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Targets of the Gal4 Transcription Activator in Functional Transcription Complexes

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Although biochemical and genetic methods have detected many activator-transcription factor interactions, the direct functional targets of most activators remain undetermined. For this study, photo-cross-linkers positioned within the Gal4 C-terminal acidic activating region were used to identify polypeptides in close physical proximity to Gal4 during transcription activation in vitro. Of six specifically cross-linked polypeptides, three (Tra1, Taf12, and Gal11) are subunits of four complexes (SAGA, Mediator, NuA4, and TFIID) known to play a role in gene regulation. These cross-linking targets had differential effects on activation. SAGA was critical for activation by Gal4, Gal11 contributed modestly to activation, and TFIID and NuA4 were not important for activation under our conditions. Tra1, Taf12, and Gal11 have also been identified as cross-linking targets of the Gcn4 acidic central activating region. Our results demonstrate that two unrelated acidic activators converge on the same set of functional targets.

Transcription activators are necessary for the precise regulation of gene expression in response to a variety of cellular signals. Many eukaryotic activators directly or indirectly facilitate recruitment of the transcription machinery to a promoter by enhancing the binding of chromatin remodeling factors, by counteracting the action of specific repressors, and/or by directly interacting with the transcription machinery (23, 26, 41). Acidic activating regions are common in eukaryotes and typically contain one or more critical hydrophobic residues and an abundance of acidic side chains. Strong acidic activators can be as short as 30 residues and are relatively insensitive to mutagenesis, and truncations of the activation regions result in a progressive loss of activity (20, 28). These results suggest that acidic activators do not fold into a typical structured protein domain. In agreement with this proposal, structural studies of the activators c-*myc*, CREB, and VP16 demonstrated that these activating regions are unstructured in the absence of an interacting partner (30, 42, 47). Sequence comparisons, the isolation of activators from random sequences, mutagenesis, and selection for mutations which increase function have not revealed any obvious similarity among the primary sequences of acidic activators, apart from being rich in acidic and hydrophobic residues (13, 19, 28). A central question in gene regulation is how these diverse activators function to stimulate transcription through the common set of factors comprising the transcription machinery.

Yeast Gal4, one of the first known acidic activators, is responsible for the regulation of genes involved in galactose catabolism (41). In the absence of galactose, Gal4 is inhibited by the repressor Gal80. Upon the addition of galactose, Gal4 is released from this repression by the activation of Gal3, which blocks the repressive function of Gal80 (27). Gal4 contains an N-terminal DNA binding and dimerization domain (residues 1 to 147), an N-terminal activating region (residues 148 to 196), and a C-terminal activating region (residues 768 to 881) (28). The most acidic part of the C-terminal activating region (residues 840 to 881) is the minimal segment required for strong activator function in vivo (28). The Gal4 activator functions to stimulate transcription in all eukaryotes tested, from yeast to humans, and activates transcription when fused to a heterologous DNA binding domain (5, 41). The Gal4 DNA binding domain has no activation activity when separated from the activating regions, although mutations within this domain have been reported to reduce activation in vivo (12).

Many direct activation targets of Gal4 have been proposed, based on a variety of biochemical, genetic, and molecular studies. In vivo assays have demonstrated that the acetyltransferase/coactivator complex SAGA, which is required for Gal4 induction, is the first detectable factor to be recruited to the *GAL1/10* upstream activation sequence upon galactose induction (3, 7). In agreement with these studies, in vivo fluorescent resonance energy transfer (FRET) assays suggest that Gal4 and the SAGA subunit Tra1 are in close proximity only after induction (4). In vitro protein-protein interaction studies have demonstrated binding of Gal4 to TATA-binding protein (TBP), TFIIB, Swi/Snf, Mediator, and SAGA (1, 22, 37, 51). In Mediator, Gal4 binds to the Srb10 and Gal11 subunits, binding Gal11 in two separate regions. Other acidic activators such as yeast Gcn4 have also been found to interact with numerous polypeptides in vitro (6, 15, 35). The short nonconserved sequences of acidic activators, coupled with the observed in vitro binding of many polypeptides, raise the question of how these factors specifically recognize their relevant targets. Nearly every general transcription factor and coactivator complex has been proposed as a direct activator target, but in only a few cases has the functional relevance of these interactions been

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demonstrated (4, 6, 16, 18, 39, 49). One limitation of many previous studies is that the activator targets were not defined in functional transcription complexes, but instead were identified using isolated factors or individual subunits of large complexes.

For this work, we used site-specific photo-cross-linkers inserted within the Gal4 C-terminal activating region to identify polypeptides in close proximity to the activator while the activator stimulates transcription. This approach revealed six polypeptides that cross-link to the activating region. Three of these polypeptides (Tra1, Gal11, and Taf12) are subunits of four complexes previously implicated in gene regulation, namely, Mediator, SAGA, NuA4, and TFIID. Remarkably, these three cross-linking targets were also identified in a cross-linking assay with the acidic activator Gcn4 (17). Thus, two activators with unrelated sequences interact with the same set of three targets during transcription activation. Unexpectedly, we found that Ste12, which is itself a gene-specific transcription factor, is also a specific target of Gal4. Functional studies demonstrate that these Gal4 cross-linking targets make differential contributions to activation by Gal4.

MATERIALS AND METHODS

Fusion protein construction and purification. Activator fusions were created by recombinant PCR and cloned into the bacterial T7 expression vector pet21a (EMD Biosciences). Cysteines were introduced by site-directed mutagenesis. These expression plasmids were transformed into BL21(DE3)RIL cells (Stratagene), and transformants were grown in 1 liter of YT medium (0.8% Bacto Tryptone, 0.5% yeast extract, 0.5% NaCl) to an optical density at 600 nm of 0.5 and induced for 4 h at 23°C with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were harvested, and pellets were frozen on dry ice. Cells were lysed by resuspension in 25 ml/liter of Gal4 buffer (20 mM HEPES, 300 mM NaCl, 10% glycerol) and then sonicated. Cell debris was pelleted and resuspended in 12 ml/liter denaturing Gal4 buffer (Gal4 buffer plus 6 M urea) for 1 h to solubilize the activator, and insoluble material was pelleted. Denatured His-tagged Gal4-Gcn4 was purified on TALON beads (Clontech). Purified protein was diluted to 0.3 mg/ml in denaturing Gal4 buffer with 0.05% NP-40 and 5 mM dithiothreitol (DTT). Renaturation was done by step dialysis with four buffer changes (Gal4 buffer with 0.05% NP-40, 5 mM DTT, and either 1 M urea, 0.5 M urea, 0.2 M urea, or 0 M urea) over 10 h.

