Proteomic Analysis Demonstrates Activator- and Chromatin-specific Recruitment to Promoters*‡§1,2

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Background: Quantitative proteomic analyses of transcription complexes were performed to compare different activators on naked and chromatin templates.

Results: Gal4-VP16 and Gal4-Gcn4 recruit SAGA, NuA4, and Swi/Snf but to different extents. Chromatin suppresses basal factor binding, leading to enhanced activation.

Conclusion: Different activators have distinct preferences in coactivators.

Significance: Quantitative mass spectrometry is a useful tool for analyzing transcription mechanisms.

In-depth characterization of RNA polymerase II preinitiation complexes remains an important and challenging goal. We used quantitative mass spectrometry to explore context-dependent Saccharomyces cerevisiae preinitiation complex formation at the HIS4 promoter reconstituted on naked and chromatinized DNA templates. The transcription activators Gal4-VP16 and Gal4-Gcn4 recruited a limited set of chromatin-related coactivator complexes, namely the chromatin remodeler Swi/Snf and histone acetyltransferases SAGA and NuA4, suggesting that transcription stimulation is mediated through these factors. Moreover, the two activators differentially recruited the coactivator complexes, consistent with specific activator-coactivator interactions. Chromatinized templates suppressed recruitment of basal transcription factors, thereby amplifying the effect of activators, compared with naked DNA templates. This system is sensitive, highly reproducible, and easily applicable to mapping the repertoire of proteins found at any promoter.

Interactions between proteins and their nucleic acid-binding sites form the basis of almost all nuclear processes, including assembly and remodeling of chromatin, subnuclear positioning of DNA and RNAs, and genetic information transfer events such as replication and transcription. The eukaryotic RNA polymerase II (RNAPII) promoter is a particularly well studied platform for protein recruitment. These DNA sequences recruit the transcription machinery to the correct locations throughout the genome so that RNA synthesis occurs in a site-selective manner (1). Studies of RNAPII promoters have identified multiple core DNA elements that individually or together direct proper assembly and orientation of the transcription pre-initiation complex (PIC) (1, 2). These basal promoter sequences are usually found within 100 bp of the transcription start site. However, no single DNA sequence element is found at all core promoters, suggesting that multiple types of PICs could be present in the cell. In addition to the core promoter sequence elements, regulatory transcription factors can bind nearby to augment or inhibit core promoter usage. For example, upstream activating sequences in yeast are often found within a few hundred base pairs upstream of the core promoter. The mammalian counterparts, called enhancers, are often kilobases away from the transcription start site and can function both upstream and downstream of the core promoter (3, 4).

Selective and efficient initiation from promoters in vitro minimally requires the basal transcription factor (TF) complexes TFIIIB, TATA-binding protein (the TBP subunit of TFIIID), TFIIIE, TFIIF, and TFIIH, which are highly conserved from yeast to human. Enhancers and upstream activating sequences bind gene-specific activator proteins, which in turn recruit coactivator complexes that collectively stimulate transcription beyond basal levels. These coactivators may include the TBP-associated factors of TFIIID, histone-modifying complexes, chromatin-remodeling complexes, and Mediator (a large multiprotein complex brought to the promoter by DNA-bound activators, which in turn recruit the core polymerase machinery) (5). In total, ~70 subunits of the Saccharomyces cerevisiae PIC have been identified, although isolation of pure...
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PICs has proven difficult (6, 7). Classical methods involve purification of specific biochemical activities through several steps of chromatography, followed by reconstituted *in vitro* transcription or DNA binding assays (2).

Recently, quantitative mass spectrometry has been used in conjunction with immobilized naked DNA templates incubated in nuclear extracts to characterize PICs (6, 7) and to identify DNA sequence-specific factors (8–10). Here, we utilized quantitative mass spectrometry to analyze PICs formed in the context of different transcription activators and on naked or chromatinized DNA templates. The activators Gal4-VP16 and Gal4-Gcn4 recruit the coactivators SAGA, NuA4, and Swi/Snf, but to different extents, suggesting that each activation domain has a preferred set of interactions. On naked DNA templates, levels of basal factors and Mediator are not strongly increased by activators. However, chromatin suppresses their association, resulting in a greater dependence on activators for Mediator binding and PIC assembly.

