The histone H4 N-terminal tail has long been regarded as a major regulator in chromatin structure and function. Although the underlying mechanism has not been unraveled, an emerging body of evidence supports that H4 tail and its post-translational modification function as a recruitment motif for key factors required for proper regulation of chromatin transcription. To investigate these aspects, we have generated HeLa cell lines that constitutively express ectopic H4 tail domain for biochemical purification of proteins associated with H4 tail. We found that expressed H4 tails stably associate with sets of transcription regulatory factors and histone methyltransferases distinct from those that associate with histone H3 tails. Importantly, point mutations of four major lysine substrates to block cellular acetylation of ectopic H4 tail significantly inhibited the association of histone methyltransferases and sets of transcription-activating factors, supporting a major role of acetylation on recruitment-based action of H4 tail during transcription. Further, our transcription analysis revealed that the proteins associated with wild-type/acetylated H4 tail, but not with mutant/unacetylated H4 tail, can enhance p300-dependent chromatin transcription. Taken together, these findings demonstrate novel roles for H4 tail and its acetylation in mediating recruitment of multiple regulatory factors that can change chromatin states for transcription regulation.

The DNA in eukaryotic cells is hierarchically packaged by four core histones to form a highly dynamic structure of chromatin (1–4). The histone N termini (called histone “tails”) that protrude from the surface of the nucleosome are key elements in the regulation of the functional state of chromatin. The most important character of histone tails is their reversible post-translational modifications, which are linked to both activation and repression of chromatin transcription (5–7). Histone acetylation is one of the major modifications that are introduced on specific lysine residues of all four core histones and generally associated with transcriptional activation (8, 9). In particular, given that the H3–H4 tetramers interact with the central part of nucleosomal DNA, H3–H4 tail domains and their acetylations have been linked to the major arbiters for transcriptional activation (10). Indeed recent studies using recombinant chromatin templates clearly established a critical role of acetylations of H3–H4 tails in p300-mediated transcription based on the transcription-inhibiting effects of substitution of major lysine substrates or deletion of H3–H4 tails (11). Histone methylation is another modification that occurs particularly in lysine and arginine residues of histones H3 and H4 with remarkable specificity (12, 13). Although it remains unclear to what extent, histone H3 and H4 methylation has been recognized as a key player for the precise regulation of chromatin function. In sharp contrast with histone acetylation, histone methylation can result in either activation or repression of chromatin transcription, depending on the modification site within histones (14–17).

Although studies investigating a possible effect of H3–H4 tails in transcription emphasize their important role in regulating gene transcription, the precise mechanism of action of H3–H4 tails during transcription still remains unclear. In many cases, H3–H4 tails exposed outside of the nucleosome core are found to serve as binding sites for distinct sets of factors that facilitate chromatin remodeling, and this altered chromatin state in turn regulates the transcriptional machinery to initiate gene transcription. The addition of chemical moieties on H3–H4 tails has been proposed to have specific effects on this recruitment-based action of H3 and H4 tails in transcription (18–20). Consistent with this model, recent studies demonstrated that particular modifications of histone tails can facilitate recruitment of regulatory proteins to distinct genomic regions by recognizing specific protein domains such as bromo- and chromo-domains (21–27).

As part of an effort to understand the mechanism of action of histone tails and their modifications in transcription, we recently have developed the protocol to purify histone tail-interacting proteins from living cells by constitutively expressing histone tail domains (28). Following this new protocol, we successfully purified and identified sets of histone modifying and transcription regulatory factors stably associated with ectopic H3 tails. Additionally, our transcription assays showed that the H3 tail-associated factors significantly enhance p53-dependent, p300-mediated transcription from chromatin template.
More importantly, it appears that cellular methylations of H3 tails at Lys-9 and Lys-27 are required for the association of repressive factors such as HP1, HDAC5/9, Mi-2b, G9a, and TIF1β. Thus our observations bear an important implication on a possible coupling between H3 tail-mediated factor recruitment and chromatin transcription. Given this selective interaction of H3 tails with regulatory proteins, it would be interesting to check if other histone tails also recognize specific signaling components to give similar outcomes in chromatin transcription.