PEAS incorporation. One milliliter (150 to 250 μg) of renatured Gal4-Gcn4 was exchanged into labeling buffer (Gal4 buffer with no DTT or NP-40) using NAP-10 columns (Amersham) and was concentrated to ~400 μl with an Ultrafree Biomax4-5K concentrator (Millipore). The photo-cross-linker PEAS (Molecular Probes) was labeled with ^{125}I (350 to 600 mCi/ml; Amersham) in Iodogen tubes (Pierce) containing 80 μl 0.1 M sodium phosphate (pH 7.4), 7.6 nmol PEAS, and ~1.5 mCi ^{125}I per 60 μg Gal4-Gcn4. After 4 min, the reaction was transferred to a tube containing 50 mM tyrosine, for a final concentration of 2 mM, to scavenge unreacted ^{125}I. This mixture was combined with ~400 μl (~60 μg) Gal4-Gcn4 and allowed to react for 30 min at 23°C. Unreacted ^{125}I was removed from ^{125}I-PEAS–Gal4-Gcn4 by buffer exchange over NAP-5 columns (Amersham) into 1 ml of labeling buffer. Recovery was about 90%, with a specific activity of 5 to 10 μCi/μg protein. Labeled protein was divided into aliquots and stored at −70°C. Attempted labeling of a non-cysteine-containing derivative gave at least 10-fold lower ^{125}I incorporation, showing that PEAS labeling was specific for cysteine derivatives. All labeled proteins were found to be active for in vitro transcription activation.

Photo-cross-linking, immunoprecipitation, and transcription assays. Preinitiation complex (PIC) formation on immobilized templates was performed as previously described (45), except that nuclear extracts were dialyzed to remove DTT. Approximately 180 μg of ^{125}I-PEAS–Gal4-Gcn4 was used per reaction. After being washed, PICs were exposed to UV irradiation (365 nm; 21,700 μW/cm^2) under a UV lamp (UVP model B-100AP) for 1 min and then digested with 60 units PstI (New England Biolabs). Samples were treated with 50 mM DTT, and labeled targets were visualized after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using a phosphorimager (Molecular Dynamics). For immunoprecipitations, cross-linked PICs were first treated with 50 mM DTT and then dissociated from the promoter with 1 M potassium acetate. Flag-tagged complexes were precipitated with 20 μl anti-Flag M2 agarose beads (Sigma). Transcription assays were performed as previously described (45), except that plasmids or immobilized templates contained the *HIS4* core promoter with either one or three upstream Gcn4 DNA binding sites, as indicated.

Anti-Flag depletion assays. Nuclear extracts were made as described at www.fhcrc.org/science/labs/hahn/. For depletion, 150 μl nuclear extract (40 to 50 μg/μl) was dialyzed for 1 hour at 4°C against buffer C with 75 mM ammonium sulfate and protease inhibitors to remove DTT. The dialyzed extract was incubated with 20 μl anti-Flag M2 agarose beads (Sigma) for 1 hour at 4°C. Extracts were then transferred to new beads for an additional hour. After depletion, anti-Flag beads were washed with 1× transcription buffer plus 0.3 M potassium acetate. Crude SAGA was eluted with 0.15 mg/ml 3× Flag peptide (Sigma) in buffer C plus 75 mM ammonium acetate. Depletions were quantitated by Western analysis using a LI-COR Bioscience Odyssey infrared imaging system.

RNA purification and primer extension. RNAs were purified from yeast essentially as described at www.fhcrc.org/science/labs/hahn/. Yeast was grown overnight at 30°C in rich medium containing 2% raffinose. For galactose-induced samples, the medium was supplemented with galactose to a final concentration of 2% and cells were grown for an additional 30 or 60 min. Cells (2×10^9) were harvested, washed once with 5 ml cold H_2O, resuspended in 400 μl cold TES (10 mM Tris, pH 7.5, 10 mM EDTA, and 0.5% SDS), and frozen in liquid nitrogen. RNAs were extracted and purified using acid phenol. Expression was assayed by primer extension, using 20 μg RNA for *GAL1* and 40 μg RNA for *GAL10* and *ACT1*. The primers used were GAL1-1 (CCTTGACGTTAAAGTATAGAGG), GAL10-1 (CAATGTATCCAGCACCACCTGT) (21), and ACT1-1 (AACCGT TATCAATAACCAAAGC) (33). *GAL1* and *GAL10* expression was quantitated and normalized to *ACT1* expression.

Chromatin immunoprecipitation. Chromatin immunoprecipitation assays were performed essentially as described previously (29), with a few modifications. The Ste12-Flag$_3$ yeast strain was grown in rich medium (300 ml) containing 2% raffinose. For galactose-induced samples, the medium was supplemented with 2% galactose for 60 min prior to cross-linking. For PCR, the following primers were used: RPS5F, CCTTCGCCGCAGGCTTAGTGGAGGTC; RPS5R, GTGTCAGACATCTTTGGAATGGTCGGT; GAL1F, GGAACTTT CAGTAATACGCTTAACTGC; and GAL1R, TTAATTTGAAGGTTTGTGG GGCCAG. Titrations of input and immunoprecipitated DNAs were used in the PCRs to ensure linearity. The ratio of immunoprecipitate (IP) to input was calculated. At least three independent PCRs were performed using two independently purified chromatin preparations.

RESULTS

Gal4 activating region with attached cross-linker functions in transcription activation. To directly identify polypeptides in close physical proximity to Gal4 during transcription activation, the ^{125}I-radiolabeled photo-cross-linker PEAS was attached to cysteines positioned within the Gal4 C-terminal activating region (AR). When irradiated with UV, PEAS covalently cross-links to nearby polypeptides within 14 Å of the cysteine alpha carbon. Initially developed to identify targets of the bacterial activator catalytic activator protein (CAP) during transcription (9, 36), PEAS has been recently used to identify protein-protein interactions among general transcription factors within the PIC (8, 50).

To simplify our initial studies with Gal4, only the minimal acidic AR (residues 840 to 881) was used. Since the Gal4 DNA binding domain (DBD) contains six cysteine residues, the Gal4 AR was fused to the N terminus of the Gcn4 DBD to produce a cysteine-free activator (Fig. 1A). A six-histidine tag was added to the N terminus for protein purification along with amino acids 1 to 17 of Gcn4 as a linker. Recombinant Gal4-Gcn4 activated multiround transcription three- to sixfold and single-round transcription about twofold from a *HIS4* core promoter template containing a single upstream Gcn4 binding site (Fig. 1B). The Gal4 activation function is not altered by fusion to the Gcn4 DBD, since an alanine substitution at Gal4 residue F869 reduces transcriptional activation twofold (not

