Direct Association of p300 with Unmodified H3 and H4 N Termini Modulates p300-dependent Acetylation and Transcription of Nucleosomal Templates*

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The nature of histone acetylation events in active chromatin is an important issue in transcriptional regulation. We have systematically analyzed the ability of p300, either alone or in response to an interacting activator, to acetylate specific recombinant histones in the context of free histones, histone octamers, or nucleosomal arrays. Our results indicate that p300 has an intrinsic ability to acetylate all core histones but that the level and specificity of histone acetylation is indeed context-dependent. Thus, H3 and H4 are preferentially acetylated in free octamers, whereas all histones are nearly equally acetylated, in an activator-dependent manner, in chromatin. Moreover, H3 and H4 show H2A and H2B tail-independent acetylation in chromatin, whereas maximal H2A and H2B acetylation in this context is dependent upon H3 and H4 tails (but not their acetylation). In further support of an apparent intrinsic preference of p300 for the H3 and H4 tails, as well as an important role for direct interactions of p300 with unacetylated H3 and H4 tails in both acetylation and transcription, we have shown that p300 selectively acetylates isolated H3 and H4 tails, that p300 strongly and selectively binds to free unacetylated H3 and H4 tails, and that p300-mediated acetylation of nucleosomal histones and transcriptional activation are selectively inhibited by isolated (unacetylated) H3 and H4 tails.

Chromatin structure plays a major role in all aspects of DNA function in eukaryotes (1, 2). The nucleosome core particle, the fundamental repeating unit of chromatin, consists of a core histone octamer surrounded by 146 bp of DNA wrapped in a left-handed superhelix (3–5). Recent studies have revealed that chromatin structure is highly dynamic, with structural changes that result in alterations in gene activity (6, 7). These structural alterations are mediated largely by posttranslational modifications of the flexible N-terminal amino acids of the core histones and by ATP-dependent transitions in nucleosome structure and position (8, 9).

Covalent modifications of nucleosomal histone tails have proved to be of major importance for various nuclear processes (10–13), and the best characterized of these modifications is the post-translational acetylation of specific lysine residues (14). Transcriptionally active regions of chromatin have long been correlated with the presence of hyperacetylated histones, whereas silent regions are correlated with the presence of hypoacetylated forms (1, 15). Recent studies have indicated that steady-state levels of histone acetylation are maintained by a balanced equilibrium of histone acetylation and deacetylation (16–18). Acetylation may alter internucleosomal interactions and thus alter higher order chromatin structure (4). However, the histone code hypothesis proposes that different combinations of posttranslational modifications may function as recognition signals for proteins that regulate transcription more directly (10, 11, 19).

p300 and its paralog, CBP, are well known multifunctional coactivators that mediate the action of a variety of transcription factors (20) and possess intrinsic histone acetyltransferase (HAT) activities (21, 22). They can be recruited to promoters by direct interactions with DNA binding transcription factors (20) and function with other cofactors such as p300/CBP-associated factor (PCAF) and the p160/SRC family of proteins (20). p300 contains a highly conserved bromodomain that is found in many chromatin-associated proteins (23–25). Although functional correlations between bromodomains and histone modifications remain to be firmly established, recent studies (26–30) have shown that bromodomains can serve as histone recognition motifs for cofactor association with histone tails. Our recent work (31) has indicated that the removal of histone H3 and H4 tails or lysine-to-arginine substitutions at major acetylation sites impairs p300-dependent transcriptional activation by Gal4-VP16. Given that HAT-containing coactivators also can functionally modify various (non-histone) regulatory proteins (32), these observations provide the first proof of a direct link between coactivator function and histone acetylation per se and thus extend earlier studies showing only correlations between these events.

In this study, we generated a variety of histone-containing substrates and systematically examined functional interactions with p300. Our results reveal context-dependent differences in histone acetylation that are relevant to an understanding of the mechanisms by which p300-mediated histone acetylation affects transcription. In addition, the free histone tails, notably those of histone H3 and H4, are shown to modulate p300 activities in a manner that is largely independent of their acetylation state. p300 is shown to bind directly to unacetylated H3 and H4 tails but not to H2A and H2B tails. These results support the idea of an activator-independent, histone H3 and H4 tail-mediated chromatin binding activity of p300, which may contribute to functional effects of H3 and H4 tails in transcriptional regulation.