In the present study, we extended our efforts to characterize H4 tail-interacting factors purified from cells that continuously express the epitope-tagged H4 tail domain. This approach allowed us to purify and identify multiple regulatory factors that specifically associate with H4 tails in vivo. Significantly, in support of chromatin-specific action of these H4 tail-associated factors, purified factors can activate p53-dependent, p300-mediated transcription from chromatin templates, but not from DNA template. More importantly, similar experiments with proteins purified with lysine-mutated (unacetylated) H4 tails showed no stimulatory effect on transcription, clearly highlighting the requirement of acetylation of ectopic H4 tails for the association of active factors.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**—The cDNA corresponding to the first 40 amino acids of human H4 was generated by PCR amplification using the sense primer 5’-GGCTAGATATCACTAGCAACCTCAAACAG and the antisense primer 5’-GTCGT CCTTGTAATCGCCGTCCTCATCTTGACGAG. The resulting PCR fragment was digested with NotI and EcoRI and subcloned into the same sites of pBS SK+ (pBS-1xnH4). The plasmid (pBS-2xnH4) containing two copies of H4 tail cDNA was generated by inserting the NsiI-EcoRI-digested H4 tail cDNA fragment into PstI-EcoRI-digested pBS-1xnH4. The NsiI-EcoRI fragment containing two copies of H4 tail from pBS-2xnH4 was inserted into PstI-EcoRI-digested pBS-2xnH4 to generate the plasmid containing four copies of H4 tail (pBS-4xnH4). The plasmid for mammalian expression was generated by inserting the EcoRI-NotI digested four copies of H4 tail fragments from pBS-4xnH4 into the same sites of pIRES, which has been modified to carry the FLAG-HA epitope at the 5’-end of the inserts (pFHnH4-iresneo). For the plasmid encoding the mutant H4 tail domain, the same procedure was followed except that Lys-5, Lys-8, Lys-12, and Lys-16 of the original H4 cDNA were first mutated to arginine by using the QuikChange Site-Directed Mutagenesis kit (Stratagene). All mutations were confirmed by DNA sequencing.

**Purification and Mass Spectrometry Analysis of the H4 Tail-associated Factors**—To generate the stable cell lines that express human H4 tail domains, HeLa cells were transfected with pHnH4-IRESneo using Lipofectamine (Invitrogen) and selected with G418 (500 µg/ml) for 2 weeks. Selected cells were grown in suspension culture (20 liters) at 37 °C to the density of 1.3 × 10⁶/ml, and nuclear extracts were prepared from cells as described (29). For the purification of H4 tail-associated factors, 200 mg of nuclear extract was applied to a P11 ion exchange column (Whatman). The P11 BC1000 fractions containing expressed H4 tails were dialyzed against BC300 and applied to anti-FLAG M2 affinity chromatography (Sigma). The H4 tail-associated factors were eluted from the affinity resin by using FLAG peptide (200 ng/ml) after washing with BC300 containing 0.1% Nonidet P-40. The purified proteins were analyzed by data-dependent tandem mass spectrometry as described recently (30). Peptides were identified by searching tandem mass spectrometry spectra against a human protein sequence data base using SEQUEST as described previously (31). Data were analyzed using a suite of software tools, including INTERACT (32), PeptideProphet (33), and ProteinProphet (34), to determine whether peptide and protein assignments were correct.

**Preparation of Histone Octamers**—Expression of intact and tailless histones and preparation of histone octamers were essentially as described (11, 35). Mutant H4 with a substitution of arginine 3 to glutamine was constructed by using the QuikChange mutagenesis kit (Stratagene) as recently described (11). The quantity and purity of histone preparations were analyzed by SDS-PAGE.

**Antibodies**—Antibodies used for Western analysis were as follows: anti-FLAG, anti-Lamin A/C, and anti-β-actin antibodies were from Sigma; anti-HA3 and anti-Nucleolin antibodies were from Santa Cruz Biotechnology; anti-Tubulin antibody was from Cell Signaling Technology; anti-p32 antibody was from CeMines; anti-CAPA-R antibody was from Bethyl; anti-PRMT5, anti-asymmetric dimethyl H4-R3, anti-acetyl H4-K5, anti-acetyl H4-K8, anti-acetyl H4-K12, and anti-acetyl H4-K16 antibodies were from Upstate; and anti-symmetric dimethyl H4-Arg-3 antibody was from Abcam. Anti-BAF170, anti-TRAP150, and anti-BAF53 antibodies were kindly provided by R. G. Roeder. Anti-monomethyl H4-K20, anti-dimethyl H4-K20, and anti-trimethyl H4-Lys-20 antibodies were given by J. C. Rice.

**Histone Methyltransferase and in Vitro Transcription Assays**—HMT assays were performed with 1 µg of recombinant histone octamers as recently described (28, 36) by incubating the histone octamers with the H4 tail-associated factors for 1 h at 30 °C in HMT reaction buffer (100 mM HEPES at pH 7.8, 300 mM KCl, 2.5 mM EDTA, 25 mM dithiothreitol, 50 mM sodium butyrate) in the presence of 2.3 µM [3H]SAM (Amersham Biosciences) or 50 µM cold SAM (Sigma). Standard p53-dependent transcription assays were performed as reported recently (28) by adding H4 tail-associated factors together with p300 and acetyl-CoA.