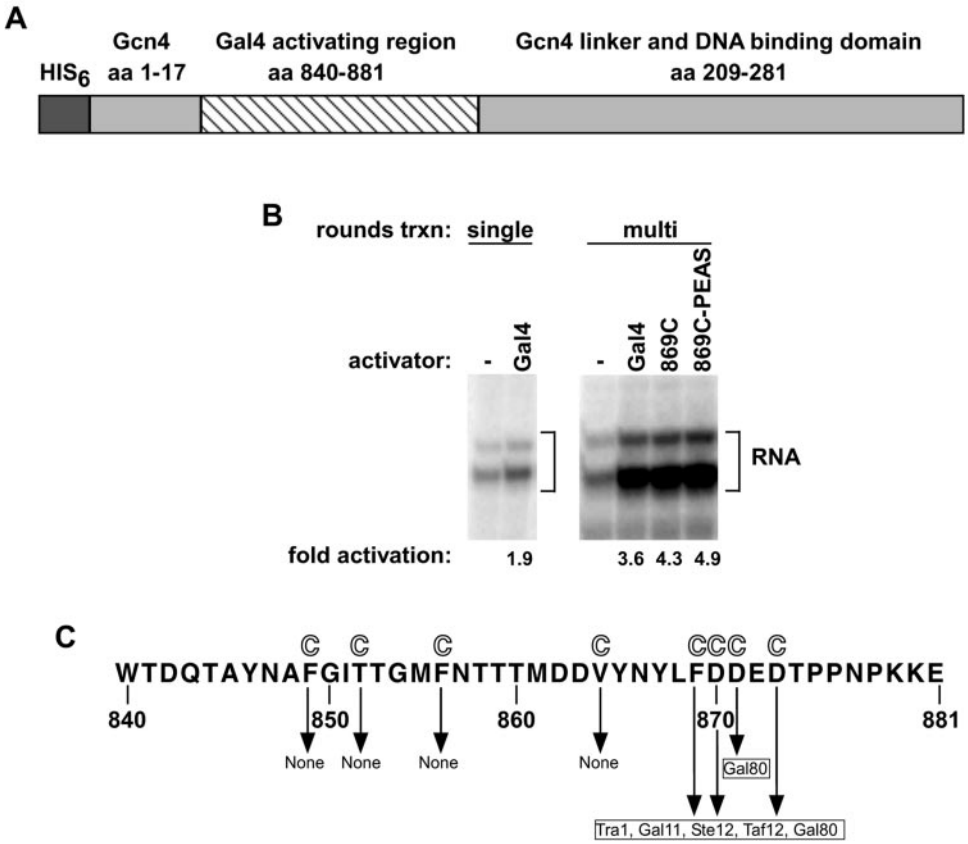

FIG. 1. Gal4-Gcn4 fusion and summary of PIC cross-linking results. (A) The minimal C-terminal Gal4 activating region was fused to the Gcn4 DNA binding and dimerization domains as indicated, with an N-terminal six-histidine tag connected via a 17-residue linker. (B) Transcription assays with Gal4-Gcn4. Single- and multiround plasmid transcription assays were performed at the *HIS4* core promoter with a single upstream Gcn4 binding site, and transcription (trxn) was assayed by primer extension. Transcription is shown using either no Gal4-Gcn4 activator (−), a noncysteine derivative (Gal4), or the F869C derivative without (869C) or with (869C-PEAS) ^{125}I-PEAS labeling. The amount of activation is indicated. (C) Cysteine substitutions were introduced within the Gal4 activating region. The eight mutants used for cross-linking are shown above the Gal4 sequence, with the cross-linking results summarized below.

shown), similar to previous results obtained with this minimal activating region fused to the Gal4 DNA binding domain (51). To screen for positions where PEAS insertion would not alter the function of the Gal4 activating region, mutagenesis was used to individually replace 15 amino acids in the activation region with cysteine. Of these recombinant cysteine derivatives, the eight most stable and active proteins were labeled at cysteine with ^{125}I-PEAS for use in cross-linking (Fig. 1C). These labeled Gal4-Gcn4 derivatives all retained the ability to activate transcription in vitro (e.g., see Fig. 1C, 869C-PEAS).

Cross-linking targets of the Gal4 C-terminal activating region. Labeled activator derivatives were incubated with nuclear extract and the yeast *HIS4* promoter template, which was immobilized to magnetic beads. After PIC formation, the PICs were exposed to UV light for 1 min to activate the cross-linker. Control reactions were not UV treated. The immobilized templates were digested with PstI at a site upstream from the Gcn4 binding site, releasing the PICs from the beads. DTT was then added to reduce the disulfide bond linking PEAS to Gal4-Gcn4 and to transfer ^{125}I-PEAS to the cross-linked targets. Cross-linked proteins were visualized by SDS-PAGE and autoradiography. Four of the Gal4-Gcn4 derivatives did not detectably cross-link to any targets, an example of which is shown in Fig. 2A (T852C) and summarized in Fig. 1C. Gal4-Gcn4 D871C reproducibly cross-linked to only a single polypeptide, p48. F869C cross-linked strongly and reproducibly to six targets (designated p48, p58, p64, p80, p120, and p>300), while the D870C and D873C mutants cross-linked more weakly to these same polypeptides (Fig. 2A). The strong cross-linking observed at F869C is consistent with the central role of this region in the function of the minimal Gal4 activating region (51).

Because some activators can stabilize the Scaffold complex, the complex of general transcription factors left behind at promoters after initiation (52), the Gal4-Gcn4 derivatives were tested for cross-linking in the Scaffold complex and in PICs formed during a second round of initiation. Scaffolds were formed by the addition of nucleotides to PICs to dissociate Pol II, TFIIB, and TFIIF, and the resulting complexes were washed and irradiated for 1 min. Second-round PICs were formed by incubation of the Scaffold complex with a nuclear extract from an *srb2*Δ strain that is unable to form PICs in the absence of a functional Scaffold complex (52). No differences in the cross-linking pattern were observed for either the Scaffold complex or second-round PICs compared to cross-linking in PICs (Fig. 2B). The simplest interpretation of this experiment is that at this promoter, the Gal4 activating region con-