**EXPERIMENTAL PROCEDURES**

**Immobilized Promoter Templates**—Immobilized promoter templates were prepared as described (6, 11). A 400-bp fragment containing upstream vector sequence, one Gal4-binding site, and 71 bp of the yeast HIS4 promoter centered around the TATA box was PCR-amplified from pSH515 with primers p965 (5′-biotin-TAATGCAGCTGACGACAGG-3′) and pBK8 (5′-ATGTATGTACAACACACATCGG-3′). The “promoterless” template was created by PCR amplifying a 306-bp fragment that ends 14 bp downstream of the Gal4 site, and 71 bp of the yeast HIS4 promoter centered around the TATA box. This DNA was linked to streptavidin-coated magnetic beads via 5′-biotin on the top strand. The “promoterless” template was created by PCR amplifying a 306-bp fragment that ends 14 bp downstream of the Gal4 site, and 71 bp of the yeast HIS4 promoter centered around the TATA box. This DNA was linked to streptavidin-coated magnetic beads via 5′-biotin on the top strand. For chromatinized templates, DNA was generated by PCR amplifying a 306-bp fragment that ends 14 bp downstream of the Gal4 site with 71 bp of the yeast HIS4 promoter centered around the TATA box (supplemental Fig. S1A) as expected (11). The template used here contains the HIS4 core promoter and a single Gal4-binding site positioned 55 bp upstream of the TATA box (supplemental Fig. S1A). This DNA was linked to streptavidin-coated magnetic beads via 5′-biotin on the top strand. The template was incubated with *S. cerevisiae* nuclear extract and washed to reduce nonspecifically bound proteins. To ensure that isolated complexes were PICs that had not initiated transcription, extracts were first depleted of ATP, and binding reactions were done in the presence of a-amanitin. The promoter and associated proteins were eluted by restriction enzyme cleavage at a PsI site 20 bp upstream of the Gal4 site. The negative control DNA fragment was identical except that it lacked the HIS4 basal promoter (supplemental Fig. S1A). Immunoblotting showed that TBP, Rpb3 (RNAPII subunit), Tfa1 (TFIIE subunit), Tfb1 (TFIH subunit), and Tfg2 (TFIIF subunit) were strongly enriched on the HIS4 template (supplemental Fig. S1B) as expected (11).

**RESULTS**

**Proteomic Analysis of the HIS4 Promoter on a Naked Template**—The HIS4 promoter has been used previously for immobilized template pulldown experiments to study PIC assembly and activity. Building upon the work of Ranish et al. (11), the template used here contains the HIS4 core promoter and a single Gal4-binding site positioned 55 bp upstream of the TATA box (supplemental Fig. S1A). This DNA was linked to streptavidin-coated magnetic beads via 5′-biotin on the top strand. The template was incubated with *S. cerevisiae* nuclear extract and washed to reduce nonspecifically bound proteins. To ensure that isolated complexes were PICs that had not initiated transcription, extracts were first depleted of ATP, and binding reactions were done in the presence of a-amanitin. The promoter and associated proteins were eluted by restriction enzyme cleavage at a PsI site 20 bp upstream of the Gal4 site. The negative control DNA fragment was identical except that it lacked the HIS4 basal promoter (supplemental Fig. S1A). Immunoblotting showed that TBP, Rpb3 (RNAPII subunit), Tfa1 (TFIIE subunit), Tfb1 (TFIH subunit), and Tfg2 (TFIIF subunit) were strongly enriched on the HIS4 template (supplemental Fig. S1B) as expected (11).