3 The abbreviations used are: HA, hemagglutinin; BAF170, Brg1-associated factor 170; DOTI1, Disruptor of telomeric silencing-1, large protein; TAF4, TBP-associated factor 4; CRSF3, cofactor required for SP1 activation 3; TRAP150, thyroid receptor-associated protein 150; PRMT5, protein arginine methyltransferase 5; CAPERα, coactivator of activating protein-1 and estrogen receptors α; MT2A, metastasis-associated protein 2; DDX17, DEAD (Asp-Glu-Ala-Asp) box polypeptide 17; COBRA1, cofactor of BRC1a; hnRNP, heterogeneous nuclear ribonucleoprotein; BAF53, Brg1-associated factor 53; MEP50, methionyl protein 50; NAP1, nucleosome assembly protein 1; SAM, S-adenosyl-L-methyl-[3H]methionine; HMT, histone methyltransferase.
Histone H4 Tail-associated Factors

RESULTS

Biochemical Purification of H4 Tail Interacting Factors from Cell Lines—We have recently demonstrated that H3 tails are stably associated in vivo with multiple regulatory factors that can promote p53-dependent, p300-mediated chromatin transcription. Because other histone tails are also known to interact with chromatin-associated proteins in vitro, the use of a similar method to identify and characterize factors recognizing other histone tails should be a logical extension of our study. To this end, we created HeLa cell lines with FLAG and HA tags integrated at the N terminus of the gene encoding the first 40 amino acids of human histone H4. The tail domain contains the nuclear localization signal, which will allow subcellular traffic of the H4 tail from the cytoplasm to the nucleus (37).

We first checked whether the expressed tails are accumulated in the HeLa nucleus. As expected, major fractions of expressed tails were transported from the cytoplasm into the nucleus (Fig. 1B).

Nuclear extracts were prepared from cultured cells grown in suspension culture and fractionated on P11 chromatography using a step gradient with increasing salt. Western blot analysis of eluates from the column with FLAG antibody showed that the H4 tail-associated factors were present in P11 BC1000 fraction (Fig. 1A). To purify the proteins further, the H4 tail-containing P11 fraction was applied to immunoaffinity chromatography on anti-FLAG resin, and the H4 tails were eluted by FLAG peptide in complexes with their interacting factors. In parallel, we also generated a cell line with mutant H4 tail domain carrying arginine substitutions at four major acetylation sites (Lys-5, Lys-8, Lys-12, and Lys-16), which block cellular acetylation of the H4 tail domain. We found that the level of expression and nuclear localization of the mutant H4 tail is comparable with that of the wild-type H4 tail (Fig. 1B). Nuclear extracts from cultured cells were subjected to the same purification procedure employed to isolate wild-type H4 tail-associated factors. Our purification of wild-type and mutant tail-associated factors was confirmed by Western blot analysis with HA antibody (Fig. 1A, lanes 2 and 3). SDS-PAGE analysis of the purified factors revealed similar band profiles between wild-type and mutant H4 tail-associated factors, along with four additional high molecular bands in the wild-type tail-associated factors and one additional low molecular band in the mutant tail-associated factors (Fig. 2A).

FIGURE 1. Purification of H4 tail-associated factors from HeLa cells. A, schematic summary of purification of H4 tail-associated factors. Nuclear extracts from H4 tail-expressing cells were first fractionated by P11 cation exchange column. The 1.0 M KCl eluates containing ectopic H4 tails were further purified with anti-FLAG M2 antibody as described under “Experimental Procedures.” The purified proteins were separated in SDS-PAGE and subjected to Western blot analysis with anti-FLAG and -HA antibodies (NE, nuclear extract; FT, flow-through). ‘A’ and ‘M’ indicated sites of acetylation and methylation of H4 tails, respectively. Lane 1, aliquot from the mock purification; lane 2, the proteins purified from wild-type H4 tail expressing cells; lane 3, the proteins purified from mutant H4 tail expressing cells. B, nuclear localization of ectopic H4 tails. Nuclear and cytoplasmic fractions were subjected to immunoblot analysis with anti-FLAG antibody. Tubulin and Lamin A/C were used as markers for cytoplasmic and nuclear fractions, respectively. Lanes 1 and 3, wild-type H4 tails; lanes 2 and 4, mutant H4 tails.

FIGURE 2. Identification of H4 tail-associated polypeptides. A, mass spectrometric analysis of H4 tail-associated polypeptides. After a large scale isolation of H4 tail-associated polypeptides, the purified polypeptides were resolved in 4–20% gradient SDS-PAGE. The specific polypeptide bands were excised, and the protein identities were determined by mass spectrometric analysis as described under “Experimental Procedures”. Proteins associated with only wild-type H4 tails are underlined. Lane 1, the proteins purified from wild-type H4 tail-expressing cells; lane 2, the proteins purified from mutant H4 tail-expressing cells. The positions of the molecular mass markers are shown. B, Western blot analysis of the purified tail-associated proteins. H4 tail-associated proteins were separated by 4–20% SDS-PAGE, transferred to nitrocellulose, and then probed with the specific antibodies indicated on the left. Lane 1, aliquot from the mock purification; lane 2, proteins purified from wild-type H4 tail expressing cells; lane 3, proteins purified from mutant H4 tail expressing cells.
Mass Spectrometric Identification of H4 Tail-associated Factors—To identify polypeptides associated with ectopic H4 tails, we performed mass spectrometry on the purified tail-associated factors. Because several protein bands were specifically stained in wild-type or mutant H4 tail-associated factors, we paid special attention to these proteins. From our analysis of the wild-type H4 tail-associated factors, we identified 18 proteins that have previously been shown to play a role in transcription regulation. Two of these proteins are HMTs PRMT5 and DOT1L (Fig. 2A, lane 1). PRMT5 is known to methylate arginine 3 on H4 and to a lesser extent unknown sites on H3/H2A tails (38), whereas DOT1L mediates H3 methylation with substrate specificity for Lys-79 (39, 40). Because H4 tail is localized in the vicinity of H3-Lys-79 (10), it is not unlikely that H4 tails play a role in controlling H3-Lys-79 methylation, possibly through stable recruitment of DOT1L onto nucleosomes. We also identified two chromatin remodeling-related factors BAF53 and nucleolin in the H4 tail-associated factors (Fig. 2A, lane 1). Because these two factors have been shown to assist nucleosome remodeling (41, 42), our results also raise the possibility that H4 tails play a role in initial association of these activities to facilitate transcription through the nucleosome. In addition, a group of proteins related to transcriptional regulation (MTA2, BAF170, β-actin, TRAP150, TAF4, CRS53, CAPERα, Ddx17, COBRA1, and hnRNPK) were identified in the H4 tail-associated factors (Fig. 2A, lane 1).

