occurs. For example, the VP16-dependent cross-links observed here do not require the Gal4 DNA-binding domain, suggesting that the interactions can occur when the proteins are not associated with their target sites within the chromatin template. Our experiments do not address whether cross-linking efficiency is influenced, either positively or negatively, when the relevant proteins are bound to genomic sequences.

The VP16 Activation Domain Directly Interacts with SAGA in Vivo—Our results provide direct evidence for a physical interaction between the VP16 activation domain and the SAGA complex *in vivo*. We observe cross-linking to nine of the 14 subunits tested, and disruption of the SAGA complex, as occurs in the *spt20* deletion strain, essentially eliminates cross-linking to all SAGA subunits tested. This result is specific to SAGA because cross-linking to TBP and TFIIB is only very slightly affected in the *spt20* deletion strain. Importantly, cross-linking to TAF12 is reduced to near-background levels in the *spt20* strain, even though TAF12 is present at normal levels in the TFIID complex. This indicates that the vast majority of VP16-dependent cross-linking to the TAFs reflects interaction with SAGA not TFIID. This result agrees with GST pulldown experiments, showing that the Gcn4 activation domain interacts with TAFs present in both SAGA and TFIID but not with TFIID-specific TAFs (39).

Interestingly, VP16-dependent cross-linking to the Tra1 subunit is largely eliminated in the *spt20* deletion strain, even though Tra1 is also present in the NuA4 HAT complex. This result appears to conflict with the observation that the Hap4 acidic activation domain photo-cross-links to Tra1 in both SAGA and NuA4 (42). This apparent discrepancy might be due to a difference in cross-linking efficiency to the two complexes, binding specificity of the VP16 and Hap4 activation domains, or a factor present *in vivo* that inhibits an interaction between NuA4 and activators. *In vivo*, SAGA is recruited to promoters by a variety of activator proteins (17, 23, 24, 47, 61, 62), whereas recruitment of NuA4 appears to be more activator-specific (20).

Given the large number of SAGA subunits that apparently cross-link to the VP16 activation domain, we suspect that some (and perhaps many) of the subunits are not directly cross-linked to the VP16 activation domain but instead are indirectly cross-linked via other SAGA subunits. In an attempt to distinguish direct from indirect cross-linking, we reduced the overall level of cross-linking by lowering the formaldehyde concentration, reasoning that interactions requiring a single cross-link would be less affected than those requiring multiple cross-links. However, lowering the formaldehyde concentration did not differentially affect the relative cross-linking efficiency of any SAGA subunits tested, suggesting that this reasoning was incorrect. Instead, we suggest that cross-linking within the SAGA complex is much more efficient than cross-linking between SAGA and the VP16 activation domain. The SAGA complex is very stable and has numerous protein-protein interaction surfaces, whereas the association between the VP16 activation domain and the directly contacted subunit(s) is likely to be transient and involve a limited interaction surface. In addition, if cross-linking were extensive enough to form multiple cross-links between each SAGA subunit, then reducing the concentration of formaldehyde would not reduce intra-SAGA cross-linking but would reduce cross-linking to the VP16 activation domain. However, our results indicate that the VP16 activation domain does not cross-link to all SAGA subunits, and while our method cannot definitively prove a lack of a protein-protein interaction, it is noteworthy that we did not observe cross-linking to Ada2, Ada3, or Gcn5, which together comprise a subcomplex of SAGA.

However, we cannot rule out that the VP16 activation domain directly contacts multiple subunits of SAGA. Photo-cross-linking experiments that identified Tra1 as a direct target of the Hap4 activation domain were unable to examine most of the other SAGA subunits due to high background (42). However, similar experiments identified three subunits of the Swi/Snf histone remodeling complex as direct targets of Hap4 and Gcn4, and perhaps as many as four to five subunits are contacted by Pho4 and Swi5 (43). This observation suggests that activation domains, which are largely unstructured in solution and have no strictly conserved domains, might have evolved to bind to a large number of proteins but with a low affinity.

Binding of the VP16 Activation Domain to TBP and TFIIB in Vivo—Although TBP, TFIIA, and TFIIB interact with the VP16 activation domains *in vitro*, we could only confirm a direct interaction *in vivo* with TBP and TFIIB. It is likely that the VP16 activation domain interacts separately with TBP and TFIIB because TBP and TFIIB only stably associate with each other in the context of a preinitiation complex and the VP16-dependent cross-links to TBP and TFIIB do not require the Gal4-DNA binding domain (data not shown). It is also likely that the cross-link to TBP does not occur to a significant extent when TBP is part of the TFIID complex because, unlike the case for SAGA, we do not observe cross-linking to other components of the complex such as TFIID-specific TAFs. Therefore, our results suggest that TBP and TFIIB are direct targets of the VP16 activation domain *in vivo*.

How do the interactions of the VP16 activation domain to TBP and TFIIB contribute to transcriptional activity *in vivo*? The simplest model is that these interactions contribute to the recruitment of the Pol II machinery to promoters, a key limiting step *in vivo* (15, 16, 21, 22, 63). However, several considerations argue against this view. First, unlike the case for SAGA, TBP and TFIIB are not recruited by the Gal4 activator bound to its genomic sites in the absence of a TATA element (23, 24). Second, the VP16 activation domain interacts with surfaces of TBP (64, 65) and TFIIB (31, 66) that are critical for promoter binding, and mutations that abolish the interactions *in vitro* do not significantly affect the level of transcriptional activity *in vivo* (67, 68). This suggests that the VP16 activation domain may not interact with TBP and TFIIB in the context of a preinitiation complex, and in this regard our observed cross-linking occurs primarily (and perhaps exclusively) when the proteins are not bound to DNA. Our results are consistent with the idea that activation domains can function as antirepressors of the autoinhibitory activity of TBP (65). However, while our

results establish that the VP16 activation domain directly interacts with TBP and TFIIB *in vivo*, the importance of these interactions for transcriptional activity *in vivo* remain to be determined.

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