The propensity of proteins to bind DNA nonspecifically often complicates the identification of physiologically signifi-
cant interactions, particularly when proteins of interest are present at low levels relative to abundant contaminants. To identify proteins bound specifically to the \textit{HIS4} promoter relative to the no-promoter control, we used a modified version of our previous quantitative mass spectrometry approach (12). First, we implemented selective solid-phase capture of cysteine-containing peptides in conjunction with iTRAQ labeling and LC-MS/MS. Protein samples are reduced with DTT, digested with trypsin, and desalted by reversed phase chromatography. Cysteine-containing peptides are captured on thiol-activated Sepharose beads, washed, and labeled with iTRAQ stable isotope reagents. Samples from different conditions are processed in parallel, and labeled peptides are eluted with DTT, combined, and analyzed by three-dimensional high-pH reversed phase/strong anion exchange/low-pH reversed phase (RP-SAX-RP) tandem mass spectrometry. A, schematic representation of the immobilized promoter experiment shown in Fig. 2. B, schematic representation of the immobilized promoter experiment shown in Fig. 2. C, iTRAQ reporter ion region from a representative MS/MS spectra of a RPA135 peptide, detected at equal quantities in each sample, and a Gal4 peptide, detected only in the activated transcription sample.

FIGURE 1. Proteomic analysis of PICs. A, workflow for cysteine peptide capture in conjunction with iTRAQ labeling and LC-MS/MS. Protein samples are reduced with DTT, digested with trypsin, and desalted by reversed phase chromatography. Cysteine-containing peptides are captured on thiol-activated Sepharose beads, washed, and labeled with iTRAQ stable isotope reagents. Samples from different conditions are processed in parallel, and labeled peptides are eluted with DTT, combined, and analyzed by three-dimensional high-pH reversed phase/strong anion exchange/low-pH reversed phase (RP-SAX-RP) tandem mass spectrometry. B, schematic representation of the immobilized promoter experiment shown in Fig. 2. C, iTRAQ reporter ion region from a representative MS/MS spectra of a RPA135 peptide, detected at equal quantities in each sample, and a Gal4 peptide, detected only in the activated transcription sample.
teine-containing peptides; on average, cysteine represents ~1% of all amino acids in the yeast proteome, with ~85% of all proteins containing at least one cysteine. As a result, enrichment of cysteine-containing peptides provides a nearly 10-fold decrease in mixture complexity while maintaining representation of the majority of yeast proteins. Immobilization of cysteine peptides enabled iTRAQ labeling directly on the beads, minimizing sample lyophilization and other handling steps. Finally, we used our recently described automated three-dimensional high-pH reversed phase/strong anion exchange/low-pH reversed phase fractionation platform to provide efficient separation of iTRAQ-labeled peptides (13, 14). These modifications ensure high dynamic range of protein identification while simultaneously minimizing the deleterious effects of peptide ratio compression that have been associated with iTRAQ-based analysis of complex mixtures (15–17).

First, we compared proteins associated with promoterless templates with those binding to the Gal4-binding site-HIS4 promoter chimera in the presence or absence of activator (Fig. 1B). Gal4-VP16 can stimulate transcription by up to 10-fold on this promoter (11). At a depth of 13 high-pH reversed phase/strong anion exchange/low-pH reversed phase fractions, we detected almost 700 proteins on average in each run, with ~97% of the identified peptides containing at least one cysteine. Two biological replicate experiments exhibited a high degree of overlap in terms of both peptide and protein identification (67 and 70%, respectively) (supplemental Fig. S2A). We also observed good reproducibility for iTRAQ ratios across biological replicates (supplemental Fig. S2B). As expected, most proteins appeared to be nonspecifically associated as evidenced by uniform ratios across all channels (115/114, 116/114, and 116/115) (Fig. 1C and supplemental Fig. S2, B and C). Significantly, exogenous Gal4 was detected only in iTRAQ channel 116 (Fig. 1C).

To estimate each protein’s probability of being enriched either on the promoter template or in the presence of Gal4-VP16, we used a mixture modeling approach (18). The distribution of the log (base 2) of iTRAQ ratios was fitted to three Gaussian distributions: enriched, not enriched, and excluded. The probability of a protein being in the enriched or the excluded distribution was then estimated as a function of its Gaussian distributions: enriched, not enriched, and excluded.