On the other hand, mutations of tail acetylation sites (Lys-5, Lys-8, Lys-12, and Lys-16) completely abolished association of H4 tails with ten active factors BAF170, DOT1L, TRAP150, TAF4, CRS53, Nucleolin, MTA2, COBRA1, hnRNPK, and BAF53, which allows us to categorize them as acetylation-specific binding proteins (Fig. 2A, lane 2). Further analysis detected p66α, Srp30c, and p32 in the mutant H4 tail-associated factors (Fig. 2A, lane 2) but not in the wild-type tail-associated factors, implying that cellular acetylation of ectopic H4 tails has inhibitory effects on association of these repressive factors. The mass spectrometry results were further confirmed by immunoblot using available antibodies. Interestingly, although we could detect PRMT5 in both wild-type and mutant H4 tail-associated factors by mass spectrometry analysis, our Western blot analysis revealed that association of PRMT5 with H4 tail was distinctly reduced by the mutations (Fig. 2B). Control purifications performed with normal HeLa nuclear extract did not show any bands in all Western blot analysis, demonstrating that all purified proteins specifically interact with the tagged H4 tails (Fig. 2B, lane 1). Collectively, our results indicate that free H4 tail domains ectopically expressed in living cells can interact with sets of chromatin remodeling and transcription factors.

Cellular Acetylation and Methylation of Expressed H4 Tails—Because ectopic H4 tails can undergo dynamic post-translational modification via the actions of cellular modifying enzymes, we next assessed the modification status of the ectopic H4 tails by Western blot analysis. Specificity of the antibodies used in this study was confirmed by Western blot analysis using (un)modified recombinant H4 (data not shown). We first checked the acetylation of purified H4 tails by using a set of highly specific antibodies that recognize acetylation of individual lysine residues within H4 tails (Lys-5, Lys-8, Lys-12, and Lys-16). Consistent with the previous reports that cellular acetylation at Lys-8 is only introduced onto nucleosomal H4 (43, 44), our purified H4 tails were found to be acetylated at Lys-5, Lys-12, and Lys-16, but not Lys-8 (Fig. 3A, lane 2). Therefore, cellular acetylation of free H4 tails appears to be selective process rather than random process.

We next checked the methylation of the H4 tails with antibodies specific to asymmetric and symmetric dimethylation of Arg-3. We detected symmetric dimethylation of Arg-3 (Fig. 3B, lane 2), which are generally related to gene activation (45, 46). It has been shown that PRMT5 catalyzes symmetric dimethylation of H4-Arg-3, whereas another H4-Arg-3-specific methyltransferase PRMT1 results in the generation of asymmetric di-methylation (17, 38). Therefore, we assume that PRMT5 of the tail-associated factors generates methylation of Arg-3 of ectopic H4 tails. Similar analysis with mono-, di-, and tri-methylation of Lys-20 also revealed mono-methylation of Lys-20 (Fig. 3C, lane 2), which is related to both transcription activation and repression (47, 48). This result is consistent with the recent report that mono-methylated H4-Lys-20 is the major methylated form of H4-Lys-20 in the free histone fractions (44). We next extended our analysis to the mutant H4 tails. As expected, our results showed that mutations at Lys-5, Lys-8, Lys-12, and Lys-16 completely abolished their cellular acetylations (Fig. 3A, lane 3). In further analysis, we found that mutations of the acetylation sites minimally affected the degree of mono-methylation at Lys-20 (Fig. 3C, lane 3) but moderately enhanced di-methylation at Arg-3 (Fig. 3B, lane 3). Therefore, our results confirm that free H4 tails ectopically expressed in living cells exist in a specific modification state.