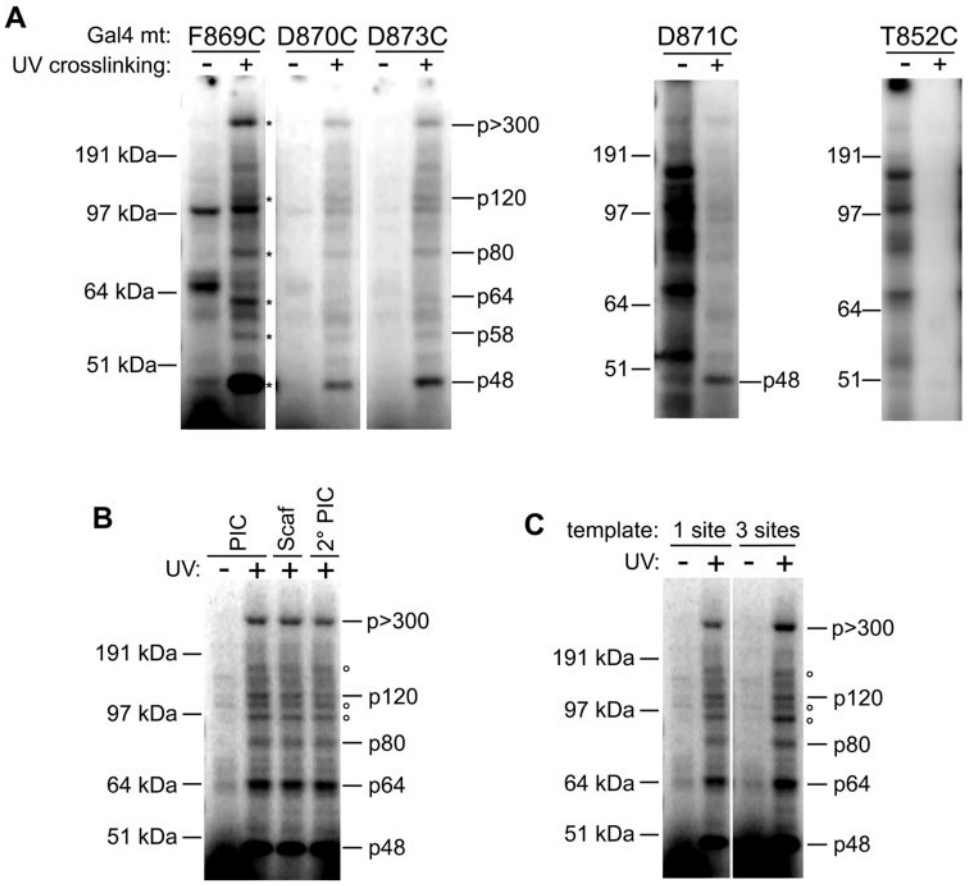

FIG. 2. Cross-linking with Gal4-Gcn4 derivatives. (A) Nuclear extract and the indicated Gal4-Gcn4 derivatives were used to form PICs on immobilized promoter templates. Promoter-bound proteins were cross-linked with UV irradiation (+), released from the beads by PstI digestion, and visualized by SDS-PAGE and autoradiography. Control PICs were not treated with UV (−). Consistent cross-linking bands are indicated by asterisks. (B) Cross-linking within PICs, Scaffold complexes, and second-round PICs. The F869C derivative was used with nuclear extract to form either PICs, Scaffold complexes, or second-round PICs. Cross-linking was performed as described in panel A. Three inconsistently observed bands are labeled with open circles. (C) Cross-linking with multiple Gcn4 binding sites. PIC cross-linking was performed as in panel A, using the F869C derivative, except that a template containing one or three Gcn4 binding sites was used as indicated.

tacts these six polypeptides during the transcription cycle and that the stimulation of PIC formation and reinitiation is mediated through some or all of these contacts. Alternatively, it is possible that the activator transiently contacts some other factor during the transcription cycle but that this interaction is not observed due to its short lifetime. This alternative model seems less likely because cross-linking at different times during PIC formation does not reveal any additional cross-linking targets (unpublished data). It should be noted, however, that our system cannot differentiate an activator dimer contacting multiple targets at a single promoter from a mixture of promoters in which each monomer contacts a single target.

Gal4 and other acidic activators have been observed to interact with numerous polypeptides in vitro, suggesting a possible low specificity in protein-protein interactions. If Gal4 cross-linking in our system was not specific, then increasing the number of bound Gal4 activators would be expected to increase the number of cross-linked polypeptides. To test this possibility, cross-linking was performed on a promoter containing three Gcn4 binding sites (capable of binding up to six activator monomers). The template with three sites bound an increased amount of Gal4-Gcn4 compared to the template with one site, and transcription from both templates was comparable (not shown). Figure 2C shows that the Gal4-Gcn4 cross-linking pattern is not altered by increasing the number of activator binding sites, indicating that cross-linking between the Gal4 AR and its targets is specific.

Identification of Gal4 cross-linking targets. To determine the identity of the cross-linked polypeptides, the cross-linking reactions were repeated with nuclear extracts from strains in which transcription factors of a similar size to that of the radiolabeled targets were triple Flag epitope tagged at the C terminus. The triple Flag tag adds approximately 5 kDa to the apparent molecular size, causing a gel mobility shift that can be observed for proteins of less than about 150 kDa. A mobility shift in the radiolabeled polypeptides is observed only when the cross-linking target is Flag tagged (8, 50). These experiments were all performed with the Gal4 derivative F869C, which produces the strongest protein cross-links.

This method identified p48, the most abundant cross-linked polypeptide, as the repressor Gal80 (Fig. 3A, lanes 2 and 4). In agreement with this result, Gal80 has previously been shown to

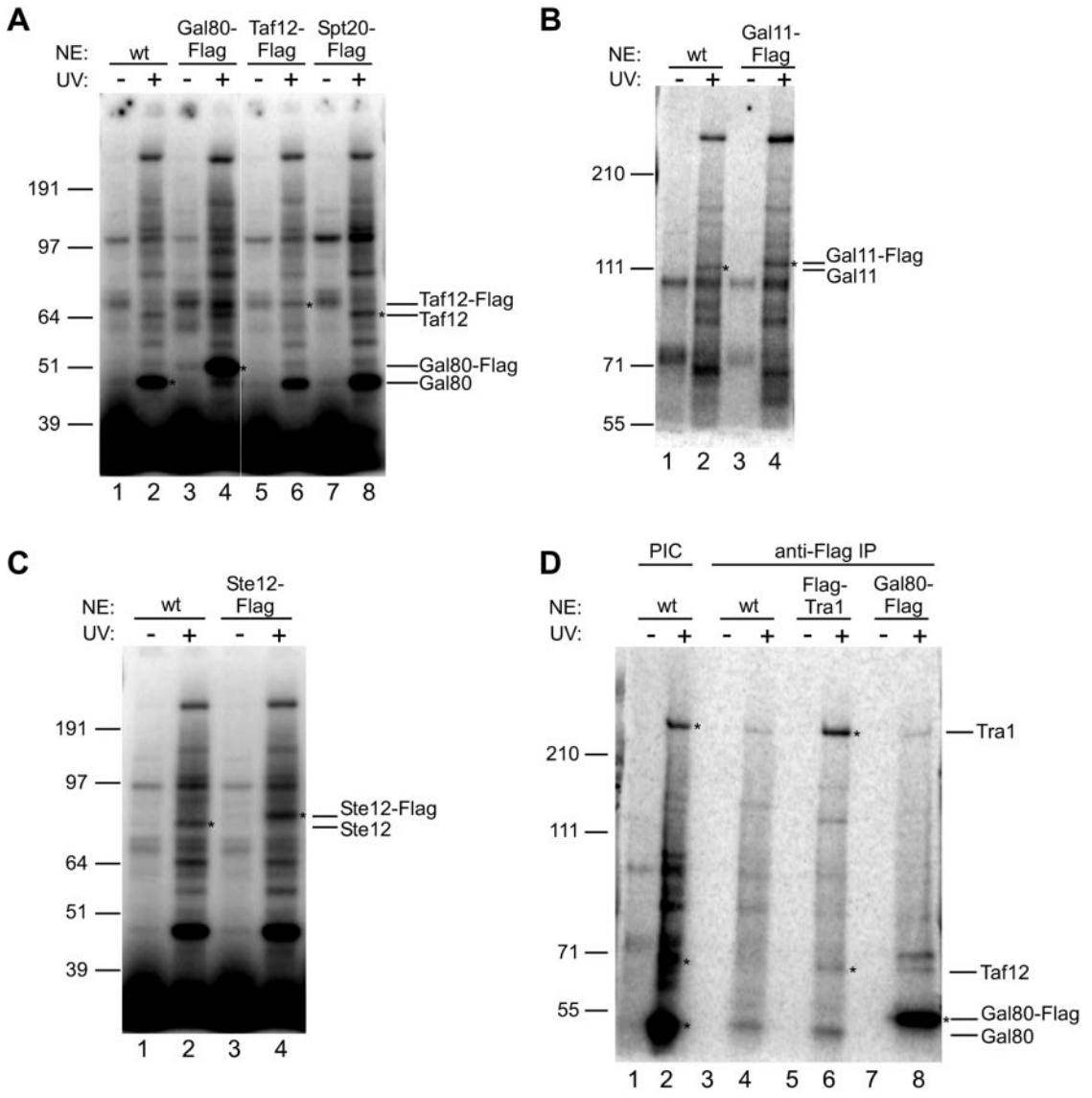

FIG. 3. Identification of Gal4 activating region cross-linking targets. Cross-linking was performed on PICs as described in the legend to Fig. 2, except that the indicated Flag-tagged nuclear extracts (NE) were used. The relevant bands in each panel are marked with asterisks. (A) Identification of p48 and p64 as Gal80 and Taf12. (B) Identification of p120 as Gal11. (C) Identification of p80 as Ste12. (D) Identification of Tra1 as the largest cross-linked polypeptide. After cross-linking, PICs were dissociated from the template with a high salt concentration and immunoprecipitated with anti-Flag beads.