For activated complexes versus the no-promoter sample (116/114), ratios of 2.0 or greater (1.0 on the log2 scale) indicated a probability of enrichment of >0.6, so these proteins were categorized as likely promoter-associated factors (supplemental Fig. S2C, left panel). The majority of the 56 proteins in this class consisted of basal initiation factors, Mediator, and the chromatin-remodeling complexes SAGA, Swi/Snf, and NuA4 (Fig. 2, A and B, and supplemental Table S1). Components of replication protein A were also enriched on the HIS4 promoter templates, as reported previously (12). A number of other proteins known to be components of the PIC, notably many TFIIH and TFIIID subunits, were identified at higher levels on the promoter, but with ratios just below the 2-fold threshold to be categorized as enriched. Some subunits within enriched complexes were not detected, but this is unlikely to be due to partial assemblies within the PIC. In some cases, the “missing” subunits lacked cysteines or had cysteines outside the detectable size range. Stochastic undersampling is a likely explanation for other cases. For example, one of the TFIIIE subunits not found by mass spectrometry was strongly enriched when assayed by immunoblotting (data not shown).

Effects of the Gal4-VP16 Activator on the PIC Proteome—A surprisingly small set of proteins was specifically recruited by Gal4-VP16 (Fig. 2C and supplemental Table S1). Virtually all came from three chromatin-related coactivator complexes: the histone H3 acetyltransferase SAGA, the histone H4 acetyltransferase NuA4, and the ATP-dependent chromatin remodeler Swi/Snf (Fig. 2D). Interestingly, subunits of many chromatin-related complexes were identified (RSC, Chd1, Isw1, Isw2, Ino80, Swr1, SAS, and Rpd3L), but these are likely to be nonspecifically associated because they were not enriched on promoter templates relative to the no-promoter control. However, a number of components of the RNAPII transcription factor TFIIIC complex were excluded in the presence of Gal4-VP16 (Fig. 2D and supplemental Table S1).

Interestingly, although basal initiation factor recruitment on a naked DNA promoter template showed a slight positive trend in the presence of Gal4-VP16, none of these proteins exceeded the 2-fold threshold for enrichment (Fig. 2E and supplemental Table S1). Immunoblotting confirmed the very modest increase in basal factor recruitment by the activator (Fig. 2F). Mediator, which has been shown genetically and biochemically to interact with activators, was also only slightly affected by Gal4-VP16 (Fig. 2D). These findings are consistent with reports suggesting that yeast Mediator associates tightly with RNAPII and may function as part of the basal initiation machinery (19–24). The minimal activator-dependent binding of basal factors is consistent with experiments showing that activators primarily stimulate reinitiation rather than the first round of transcription (25) and that this function may be the primary mode of transcription activation on naked DNA templates (11). Using our extracts and activators, we confirmed that single-round reactions gave <1.4-fold activation, whereas multiround reactions showed 2.6–4.3-fold activation (data not shown).

Comparison of Two Transcription Activators—Given the limited set of complexes recruited by Gal4-VP16, we were curious to test whether another activator would recruit the same set...
of complexes to the promoter. Gal4-Gcn4 contains the identical DNA-binding domain as in the experiment above but carries the Gcn4 activation domain (Fig. 3A) (26). Gal4-Gcn4 also stimulated transcription from the HIS4 promoter, although not as strongly as Gal4-VP16 (Fig. 3B). Immobilized template assays were performed with Gal4-Gcn4 and analyzed as described above (Fig. 3C). As before, we observed good reproducibility across biological replicates for both protein identifications and iTRAQ ratios (supplemental Fig. S3).

Gal4-Gcn4 stimulated binding of the same limited set of complexes as Gal4-VP16 (Fig. 3, D and E, and supplemental Table S2). Interestingly, although recruitment of SAGA and Swi/Snf was similar for the two activators, NuA4 subunits that were strongly enriched by Gal4-VP16 were only modestly recruited by Gal4-Gcn4 (Fig. 3, F and G). These data show that activation domains have quantitatively and qualitatively different abilities to interact with coactivators and therefore are likely to stimulate transcription through different complexes.

Proteomic Analysis of the HIS4 Promoter on a Chromatinized Template—To determine the effect of nucleosomes on PIC formation, the template used above was assembled into chromatin using recombinant yeast histones and the assembly factors Nap1 and Isw1a (27). Chromatinization was verified using recombinant yeast histones and the assembly factors recruited by Gal4-Gcn4 (Fig. 3, C, right panel). These data show that activation domains have quantitatively and qualitatively different abilities to interact with coactivators and therefore are likely to stimulate transcription through different complexes.