HMT Activities of H4 Tail-associated Factors—Because our mass spectrometry analysis revealed the presence of PRMT5...
and DOT1L, which are histone-methylating cofactors, we next checked if the purified factors can indeed establish the expected histone methylation. To confirm the specificity of the factors in tail modification, recombinant intact histone octamers and histone octamers lacking specific N-terminal tail domains were prepared by using recombinant histones expressed in bacteria (Fig. 4A). HMT assays were performed according to the protocol described recently (28, 36). As expected, our assays with the H4 tail-associated factors confirmed that H4 can be highly methylated, whereas H3 and H2A/H2B can be moderately methylated by the purified factors (Fig. 4B, lane 2). Removal of H2A tail alone or H2A/H2B tails together completely blocked methylation of H2A/H2B by the H4 tail-associated factors (Fig. 4B, lanes 5 and 8), reflecting that HMT activity (most likely PRMT5) of the purified factors can methylate H2A, but not H2B (49). A similar analysis of H3 tailless histone octamers failed to show any methylation of H3 and H2A (Fig. 4B, lane 11), indicating that H2A methylation is somehow linked to H3 tail domain. Because tailless H3 in our assay has DOT1L substrate (Lys-79) within its globular domain, it is likely that the observed methylation of H3 on wild-type histone octamers is due to PRMT5. In addition, removal of both H3 and H4 tails significantly enhanced methylation of H2A (Fig. 4B, lane 14), which demonstrates an intrinsic preference of PRMT5 in the purified factors for H4 and, to a lesser extent, H3. It is also possible that some other H4 tail-associated factors might preferentially interact with H3 and H4 tails, which will in turn inhibit PRMT5-mediated methylation of H2A tail.

Because PRMT5 is known to preferentially methylate H4-R3, H4 methylation by the purified factors was further characterized by Western blot analysis using antibodies specific for symmetric and asymmetric di-methylations of Arg-3. As shown in Fig. 4C, our analysis confirmed the presence of HMT activity specific for symmetric di-methylation of H4-Arg-3. A further analysis with H4 with Arg to Gln mutation at Arg-3 failed to show any H4 methylation (Fig. 4D, lane 4), again confirming Arg-3 to be the primary substrate in H4. The mutant H4 tail-associated factors were also subjected to HMT assay, but we could detect very little HMT activity in all cases (Fig. 4B, lanes 3, 6, 9, 12, and 15; Fig. 4C, lane 3; Fig. 4D, lanes 5 and 6). Taken together, these data demonstrate that the wild-type H4 tail-associated factors have HMT activity mainly derived from PRMT5, which has substrate specificity for H4 tail.

**Effect of H4 Tail-associated Factors on the p300-Mediated Transcription**—We have recently demonstrated that H3 tail-associated factors could stimulate p300-mediated transcription (28). Therefore we next checked if the H4 tail-associated factors have a similar effect on transcription. Transcription assays with DNA or recombinant chromatin were performed essentially as recently described (28), except that the H4 tail-associated factors were added together with acetyl-CoA and p300 (Fig. 5A). As shown in Fig. 5, transcription from chromatin template is completely dependent upon p53, p300 and acetyl-CoA (Fig. 5B, lanes 1–3), whereas transcription from histone-free DNA template is solely dependent upon activator p53 (lanes 14–16).

Consistent with the presence of sets of active factors, the H4 tail-associated factors significantly increased the level of transcription from chromatin template (Fig. 5B, lanes 3–5). In contrast, the similar experiments with DNA was unaffected by the tail-associated factors (Fig. 5B, lanes 16–18). We next examined the effect of the mutant H4 tail-associated factors in transcription. If the observed promotion of chromatin transcription was mainly accomplished by positive regulatory factors
Histone H4 Tail-associated Factors

Specific Role of Acetylation in Recognition of H4 Tail by Regulatory Factors—We recently initiated our purification and characterization of proteins capable of binding to histone tails by using ectopically expressed tail domains. In our initial study with H3 tail, it was found that H3 tail can specifically interact with multiple factors (e.g., Spt16, SRRP1, Nucleolin, CARM1, HMGB1, ASH1, and MLL3), which can up-regulate p300-mediated chromatin transcription. Significantly, similar experiments with Lys-9/Lys-27-mutated H3 tail showed that Lys-9/ Lys-27 methylation of ectopic H3 tail is required for association of repressive factors (e.g., HP1α/β/γ, G9a, Mi2b, HDAC5/9, and TIF1β), supporting recruitment-based contribution of Lys-9/ Lys-27 methylation in transcription (28). Because these results also confirm the feasibility of our approach to identify the factors associated with specific tail modifications, we extended our effort to purify and characterize factors recognizing H4 tail in the present study. The major finding of our study is that ectopic H4 tail specifically associates with sets of factors to have a positive effect in p300-mediated chromatin transcription. However, it is notable that H4 tail associates with factors different from those associated with H3 tail, implicating that H3 and H4 tails may have non-redundant functions in gene transcription (50, 51).