bind to amino acids 850 to 874 of Gal4 (27). The presence of Gal80 at the promoter was surprising, since Gal4-Gcn4 activates transcription in vitro under these conditions (Fig. 1B). To determine if Gal80 was inhibiting transcription and/or activator interactions, a *gal80*Δ strain was constructed and used in transcription and cross-linking experiments. Extracts from this strain did not have increased Gal4 activation activity in vitro, nor did the deletion significantly alter the other transcription factors cross-linked to Gal4-Gcn4 (data not shown). Since Gal4-Gcn4 binds to nearly all the immobilized templates but PIC formation occurs on approximately 10% of templates (43), one possibility consistent with our results is that Gal80 interacts with Gal4 mainly on the fraction of templates where PIC formation does not occur.

The p64 cross-linking target was identified as Taf12, a shared Taf present in both SAGA and the TBP-containing complex TFIID (Fig. 3A, lane 6). Taf12 has been identified in several studies as a potential target of both Gal4 and Gcn4 (22, 34). In contrast, Spt20-Flag (apparent gel mobility, approximately 64 kDa) does not alter the migration of any cross-linked polypeptide (lane 8). The Mediator subunit Gal11, which has been well characterized as important for the optimal expression of Gal4-regulated genes and is capable of binding Gal4 in vitro (37), was identified as the 120-kDa cross-linked polypeptide (Fig. 3B).

After testing a number of candidates for the p80 cross-linking target, a Ste12-Flag nuclear extract was used in the cross-linking assay. Ste12 was considered a potential candidate

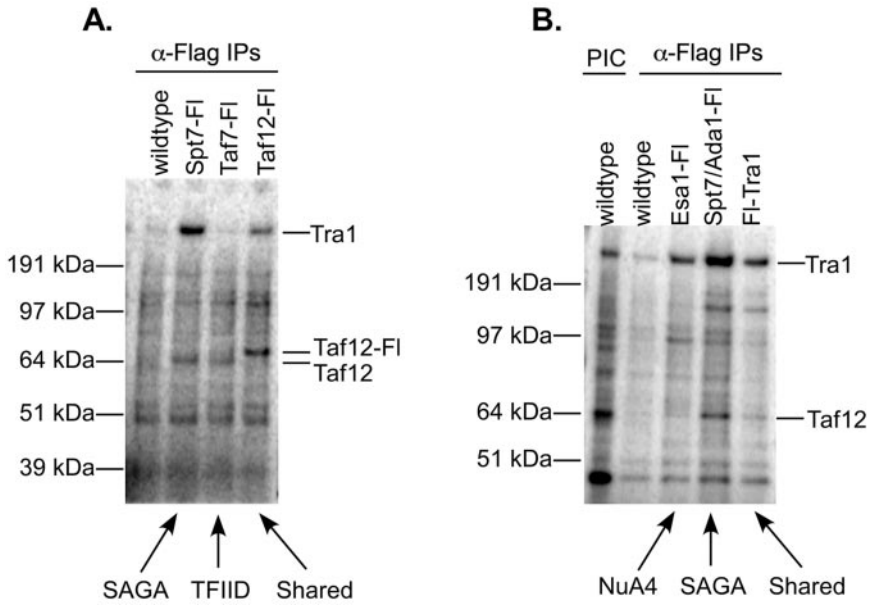

FIG. 4. Gal4 cross-linking to TFIID, SAGA, and NuA4 complexes. Cross-linking in PICs and immunoprecipitation were performed as in the legend to Fig. 3D, using the indicated Flag-tagged extracts. (A) Gal4 cross-links to Taf12 in both TFIID and SAGA. (B) Gal4 cross-links to Tra1 in both NuA4 and SAGA extracts.

because a mass spectrometry analysis of PICs formed with the VP16 activator at the *HIS4* promoter in vitro found that Ste12 bound to the promoter and that this binding was stimulated by TBP (44; J. Ranish, personal communication). Figure 3C demonstrates that Ste12 is the 80-kDa cross-linking target. Ste12 is a transcription factor involved in the response to both carbon and nitrogen starvation as well as in the pheromone response (38, 53), but it has not previously been implicated in regulation of the *GAL* regulon. Ste12 is known to activate at least some of its target genes in cooperation with other activators, including Tec1 and Mcm1 (53). When tested using a Tec1-Flag extract, Tec1 (55 kDa) was not the p58 cross-linking target (not shown). All other known PIC components or coactivator subunits in the 58-kDa mobility range (including Gal3) were also tested by this method and were not found to be cross-linking targets. Because the p58 cross-link is relatively weak and inconsistent, it was not studied further.

Due to the unusually large size of the >300-kDa cross-linked polypeptide and previous work demonstrating an interaction of Tra1 with both Gal4 and Gcn4 (4, 6), we repeated the cross-linking assay using a Flag-Tra1 extract. Since the size of this factor is outside the range in which the triple Flag epitope would produce an observable gel mobility shift, immunoprecipitation was used to test if Tra1 cross-linked to the Gal4 activating region. After cross-linking and DTT treatment, PICs were dissociated with 1 M potassium acetate and precipitated with anti-Flag–Sepharose. Anti-Flag precipitated only background levels of radiolabeled proteins from untagged, wild-type PICs (Fig. 3D, lane 4). In contrast, labeled Flag-Tra1 was specifically precipitated (lane 6), demonstrating that Tra1 is a cross-linking target. Labeled Taf12 was coprecipitated with Flag-Tra1, showing that at least some of Taf12 is cross-linked as a component of SAGA, which contains both Tra1 and Taf12 as subunits. Labeled Gal80-Flag was precipitated in a similar experiment, confirming Gal80 as a cross-linking target in our assays (Fig. 3D, lane 8).