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As seen on naked DNA templates, the addition of the Gal4-VP16 activator to chromatinized templates significantly and specifically increased binding of SAGA, NuA4, and Swi/Snf, but not other chromatin-related factors (Fig. 5, A and B, and supplemental Table S4). Interestingly, Gal4-VP16 on the chromatin templates now more strongly increased the levels of RNAPII, basal factors, and especially Mediator. Both immunoblotting (Fig. 5C) and in vitro transcription (Fig. 5D) confirmed that chromatin suppressed basal factor association, leading to stronger recruitment upon addition of the activator. These results suggest that activators help overcome chromatin-mediated repression of PIC assembly.

The activator Gal4-Gcn4 was again compared with Gal4-VP16, now using chromatinized templates (Fig. 6A and supplemental Fig. S6 and Table S5). Both activators gave similar levels of in vitro activation (Fig. 6B), and Gal4-Gcn4 recruited the same set of coactivators, namely NuA4, SAGA, and Swi/Snf (Fig. 6, C and D). The suppression of basal PIC recruitment by chromatin led to a larger -fold increase in basal factors by Gal4-Gcn4, with Mediator being particularly enhanced. Direct comparison of the two activators on chromatin showed that, as seen on naked DNA templates (Fig. 3), the levels of Swi/Snf recruitment were similar, and NuA4 appeared to be a better target for the VP16 activation domain (Fig. 6, E and F). In contrast, SAGA and Mediator were more strongly recruited by Gal4-Gcn4, perhaps suggesting additional interactions with or enhanced by the chromatin template.

**DISCUSSION**

Genome-wide analyses show that no single DNA sequence element is found at all eukaryotic promoters (28), suggesting there are likely to be multiple modes of promoter recognition and possibly even multiple types of PICs (29). Each promoter has a distinct arrangement of sequence elements that recruit basal and regulatory transcription factors, making it increasingly important to determine the associated protein components. Herein, we utilized a multiplexed quantitative proteomic strategy to assay basal and activated transcription on both chromatin and naked templates, as well as to compare two different activators. Cysteine peptide capture combined with multidimensional fractionation provides for efficient quantification over a wide dynamic range of the proteome, although ~15% of yeast proteins lack a cysteine residue and hence will not be represented in these experiments. The single-stranded DNA-binding protein Sub1 (12) is one notable example of this class of proteins not detected in our analysis. Very small or very large cysteine-containing

**FIGURE 3. Proteomic analysis of different activated transcription complexes.** A, schematic diagram of Gal4-VP16 and Gal4-Gcn4 activator proteins used in this study. Both have a common DNA-binding domain from Gal4, and each has a unique activation domain, as described previously (11). B, in vitro transcription reactions were performed in nuclear extract using the immobilized HIS4 promoter templates, which included ~150 nucleotides downstream of the transcription start site. Reactions were performed in the absence of activator or in the presence of Gal4-Gcn4 or Gal4-VP16. C, schematic representation of the immobilized promoter reactions analyzed by iTRAQ-based quantitative mass spectrometry. RP-SAX-RP, high-pH reversed phase/strong anion exchange/low-pH reversed phase. D, scatter plot of the average protein ratio for reactions with and without Gal4-Gcn4 (log_{2}(117/115)). Threshold log_{2} ratios for proteins enriched or excluded with probability ≥0.6 are +0.40 and −0.88, respectively (supplemental Fig. S3C, left panel). The peach circle represents Gal4. Other proteins are color-coded based on their membership in coactivator complexes. Gray circles represent proteins that are not members of coactivator complexes. E, NuA4, SAGA, and Swi/Snf subunits from D organized by complex. Two other NuA4 subunits (Ep11 and Yaf9) were identified only in a single replicate, but likewise were recruited more strongly by Gal4-VP16 (supplemental Table S2). F, scatter plot of the average protein ratio for Gal4-Gcn4/Gal4-VP16 (log_{2}(117/116)). Proteins are color-coded as described for D. Threshold log_{2} ratios for proteins enriched or excluded with probability ≥0.6 are +0.65 and +0.067, respectively (supplemental Fig. S3C, right panel). G, NuA4, SAGA, and Swi/Snf subunits from F organized by complex.
Peptides may also be missed. Nonetheless, except for Sub1, none of the expected transcription factors were missing from our data. Our approach is easily extended to any promoter context and can be used to analyze up to eight conditions (e.g. activators, promoters, etc.) simultaneously (30). In addition to the work described herein, we have successfully applied two- and three-dimensional high peak capacity separations for quantitative proteomic analyses in multiple bio-