* This work was supported by Grant CA42567 from the NIH (to R. G. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: HAT, histone acetyltransferase; K/R, lysine-to-arginine substitution; GST, glutathione S-transferase; CREB, CAMP-response element-binding protein; CBP, CREB-binding protein.
EXPERIMENTAL PROCEDURES

Preparation of Xenopus laevis Recombinant Histone—Mutations in the histone N termini were generated by PCR-directed mutagenesis as described previously (31). The expression and purification of histones were essentially as described by Luger et al. (33). Histone preparations were analyzed by 15% SDS-PAGE and Western blot.

Construction and Expression of X. laevis Core Histone Tails—Glutathione S-transferase (GST)-histone tail fusion proteins were made as follows: the N-terminal tail sequences corresponding to residues 1–38 for H2A, 1–33 for H2B, 1–41 for H3, and 1–36 for H4 were PCR-amplified and inserted into the EcoRI and BamHI sites of the plasmid pGEX-2T. Fusion proteins were expressed in E. coli strain BL21 and purified on glutathione-Sepharose 4B (Amersham Biosciences) following the manufacturer’s protocol. Bound material was subjected to thrombin digestion at 22 °C for 5 h and the supernatant, containing free tails, was dialyzed overnight against BC100. Tail integrity was checked by 18% SDS-PAGE (Fig. 3A) and tail concentrations were determined both by Coomassie Blue staining and by Bradford assay. Molar concentrations of tails were determined assuming masses of 5 kDa for nH2A, nH2B, and nH4 and 6 kDa for nH3.

Chromatin Assembly and Characterization—FLAG-tagged Drosophila ATP-dependent chromatin assembly factor (ACF) subunits were expressed in bacteria and purified as described previously (34). A histone/DNA weight ratio of 1.1 gave optimal assembly under our conditions.

Histone Acetyltransferase and in Vitro Transcription Assays—FLAG-tagged human p300 and Ga4-VP16 proteins were expressed and purified on M2-agarose (Sigma Chemical) according to standard procedures. HAT and transcription assays were performed as reported previously (31). For inhibition assays with free histone tails, p300 was initially recruited to the promoter template by Ga4-VP16. Histone tails were then added together with acetyl-CoA as described in the figure legends.

Histone Tail and p300 Interaction Assays—For binding assays, recombinant p300 (2 μg) was incubated with the indicated GST-tail (~1 μg) immobilized on glutathione-Sepharose beads in 200 μl of binding buffer (150 mM NaCl, 20 mM HEPES, pH 7.5, 0.25 mM EDTA, 10% glycerol, 0.05% Nonidet P-40, 1 mM phenylmethylsulfonfyl fluoride, 2 mM dithiothreitol, 5 μg/ml leupeptin, and 5 μg/ml aprotonin) in the presence or absence of acetyl-CoA for 40 min at 30 °C. After incubation, the beads were gently rotated for 3 h at 4 °C and washed four times with binding buffer. Equal amounts of beads were directly suspended in SDS sample buffer, and bound proteins were resolved by 6% SDS-PAGE and detected by Western blot with p300 antibody (Santa Cruz Biotechnology).

RESULTS

Substrate-dependent, Differential Modes of Histone Acetylation—To investigate the underlying mechanism for p300-mediated histone acetylation, we first prepared recombinant wild-type and mutant X. laevis histones (Fig. 1B) that either lacked the N-terminal histone tails (indicated by prefix m) or contained lysine-to-arginine (K/R) substitutions (indicated by prefix Ac).

These individual histones were then used to reconstitute different histone octamers: intact, H2A-tailless (nH2A), H2B-tailless (nH2B), H2A- and H2B-tailless (nH2A + nH2B), H3-tailless (nH3), H4-tailless (nH4), H3- and H4-tailless (nH3 + nH4), totally tailless (All Tails), H2A-mutant (mH2A), H2B-mutant (mH2B), H2A- and H2B-mutant (mH2A + mH2B), H3-mutant (mH3), H4-mutant (mH4), and H3- and H4-mutant (mH3 + mH4), and totally mutant (All mTails). The purity of the assembled histone octamers is shown in Fig. 1C.