Since Taf12 is a component of TFIID as well as SAGA, TFIID was immunoprecipitated after cross-linking to determine if Taf12 also cross-links to Gal4 as a component of TFIID. Nuclear extracts Flag tagged on Spt7 (a SAGA-specific subunit), Taf7 (a TFIID-specific subunit), or Taf12 (a shared SAGA and TFIID subunit) were used for cross-linking and anti-Flag immunoprecipitation (Fig. 4A). As expected, labeled Tra1 was precipitated only in the Spt7-Flag and Taf12-Flag reactions. Labeled Taf12 was specifically precipitated in the Taf12-Flag reaction and in both the Spt7-Flag and Taf7-Flag PICs. Therefore, the Gal4 activating region cross-links to Taf12 in the context of both TFIID and SAGA within the PIC. Immunoprecipitations were also performed with the Tra1-containing complexes NuA4 and SAGA. As shown in Fig. 4B, radiolabeled Tra1 is immunoprecipitated as a component of both NuA4 (Esa1-Flag) and SAGA (Spt7-Flag/Ada1-Flag), showing that Gal4 can cross-link to Tra1 in the context of both NuA4 and SAGA. In contrast, in vivo FRET studies did not observe an interaction between Gal4 and Tra1 in the absence of intact SAGA (4). However, it is possible that the FRET signal between Tra1 in NuA4 and Gal4 is obscured by other NuA4 subunits.

Differential functions of cross-linking targets in Gal4 activation. To investigate the role of the Gal4 cross-linking targets in activation, these factors were selectively eliminated, and the effect of elimination on transcription activation was examined. Extracts were made from both *gal11*Δ and *ste12*Δ strains and used for in vitro transcription and photo-cross-linking assays. Cross-linking assays showed that deletion of either factor did not affect cross-linking to any other polypeptide (Fig. 5A), demonstrating that interaction with these other factors is independent of Gal11 and Ste12. Previously, we have shown that

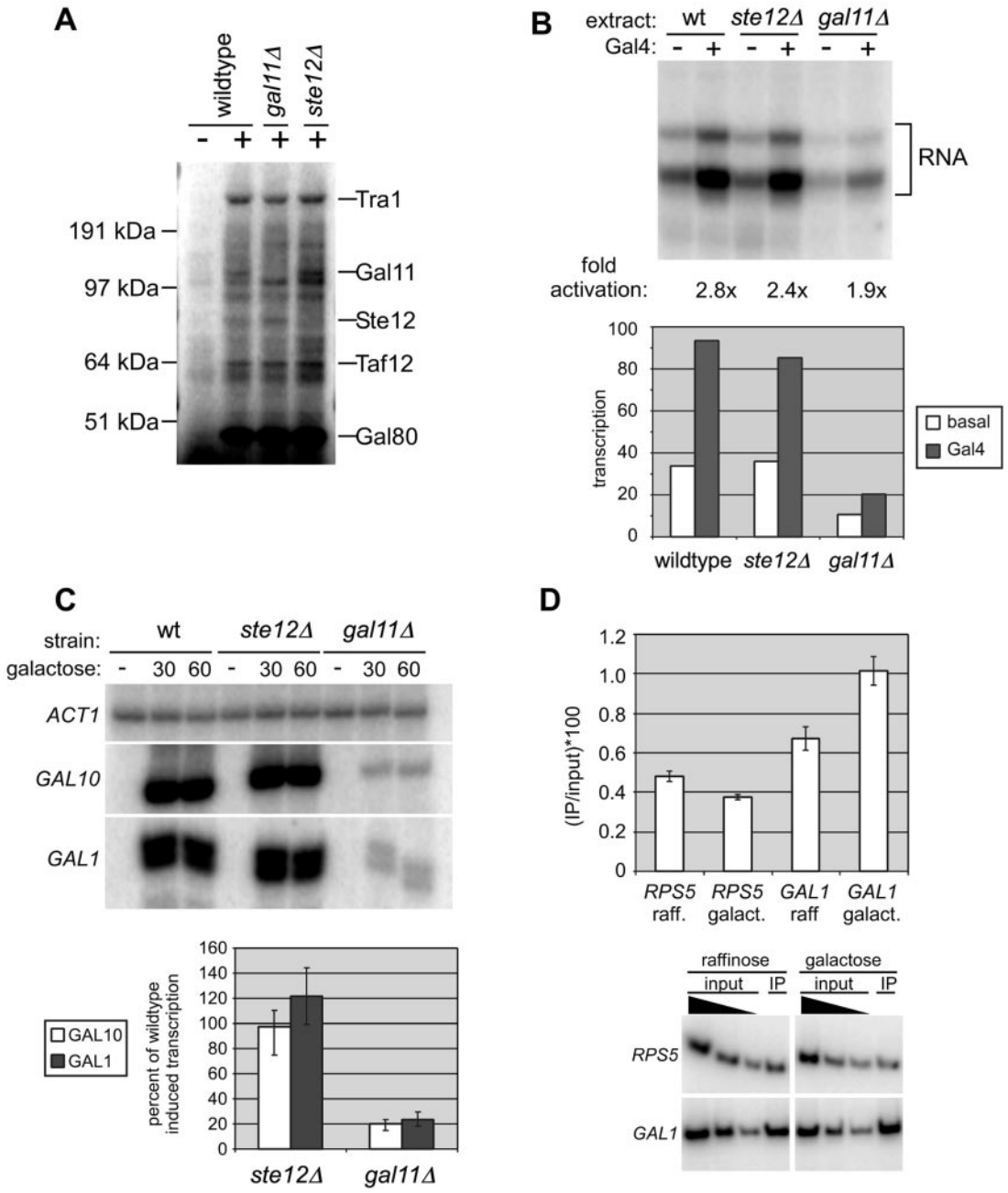

FIG. 5. Activity of Gal11 and Ste12 in Gal4 activation and recruitment of Ste12 to the *GAL1* promoter. (A) Cross-linking of other targets is independent of Gal11 and Ste12. Cross-linking assays were performed as described in the legend to Fig. 2A, using the indicated nuclear extracts. (B) Multiround transcription was performed with wild-type, *ste12*Δ, and *gal11*Δ nuclear extracts in the presence (+) or absence (−) of Gal4-Gcn4. The graph compares absolute levels of transcription for each condition. The amount of activation is indicated. (C) The yeast strains shown were grown in 2% raffinose (−) and then induced with 2% galactose for 30 or 60 min. RNAs were isolated and assayed by primer extension. Transcription was normalized to *ACT1* RNA levels. (D) Galactose-dependent ChIP shows recruitment of Ste12 to the *GAL1* promoter. Cross-linked chromatin was isolated from uninduced (raff.) or induced (galact.) cells, and Ste12-Flag cross-linking was assayed by IP with an anti-Flag monoclonal antibody followed by quantitative PCR. The graph summarizes the results from three separate experiments, an example of which is shown.