Our mass spectrometry results indicate that wild-type ectopic H4 tails are stably associated with 18 proteins, including 2 known HMTs (DOT1L and PRMT5), 5 chromatin-related proteins (BAF170, nucleolin, MTA2, BAF53, and β-actin), and 11 transcription-related factors (TRAP150, TAF4, CRSP3, TOP3, CAPERα, Ddx17, COBRA1, hnRNPK, hnRNPF, MEP50, and hnRNPA1). Another important feature of interactions between H4 tail and factors is that association of H4 tail with known activating factors (BAF170, DOT1L, TRAP150, TAF4, CRSP3, Nucleolin, MTA2, COBRA1, hnRNPK, BAF53) is lost when acetylation of H4 tail is blocked by mutation, indicating that tail acetylation is essential for the interactions. Because most dissociated factors have not been shown to interact with acetylated H4 tails, these results point to a previously unrecognized contribution of H4 acetylation to the control of factor recruitment during transcription. Further analysis with the mutant/unacetylated H4 tail-associated factors detected p66α, SRp30c, and p32 in the mutant tail-associated factors, but not in the wild-type tail-associated factors. Therefore it may be that acetylation of H4 tail also plays a negative role in binding of some repressive factors.

In checking cellular modification of ectopic H4 tail, we found methylation (at Arg-3 and Lys-20) and acetylation (at Lys-5, Lys-12, and Lys-16) of the expressed tail. Consistent with HMT activities specific for H4–R3, our mass spectrometry analysis of the purified factors identified PRMT5. However, our analysis failed to reveal the presence of any Histone acetyltransferase/HMT activities specific for H4–R3, our mass spectrometry analysis of the purified factors identified PRMT5. Another important feature of interactions between H4 tail and factors is that association of H4 tail with known activating factors (BAF170, DOT1L, TRAP150, TAF4, CRSP3, Nucleolin, MTA2, COBRA1, hnRNPK, and BAF53) associated with wild-type/acylated H4 tails, mutant/unacylated H4 tail-associated factors, which are absent from these positive factors, should not show any boost in transcription. Indeed, our results show that the mutant tail-associated factors have minimal effect on both chromatin (Fig. 5B, lanes 8–10) and DNA transcription (lanes 21–23). In transcription assays without p300 and acetyl-CoA, the H4 tail-associated factors showed no effect in transcription (Fig. 5B, lanes 11–13 and 24–26), suggesting that acetylation of chromatin templates by p300 is necessary for the effect of the tail-associated factors in transcription. Taken together, these results, in agreement with mass spectrometry results, indicate a stable association of positive regulatory factors with acetylated H4 tails is critical for transcription activities of the H4 tail-associated factors.

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(ABF170, DOT1L, TRAP150, TAF4, CRSP3, Nucleolin, MTA2, COBRA1, hnRNPK, and BAF53) associated with wild-type/acylated H4 tails, mutant/unacylated H4 tail-associated factors, which are absent from these positive factors, should not show any boost in transcription. Indeed, our results show that the mutant tail-associated factors have minimal effect on both chromatin (Fig. 5B, lanes 8–10) and DNA transcription (lanes 21–23). In transcription assays without p300 and acetyl-CoA, the H4 tail-associated factors showed no effect in transcription (Fig. 5B, lanes 11–13 and 24–26), suggesting that acetylation of chromatin templates by p300 is necessary for the effect of the tail-associated factors in transcription. Taken together, these results, in agreement with mass spectrometry results, indicate a stable association of positive regulatory factors with acetylated H4 tails is critical for transcription activities of the H4 tail-associated factors.

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that some, if not all, purified factors specifically recognize p300-mediated H4 acetylation restricted to nucleosomes within the promoter region of chromatin. This targeting of the purified factors to promoter will induce localized nucleosome-remodeling events, which will in turn facilitate preinitiation complex assembly for transcription initiation.

Functional Implications of Association of Regulatory Factors with H4 Tail—The finding that acetylated H4 tail interacts stably with factors to promote chromatin transcription raises the possibility that acetylation of H4 tail may directly be involved in recruiting regulatory factors to establish an active state of chromatin transcription. Consistent with chromatin-specific activities of the purified factors, we found nucleolin, which has a positive effect on chromatin transcription by acting as a histone chaperone for removal of H2A/H2B dimer (42). Because our recent study with H3 tail (28) also showed that nucleolin can bind to H3 tail, we speculate that initial recognition of H3/H4 tails by nucleolin facilitates stable recruitment of nucleolin into chromatin template. This stable localization of nucleolin then facilitates its action onto proximal H2A/H2B dimer. Considering that acetylation of H4 tail is necessary for association of nucleolin with H4 tail (Fig. 2), it will also be of interest to check if H3 tail acetylation can also assist the stable interaction of nucleolin with H3 tail.

Another interesting finding is the presence of PRMT5 and DOT1L in the wild-type tail-associated factors. Because H4 tail is positioned in the vicinity of the first α-helix of H3 globular domain where the H3-Lys-79 is positioned (10), it is also likely that acetylated H4 tails play a role in initial recruitments of DOT1L for H3-Lys-79 methylation, which will facilitate chromatin transcription, especially elongation process (52). Although we found that PRMT5 can associate with both wild-type and mutant H4 tails by mass spectrometry analysis, cellular association of PRMT5 with H4 tail was significantly decreased by mutations of H4 tail at four major acetylation sites. Thus it is possible that the function of PRMT5 as a positive regulator of transcription requires active acetylation of H4 tail, which is an important component of the activation of the promoter to very high transcription rates.