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**FIGURE 4.** **Proteomic analysis of chromatinized DNA templates.** A, workflow for proteomic analysis of PIC assembly on a chromatinized *HIS4* template. B, proteins that were specifically enriched on chromatinized templates are listed, along with their description and log2 ratio. C, Scatter plot of protein ratios on chromatinized versus naked templates. Log2 ratios were derived from iTRAQ reporter ions across replicate experiments; threshold log2 ratios for proteins enriched or excluded with probability $p < 0.05$ are $+0.66$ and $-1.2$, respectively (supplemental Fig. S4C). Complexes are color-coded as described in the legend to Fig. 2. D, the values from C for known transcription factors are grouped by complex.
logical contexts (12, 13, 31–33). Continued development in mass spectrometry acquisition methods (34–37) may provide further improvements in the performance of iTRAQ-based quantitative studies.

More than 50 proteins specifically associated with the HIS4 promoter template were identified (Fig. 2A and supplemental Table S1). Most of these promoter-associated proteins are known transcription factors previously identified using in vitro and cell-based experiments (2). All known basal factors, as well as multiple chromatin and coactivator complexes, were also detected. However, these are unlikely to simultaneously co-occupy a single DNA molecule, as the pathway to a productive PIC may involve multiple complexes dynamically assembling and disassembling at the promoter (29). It was estimated that ~10% of DNA molecules in immobilized template assays are bound by a transcriptionally competent PIC (25), indicating that a mixture of complexes is captured on the immobilized templates.
A surprisingly small subset of proteins was specifically enriched on naked DNA templates by activator proteins (Fig. 2D). These were almost exclusively components of three complexes: SAGA and NuA4 are histone acetyltransferases, whereas Swi/Snf is an ATP-dependent chromatin remodeler (5). Along with Mediator, these three coactivator complexes are known to cross-link directly to activation domains (38–40). Interestingly, we observed quantitatively different recruitment of these complexes when comparing two different activation domains (Fig. 3), demonstrating that specific activators can have differ-
ent targets. Somewhat surprisingly, RNAPII, the basal initiation factors, and Mediator were only modestly enriched in the presence of activator (Fig. 2E). This result indicates that coactivator recruitment is independent of the PIC and that PIC assembly is not limiting under these conditions. Consistent with these observations, Gal4-VP16 modestly stimulates a single round of transcription on naked DNA (11) while stabilizing a “scaffold” of proteins left behind at promoters after initiation to facilitate reinitiation (25). Mediator may have activator-independent functions at the HIS4 promoter (26), and its tight association with RNAPII has led to suggestions that it acts as a basal initiation factor (19–21, 23, 24). Unexpectedly, we observed that activators decreased the association of TFIIC complexes on promoters. Whether this effect is the result of competitive binding or targeted displacement remains to be determined, but this finding raises the interesting possibility that transcription activation may involve removal, as well as recruitment, of factors.

Chromatinized templates had a significant impact on our results. There was an overall reduction in PIC assembly, contrasting with a strong increase in known chromatin-binding proteins (Fig. 4). Gal4-VP16 and Gal4-Gcn4 still recruited only NuA4, SAGA, and Swi/Snf, with interesting quantitative differences between the activators (Figs. 5 and 6). On chromatin templates, the activators had a much stronger stimulatory effect on promoter binding of Mediator, basal factors, and RNAPII. This difference was also reflected in vitro, where chromatin suppressed basal transcription, resulting in a larger -fold increase in activated transcription. Because HIS4 is a strong TATA promoter, PIC assembly may occur largely independent of activators on naked templates. Competing nucleosomes may confound a requirement for an activator-Mediator interaction, as well as potentiate the effects of the chromatin-related coactivators.

The quantitative mass spectrometry approach used here will be easily amenable to studying different promoters and activators. Future experiments will determine whether the same set of factors and interactions are universal or can vary on different genes.

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