We first analyzed the ability of recombinant p300 to acetylate individual free histones (Fig. 2A) under identical conditions. Intact H2A, H2B, H3, and H4 histones each showed a comparable level of acetylation (Fig. 2A, lanes 1–4). Interestingly, K/R H2A (positions 5 and 9), H2B (positions 2, 9, 12, and 17), and H3 (positions 9, 14, 18, and 23) mutants showed acetylation levels comparable with those of wild-type free his-
Histones are identical to those described in Fig. 1. Reaction products were analyzed by 15% SDS-PAGE. The indicated mutant histones were used. Assays were identical to Fig. 2, except that free histone octamers were used. The indicated histones are identical to those described in Fig. 1. C, HAT assay with recombinant chromatin templates. In each case the indicated chromatin template was incubated with p300, [3H]acetyl-CoA (2.7 M) as described under “Experimental Procedures.” Reaction products were analyzed by 15% SDS-PAGE. The indicated mutants also were acetylated (Fig. 3—lanes 1—16). Consistent with recent observations (31, 35, 37), p300-mediated acetylation of histones within reconstituted chromatin substrates was completely dependent upon the presence of a transcriptional activator (GAL4-VP16) that is known to interact directly with p300 (Fig. 2C; data not shown). These results also indicate not only high levels of H3 and H4 acetylation but also highly enhanced levels of H2A and H2B acetylation in chromatin compared with isolated histone octamers (Fig. 2C, lanes 1 and 2 versus Fig. 2B, lanes 1 and 2). Moreover, in confirmation of previous results (31), an analysis of chromatin with histone tail deletions showed that H2A and H2B acetylation was strongly dependent upon H3 and H4 tails, whereas removal of H2A and H2B tails had no effect on H3 and H4 acetylation (lanes 3—9); in addition, an analysis of chromatin with lysine-to-arginine substitution mutations in the histone tails failed to show any significant interdependency (lanes 10—16). Hence, whereas the H3 and H4 tails are required for optimal acetylation of H2A and H2B, their ability to be acetylated at major lysine substrate sites is not. Concomitant lysine-to-arginine substitutions in all four core histone tails abolished acetylation as effectively as did removal of the tails (lane 16 versus lane 9); these results confirm the integrity of the assembled chromatin, because histones lacking tails or containing the lysine-to-arginine substitutions are readily acetylated when assayed as free histones or as DNA-free octamers (Fig. 2, A and B). Collectively, these data demonstrate distinct patterns and context-dependent interdependencies of histone acetylation by p300, with an intrinsic preference for histone H3 and H4 N termini.

**Diff**ential Repressive Effect of Free Histone Tails on P300—Dependent Acetylation—A possible interpretation of the histone acetylation results is that the H3 and H4 tails play the major role in determining p300 function during and/or after its recruitment by an activator. This possibility was investigated by analyzing whether free histone tails, especially those of H3 and H4, are efficient inhibitors of p300 function in solution. To this end, we expressed and purified intact and K/R mutant (m) core histone N termini (Fig. 3, prefix n) as GST fusion proteins (Fig. 3A, lanes 1—4 and 11—14), and then purified the tail components after removal of GST with thrombin digestion. Fig. 3A shows the Coomassie-stained patterns of electrophoretically resolved wild-type and mutant H2A, H2B, H3, and H4 tails (lanes 6—9 and 16—19, respectively).

Isolated histone tails, normalized on the basis of Coomassie staining, were then tested as substrates for p300. In contrast to what was observed with free full-length histones (Fig. 2A), p300 acetylated predominantly wild type H3 and H4 tails with a slight preference for the H3 tail (Fig. 3B, lanes 1—4). Interestingly, mutant (lysine-to-arginine substitution) H3 and H4 tails also were acetylated (Fig. 3B, lanes 7 and 8), although the levels were significantly lower than those observed for wild type free histone tails. These results, showing that free H3 and H4 tails are better acetylation substrates for p300 than are free H2A and H2B tails, indicate that acetylation of the H2A and H2B tails can be modulated by corresponding globular domains of H2A and H2B.
Next, to check whether free histone tails could act as competitive inhibitors of p300 function, they were added to a HAT assay in which chromatin templates assembled with intact core histones served as substrates (Fig. 3C). As shown in Fig. 3D, a slight inhibition of Gal4-VP16 dependent p300-mediated acetylation was observed only at the highest tested concentrations of nH2A (lanes 1–4), nH2B (lanes 5–8), or nH2A+nH2B (lanes 9–12). In contrast, nH3 and nH4 (lanes 13–20) showed a much stronger inhibition of acetylation; their inhibitory concentrations were significantly (~8-fold) lower than those for nH2A and nH2B. Furthermore, an equimolar mixture of nH3+nH4 (lanes 21–24) showed inhibitory effects comparable to those observed with equivalent masses of nH3 or nH4, indicating that there was no synergistic effect of nH3 and nH4 tails in inhibition. When an equimolar mixture of all four histone tails (lanes 25–28) was used, the inhibition seemed slightly less, suggesting that inhibition was proportional mainly to the concentration of nH3 and nH4. A control assay with GST alone (lanes 29–32) showed no inhibitory effect. In all the assays, the final concentration of free tails was kept constant.