Identification of BAF170/BAF53 subunits of human SWI/ SNF complexes in the wild-type but not the mutant tail-associated factors also supports the possible role of H4 tail and its acetylation in regulation of co-transcriptional ATP-dependent remodeling processes. Indeed recent studies showed that BAF53 is crucial for p53-dependent transcription and directly interacts with acetylated H4 tails, whereas BAF170 promotes the recruitment of many chromatin remodeling/modifying complexes (41, 53, 54). Therefore it is likely that BAF170/BAF53 in the purified factors plays a positive role in p300-mediated transcription by modulating the level of promoter-targeted nucleosome remodeling. Related, recent studies also revealed that β-actin can stably associate with BAF53 within the same complex for its function in transcriptional activation (55–57). Because β-actin can also bind to RNA polymerase II and facilitate transcription initiation (58), it is possible that β-actin in conjunction with BAF53 functions as a platform for the localization of chromatin remodeling activities and the co-transcriptional recruitment and/or tethering of general transcription factors. Therefore, our observations bear an important implication on a possible coupling between acetylation of H4 tail and sequential recruitment of chromatin remodeling factors for optimal transcription.

It is also worth noting that our purification of the H4 tail-associated factors did not show any interaction between acetylated H4 tail and bromodomain-containing factors. One possible explanation is that binding properties of bromodomain to acetylated H4 tail could be influenced by other neighboring modifications such as methylations at Arg-3 and Lys-20. Therefore, it will be of interest to check if mutations of Arg-3 and Lys-20 can assist bromodomain interaction with ectopic H4 tail. An alternative explanation is that association of bromodomain-containing proteins with H4 tail may rely on the presence of the acetylation at Lys-8, which is absent in our expressed H4 tail. Experiments are currently ongoing to examine whether a single acetylation at Lys-8 is sufficient for bromodomain interaction with H4 tail or if any distinct pattern of acetylation of H4 tail is required. Because we also failed to detect bromodomain-containing factors in H3 tail-associated factors in our recent study (28), we speculate that bromodomain-histone tail interactions are a highly specific determinant for gene regulation, which can be significantly affected by chromatin environments.

In conclusion, we purified regulatory factors associated with H4 tail and show that these factors contribute to p300-mediated transcriptional activation of chromatin. Our finding that positive regulatory factors bind to H4 tails in acetylation-dependent manner also provides a mechanism for the establishment of transcriptional activation by acetylation of H4 tail. Therefore, identification of key H4 tail-associated factors that play a major role in potentiating chromatin transcription will be essential to fully understand the action of acetylated H4 tail in transcription. It also will be of interest to determine if any specific pattern of H4 acetylation performs unique functions for recruitment/association of specific factors.