deletion of the Mediator subunit Pgd1 also eliminated Gal11 binding within PICs but did not specifically affect the binding of other Mediator subunits such as Srb4 and Sin4 (45). Transcription assays using the *gal11*Δ extract showed that the deletion of Gal11 reduced basal transcription three- to fivefold (Fig. 5B), consistent with the effect of deleting other components of the Sin4 subcomplex of Mediator (45). Although the *gal11*Δ extract showed about fivefold lower levels of activated transcription than the wild type, the amount of activation was only moderately reduced due to the decrease in basal tran-

scription caused by the removal of Gal11. Thus, Gal11 contributes to the overall level of basal and activated transcription but is not essential for activation to occur under our in vitro conditions. As shown previously, Gal11 is important for Gal4-mediated activation in vivo (Fig. 5C). Induced levels of *GAL1* and *GAL10* transcription in vivo were reduced fivefold in the *gal11*Δ strain, as measured by primer extension, close to the decrease observed in vitro. In contrast, the deletion of *ste12* did not significantly affect Gal4-mediated activation either in vitro or in vivo, and no growth defects were observed on glucose or galactose medium (Fig. 5B and C; data not shown). Surprisingly, several Gal4-regulated genes, including *GAL1*, do contain a Ste12 binding site (TGAAACA/G). Chromatin immunoprecipitation analysis revealed a consistent 50% increase in Ste12 binding at the *GAL1* promoter, but not at the Ste12-independent promoter *RPS5*, under Gal4-inducing conditions (Fig. 5D), suggesting that Ste12 may be an in vivo target of Gal4. Although Ste12 does not appear to play an essential role in Gal4 function, the possibility remains that Ste12 function may be redundant with that of other coactivators.

Since Taf12 and Tra1 are essential for yeast viability, their contribution to Gal4 activation in vitro was tested by selectively depleting TFIID, SAGA, or NuA4 from nuclear extracts. To effectively deplete SAGA and TFIID, strains containing epitope tags on two subunits of each complex were used. SAGA was depleted to <5% of native levels using a Spt7-Flag, Ada1-Flag double-tagged extract (not shown). The transcription activity of the nontagged extract was not significantly affected by treatment with anti-Flag beads (Fig. 6A). In contrast, depletion of SAGA from the tagged strain almost completely eliminated Gal4 activation, demonstrating that SAGA is essential for activation in our in vitro system. Activation could be rescued by the addition of a SAGA preparation purified from a Spt7-Flag strain (Fig. 6A). These results are consistent with in vivo work showing the importance of SAGA function for Gal4 target genes (3, 24) and suggest a model in which SAGA recruitment to Gal4 target genes is facilitated by an interaction between Gal4 and Tra1 and/or Taf12.

In similar assays, TFIID or NuA4 was depleted using strains with epitope tags on the TFIID-specific subunits Taf3 and Taf7 or on the NuA4-specific subunit Esa1. Extracts were depleted of at least 95% of the indicated factor. In contrast to the results for SAGA depletion, neither NuA4 nor TFIID depletion significantly impaired activation by Gal4-Gcn4 (Fig. 6B). Extracts depleted of TFIID did show a lower level of basal transcription. This decrease was likely due to a partial depletion of TBP from the extracts, since basal transcription could be restored by the addition of recombinant TBP (not shown). The TFIID-depleted extracts were not impaired for activation by Gal4-Gcn4 compared to undepleted extracts (Fig. 6B). The lack of TFIID dependence is consistent with in vivo results showing that the *HIS4* promoter is TFIID independent in vivo (48). The depletion of NuA4 by Flag-tagged Esa1 had no effect on basal or activated transcription (lanes 9 to 12). This result is consistent with the finding that although Esa1 is recruited to many protein coding genes, only a subset of genes depend on NuA4 for optimal expression (46).

DISCUSSION

Activators can stimulate transcription in part by direct interaction with the general transcription machinery, as many activators have been observed to stimulate transcription in the absence of chromatin or other known repressors of transcription (32, 40). The identity of the direct activator targets and the mechanisms whereby these activator-target interactions stimulate transcription remain important questions. It is also unclear whether activators typically have multiple targets within the transcription machinery, if these targets are functionally redundant, and if a small set of common targets are recognized by multiple activators. Although many components of the transcription machinery have been proposed as activator targets, in only a few cases has a functional target been conclusively identified.

Bacterial activators such as CAP contain a surface required for activation which is structured in the absence of any target. For example, CAP activating region 1 interacts with RNA Pol subunit α by a weak protein-protein interaction that does not involve a conformational change in either the activator or target (2). In contrast, the common class of eukaryotic acidic activators likely do not have a defined structure in the absence of a target. This may contribute to the numerous protein-protein interactions observed in vitro with components of the transcription machinery, chromatin remodeling factors, and other proteins unrelated to transcription (31).

In this work, we have used a different approach to define eukaryotic activator targets by probing for polypeptides in close physical proximity to the activating region during the process of activation. This approach applied to the minimal Gal4 C-terminal activating region has narrowed the list of proposed targets to four proteins (Taf12, Tra1, Gal11, and Ste12), three of which are components of four complexes (TFIID, SAGA, NuA4, and Mediator) previously implicated in transcriptional regulation. The number of factors cross-linking to the Gal4 AR was significantly smaller than that proposed by previous studies using protein-protein interaction assays outside the context of transcription. One possibility is that these other interactions do not normally occur during transcription and are only observed in vitro using isolated proteins or factors. Alternatively, the Gal4 AR may interact with additional factors during transcription, but the conditions necessary for these interactions may not be present in our in vitro system. For example, factors that interact with chromatin may not bind stably to the immobilized templates used for this study. The activator used here, consisting of the minimal Gal4 AR fused to the heterologous Gcn4 DNA binding domain, activates transcription in vitro at least as well as the same AR fused to the Gal4 DNA binding domain (not shown).

Although the Ste12-Gal4 interaction was unexpected, Ste12 binding to PICs formed on the *HIS4* promoter in vitro is stimulated by the Gal4 AR (data not shown). In addition, an examination of Gal4-regulated promoters revealed that both *GAL1* and *GAL3* contain an intact Ste12 binding site, while three other Gal4-regulated genes (*MTH1*, *FUR4*, and *PCL10*) have a Ste12 element with a single base pair substitution in their promoters. In vivo binding assays at *GAL1* revealed an increase in Ste12 binding under Gal4-inducing conditions, supporting a role for Ste12 as an authentic in vivo Gal4 target.