Competition assays with point mutated (lysine to arginine) histone tails (Fig. 3, prefix mn) were also performed. As shown in Fig. 3E, mnH2A (lanes 1–4), mnH2B (lanes 5–8), and a mixture of mnH2A and mnH2B (lanes 9–12) all failed to abolish or significantly reduce the acetylation of chromatin, whereas mnH3 (lanes 13–16), mnH4 (lanes 17–20), and a mixture of mnH3 and mnH4 (lanes 21–24) all diminished the HAT activity of p300 to an extent comparable to that observed with wild-type histone tails. As expected from these results, a mixture of all four mutant histone tails (lanes 25–28) also inhibited p300 activity. Furthermore, identical assays using free histone octamers as a substrate showed a comparable inhibitory effect of H3 and H4 tails on p300 HAT activity, although slightly higher concentrations of the tails were required (data not shown). The results from these competition assays strongly support the notion of a preferential association of p300 with H3 and H4 histone tails in a chromatin context, and in a manner that is independent of their ability to be acetylated.

**Free H3 and H4 Tails Inhibit p300-mediated Transcription**. We next examined the effect of ectopic free histone tails on p300-mediated transcription activation. Transcription assays with recombinant chromatin templates containing Gal4 binding sites upstream of core promoter sequences were carried out as described previously (31), except that core histone tails were added together with acetyl-CoA (Fig. 3C). Transcription from this template is completely dependent upon an activator (Gal4-VP16), p300, and acetyl-CoA (Fig. 4A) (31). As shown in Fig. 4B, nH2A (lanes 1–4), nH2B (lanes 5–8), and a mixture of nH2A and nH2B (lanes 9–12) showed only a slight inhibitory effect on transcription at the highest concentrations tested. In
contrast, a significant inhibitory effect on transcription was observed in the presence of comparable concentrations of nH3 (lanes 13–16) or nH4 (lanes 17–20). In addition, the experiments with a mixture of nH3 and nH4 (lanes 21–24) tails or of all four histone tails (lanes 25–28) failed to reveal any synergistic or cooperative effects of the tails on this inhibition.

We also examined whether free mutant histone tails with lysine-to-arginine substitutions could block the transcription activation by p300. Consistent with the results of the HAT competition assays, mutant H3 and H4 tails, alone or together, showed strong inhibitory effects on the p300-dependent transcription (Fig. 4C, lanes 13–24). By contrast, mutant H2A and H2B tails, alone or together, minimally affected transcription at the highest concentrations tested (Fig. 4C, lanes 1–12). A control analysis showed that comparable concentrations of GST alone did not affect transcription (Fig. 4C, lanes 29–32). Taken together, these results suggest that association of p300 with N-terminal tails of H3 and H4 can regulate p300-mediated transcription in a manner that is largely independent of their acetylation at natural acetylation sites.

**Preferred Interaction of p300 with H3 and H4 Tails in Vitro**—To examine how the histone tails influence p300 function more directly, we analyzed the ability of intact and mutated (Fig. 3A, K/R substitutions) histone tails to interact with a fixed concentration of p300. Equimolar amounts of individual GST-tail fusion proteins (Fig. 3A, lanes 1–4 and 11–14) were prebound to GST beads and incubated with p300. After extensive washing of the beads, bound proteins were eluted and analyzed by Western blot with p300 antibody. To investigate the possible involvement of histone tail acetylation in p300 interactions, the assays were conducted in the absence or presence of acetyl-CoA. As shown in Fig. 5A, p300 binding was highly selective for intact H3 and H4 tails (lanes 5 and 6) relative to intact H2A and H2B tails (lanes 3 and 4). GST alone showed no binding (lanes 2 and 12). Interestingly, the binding of intact H3 and H4 tails, which lack any acetylated residues because of expression in bacteria, was independent of the presence of any accompanying acetylation.