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REFERENCES

1. Van Holde, K. E. (1988) Chromatin, Springer-Verlag, New York
2. Workman, J. L., and Kingston, R. E. (1998) Annu. Rev. Biochem. 67, 545–579
3. Kornberg, R. D., and Lorch, Y. (1999) Cell 98, 285–294
4. Hansen, J. C. (2002) Annu. Rev. Biophys. Biomol. Struct. 31, 361–392
5. Berger, S. L. (2002) Curr. Opin. Genet. Dev. 12, 142–148
6. Cosgrove, M. S., and Wolberger, C. (2005) Biochem. Cell Biol. 83, 468–476
7. Kouzarides, T. (2007) Cell 128, 693–705
8. Grunstein, M. (1997) Nature 389, 349–352
9. Struhl, K. (1998) Genes Dev. 12, 599–606
10. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) Nature 389, 251–260
11. An, W., Palhan, V. B., Karymov, M. A., Leuba, S. H., and Roeder, R. G. (2002) Mol. Cell 9, 811–821
12. Shilatifard, A. (2006) Annu. Rev. Biochem. 75, 243–269
13. Zhang, Y., and Reinberg, D. (2001) Genes Dev. 15, 2343–2360
14. Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B. E., Emre, N. C., Schreiber, S. L., Mellor, J., and Kouzarides, T. (2002) *Nature* 419, 407–411.
15. Schubeler, D., MacAlpine, D. M., Scalzo, D., Wirbelauer, C., Kooperberg, C., van Leeuwen, F., Gottschling, D. E., O'Neill, L. P., Turner, B. M., Delrow, J., Bell, S. P., and Groudine, M. (2004) *Genes Dev.* 18, 1263–1271.
16. Schotta, G., Lachner, M., Sarma, K., Ebert, A., Sengupta, R., Reuter, G., Reinberg, D., and Jenuwein, T. (2004) *Genes Dev.* 18, 1251–1262.
17. Wang, H., Huang, Z. Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Tempst, P., and Zhang, Y. (2001) *Science* 293, 853–857.
18. Strahl, B. D., and Allis, C. D. (2000) *Nature* 403, 41–45.
19. Turner, B. M. (2002) *Cell* 111, 285–291.
20. Jenuwein, T., and Allis, C. D. (2001) *Science* 293, 1074–1080.
21. Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C., and Kouzarides, T. (2001) *Nature* 410, 120–124.
22. Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S., and Zhang, Y. (2002) *Science* 298, 1039–1043.
23. Lachner, M., O’Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001) *Nature* 410, 116–120.
24. Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D., and Grewal, S. I. (2001) *Science* 292, 110–113.
25. Wysocka, J., Swigut, T., Milne, T. A., Dou, Y., Zhang, X., Burlingame, A. L., Roeder, R. G., Brivanlou, A. H., and Allis, C. D. (2005) *Cell* 121, 859–872.
26. Pray-Grant, M. G., Daniel, J. A., Schiltz, D., Yates, J. R., III, and Grant, P. A. (2005) *Nature* 433, 434–438.
27. Zeng, L., and Zhou, M. M. (2002) *FEBS Lett.* 20, 124–128.
28. Heo, K., Kim, B., Kim, K., Choi, J., Kim, H., Zhan, Y., Ranish, J. A., and An, W. (2007) *J. Biol. Chem.* 282, 277, 862–868.
29. Galarneau, L., Nourani, A., Boudreault, A. A., Zhang, Y., Heliot, L., Allard, S., Savard, K., Zhu, Y., and Kouzarides, T. (2006) *Science* 313, 120–124.
30. van Leeuwen, F., Gafken, P. R., and Gottschling, D. E. (2002) *Cell* 109, 745–756.
31. Phelan, M. L., Sif, S., Narlikar, G. J., and Kingston, R. E. (1999) *Mol. Cell.* 3, 247–253.
32. Angelov, D., Bondarenko, V. A., Almagro, S., Menoni, H., Mongelard, F., Hans, F., Mietton, F., Studitsky, V. M., Hamiche, A., Dimitrov, S., and Bouvet, P. (2006) *EMBO J.* 19, 1669–1679.
33. Santos-Rosa, H., Schneider, R., Groudine, M., and Clarke, S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 1237–1241.
34. Karachentsev, D., Druzhinina, M., and Steward, R. (2007) *Dev. Biol.* 304, 46–52.
35. Angelov, D., Bondarenko, V. A., Almagro, S., Menoni, H., Mongelard, F., Hans, F., Mietton, F., Studitsky, V. M., Hamiche, A., Dimitrov, S., and Bouvet, P. (2006) *EMBO J.* 19, 1669–1679.
36. Sobel, R. E., Cook, R. G., Perry, C. A., Annunziato, A. T., and Allis, C. D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 1237–1241.
37. Talaszt, H., Lindner, H. H., Sarg, B., and Helliwell, W. (2005) *J. Biol. Chem.* 280, 38814–38822.
38. Sims, J. K., Houston, S. I., Maszynskin, T., and Rice, J. C. (2006) *J. Biol. Chem.* 281, 12760–12766.
39. Ancelin, K., Lange, U. C., Hajkova, P., Schneider, R., Bannister, A. J., Kouzarides, T., and Surani, M. A. (2006) Nat. Cell Biol. 8, 623–630.
40. Dacwag, C. S., Ohkawa, Y., Pal, S., Sif, S., and Imbalzano, A. N. (2007) *Cell Biol.* 27, 384–394.
41. Bell, S. P., and Groudine, M. (2004) *Cell Biol.* 27, 1041–1053.
42. Wang, H., Huang, Z. Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Tempst, P., and Zhang, Y. (2001) *Science* 293, 853–857.
43. Sekiguchi, J., and Zilouchian, S. (1998) *FEBS Lett.* 434–438.
44. Talaszt, H., Lindner, H. H., Sarg, B., and Helliwell, W. (2005) *J. Biol. Chem.* 280, 38814–38822.
45. Dacwag, C. S., Ohkawa, Y., Pal, S., Sif, S., and Imbalzano, A. N. (2007) *Mol. Cell Biol.* 27, 384–394.
46. Richard, S., Morel, M., and Cleroux, P. (2005) *Biochem. J.* 388, 379–386.
47. Talaszt, H., Lindner, H. H., Sarg, B., and Helliwell, W. (2005) *J. Biol. Chem.* 280, 38814–38822.
48. Sims, J. K., Houston, S. I., Maszynskin, T., and Rice, J. C. (2006) *J. Biol. Chem.* 281, 12760–12766.
49. Ancelin, K., Lange, U. C., Hajkova, P., Schneider, R., Bannister, A. J., Kouzarides, T., and Surani, M. A. (2006) Nat. Cell Biol. 8, 623–630.
50. Dacwag, C. S., Ohkawa, Y., Pal, S., Sif, S., and Imbalzano, A. N. (2007) *Mol. Cell Biol.* 27, 384–394.
51. Talaszt, H., Lindner, H. H., Sarg, B., and Helliwell, W. (2005) *J. Biol. Chem.* 280, 38814–38822.
52. Sims, J. K., Houston, S. I., Maszynskin, T., and Rice, J. C. (2006) *J. Biol. Chem.* 281, 12760–12766.