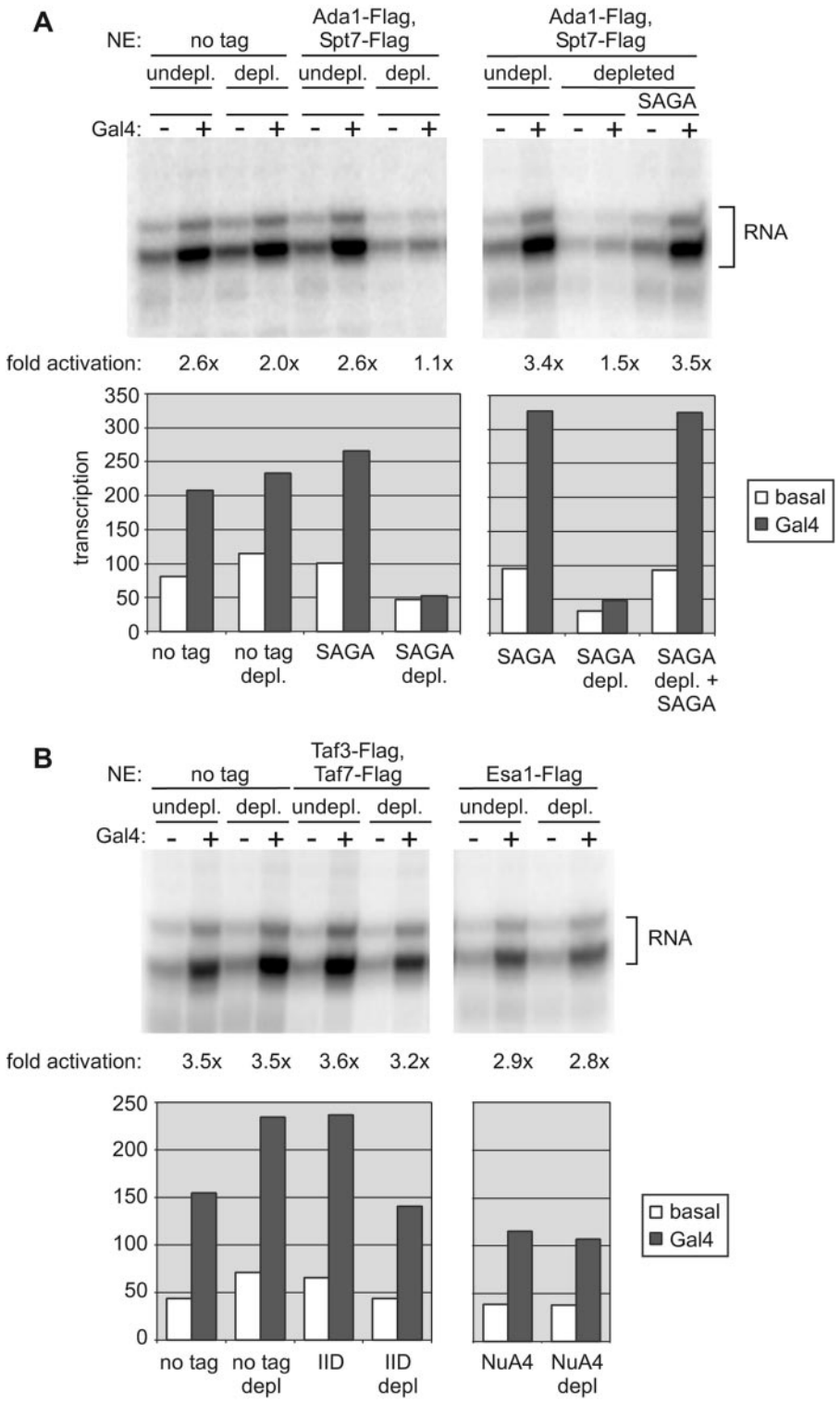

FIG. 6. Function of SAGA, TFIID, and NuA4 in transcription activation in vitro. (A) SAGA is essential for activation by Gal4-Gcn4. Untagged and Spt7-Flag/Ada1-Flag double-tagged extracts were depleted with anti-Flag beads. Depleted and undepleted extracts were tested in multiround transcription assays either with (+) or without (−) Gal4-Gcn4. A typical experiment in which SAGA purified from a Spt7-Flag extract was added is also shown. The graphs compare absolute levels of transcription under all conditions, and the amount of activation is indicated. (B) TFIID and NuA4 depletion. Untagged and Taf3-Flag/Taf7-Flag- and Esa1-Flag-tagged extracts were assayed as in panel A.

However, functional studies both in vivo and in vitro have not identified a Ste12 contribution to Gal4-mediated activation. It is possible that Ste12 is functionally redundant with another factor or is required only under certain conditions.

Both Taf12 and Tra1 are components of SAGA, a factor required for Gal4 activation in vivo. Both of these factors, along with a number of other general factors, were previously found to interact with Gal4 when the activator was mixed with extracts or purified factors (1, 22, 51). Mutations in Taf12 and Tra1 have also been found to reduce activation by other acidic activators (6, 34). We found that within a PIC, Gal4 cross-linked to Taf12 in both SAGA and TFIID and cross-linked to Tra1 in SAGA as well as in the NuA4 complex. This suggests that the activator interaction surfaces of both Taf12 and Tra1 are accessible in multiple complexes. Interestingly, neither TFIID nor NuA4 is required for Gal4-induced transcription. This demonstrates that not all activator-target interactions contribute equally to activation. The Gal4-regulated and *HIS4* promoters are largely TFIID independent in vivo, and it has been proposed that TFIID dependence is determined largely by the core promoter region (10, 25). In our in vitro system, TFIID is clearly present on a fraction of PICs, as determined by Western analysis (not shown) and Gal4 cross-linking, but it does not contribute to transcription, as demonstrated by the depletion of TFIID from extracts. The NuA4 histone acetyltransferase (HAT) complex is not predicted to be required in the absence of nucleosomes (14).

Gal11 has been well established as important for the maximal expression of Gal4-regulated genes. Gal4 and Gcn4 both bind multiple regions of Gal11 in vitro (37). Gal11 is a component of the Mediator tail domain composed of Gal11, Pgd1, Med2, and Sin4. In vitro studies showed that disruption of this tail domain by mutation of Sin4 or Pgd1 decreased the stability of Mediator and disrupted the stability of the Scaffold complex after transcription initiation (45). This instability contributes in part to the effect of Gal11 on multiround transcription. Consistent with our cross-linking results, it was observed in one study that the in vivo recruitment of Mediator could occur in the absence of SAGA (7). Although there is a modest Gal11 requirement for activation in our in vitro system, it is not essential. These results further demonstrate that not all activator targets contribute equally to transcription. Recently, it was found that efficient activation of chromosomally integrated genes required artificial recruitment of more than one factor (11). In accordance with these findings, the Gal4 activating region appears to make contacts with multiple factors in our in vitro system, and each of these interactions makes a differential contribution to the activated level of transcription, varying from critical to not functionally important.

In a parallel study, we inserted PEAS within the central Gcn4 activating region and identified cross-linked proteins within PICs, Scaffold complexes, and second-round PICs (17). Remarkably, this Gcn4 activating region also cross-links to Tra1, Gal11, and Taf12, although cross-linking to Ste12 was not observed. Like Gal4, Gcn4 cross-links to Taf12 and Tra1 in multiple complexes. For Gcn4, the interaction with SAGA and Gal11 was functionally important for activation, with Gal11 contributing modestly to activation and SAGA being essential for activation. Thus, two acidic activators converge on an overlapping set of targets within the PIC which differentially contribute to activation. Since Gal11, Taf12, and Tra1 have no obvious sequence similarity, it is unclear what features common to all three factors allow recognition by these two activators. It is also surprising that two activators have the same set of overlapping targets, since these two activators have no obvious primary sequence similarity other than being enriched in acidic and hydrophobic residues. It remains to be determined if other activators of this class also recognize this shared set of three factors. Additionally, natural activators such as Gcn4 and Gal4 often have multiple activating regions. It will be of interest to determine if multiple activating regions within the same protein contact an overlapping set of targets and if the presence of multiple activating regions can influence the interaction of factors with each individual activating region. In bacteria, for example, the two activating regions of CAP have previously been shown to each interact with a unique region of the RNA polymerase α subunit (36). The methods used here can be extended to examine this question as well as to examine targets of other classes of activators.

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