To investigate the possible role of lysine residues at the major acetylation sites for regulating p300-tail interactions, we conducted identical binding assays with mutant histone tails bearing lysine-to-arginine substitutions (Fig. 5, prefix m) fused to GST. As with unmodified tails, positive interactions were detectable exclusively with H3 and H4 tails (Fig. 5A, lanes 13–16), although the absolute levels of bound p300 were 2–3-fold lower than those observed with wild-type counterparts (lanes 5 and 6 versus 15 and 16). In this case, however, there was an apparent effect of acetyl-CoA on p300-mutant histone tail interactions, as reflected by moderate 1.8–3-fold increases in the binding of p300 to mutant H3 and H4 tails (lanes 15 and 16 versus lanes 19 and 20). This suggests that, unlike the major lysine substrates, the acetylation of minor lysine residues may functionally contribute to p300 recruitment by acetylation when the major lysine substrates are absent. The binding efficiency with the different histone tails and the effect of acetylation on the interactions are quantitatively presented in Fig. 5B as average percentages of p300 retention on beads from three separate experiments. Taken together, these results, in agreement with the tail competition assays, indicate a stable association of p300 with histone H3 and H4 tails that is acetyl-
lation-independent and functionally significant for p300-mediated transcriptional activation.

**DISCUSSION**

We recently demonstrated a direct link between the presence and acetylation status of histone H3 and H4 tails and p300-dependent transcription from chromatin templates (31), but the underlying mechanisms remain to be unraveled. In the present work, and through systematic analyses with purified wild type and mutant recombinant histones, we describe discrete patterns of p300-mediated acetylation with different histone substrates and a selective, acetylation-independent interaction of p300 with H3 and H4 tails. Our data clearly underscore an intrinsic preference of p300 for physical and functional interactions with H3 and H4 tails, and possible roles of their interaction in transcriptional regulation are discussed below.

**Substrate and Context Effects on p300-mediated Histone Acetylation**—As a first step toward understanding the mechanisms, we investigated the histone acetylation activity of p300 with various substrates. One important observation, both confirming and extending previous results, is the effect of context on histone acetylation by p300. Thus, whereas all histones are good substrates when tested individually, H3 and H4 are the preferred substrates when free histone octamers are assayed at a limiting p300 concentration. By contrast, at the same limiting p300 concentration, all histones are acetylated in chromatin in response to a transcriptional activator.

Context effects are also evident from studies with histone tail deletions. Thus, the joint deletion of H3 and H4 tails results in increased H2A and H2B acetylation in free histone octamers but, most significantly, decreased H2A and H2B acetylation in chromatin. In contrast, joint deletion of H2A and H2B tails has no significant effect on acetylation of H3 and H4 tails either in free octamers or in chromatin. Similar studies with histones containing lysine-to-arginine substitutions at the major acetylation sites indicated some modest interdependencies of acetylation events in free histone octamers but almost none in chromatin substrates. The latter results with histone tails that are largely intact has important implications for acetylation-independent binding of p300 to chromatin (see below).

In almost all cases, these results are most simply explained by an intrinsic preference of p300 for H3 and H4 substrates and by competitive substrate interactions. This conclusion is supported by the observation that isolated H3 and H4 tails are both preferentially acetylated (by p300) relative to isolated H2A and H2B tails (Fig. 3B). In contrast, the context-dependent requirement of H3 and H4 tails for maximal H2A and H2B acetylation within chromatin must have another basis. This could reflect structural differences between tails within free and nucleosomal histones or the stabilization and enhanced catalytic activity of p300 on chromatin through interactions either with Gal4-VP16 (35) or (via its bromodomain) with unacetylated (27) or acetylated (26, 28–30) H3 and H4 tails.

**Functional Implications of H3 and H4 Tail-dependent Association of p300 with Chromatin**—Competition studies (Figs. 3 and 4) allowed an analysis of the effect of each histone tail on p300 activity and showed a dosage-dependent repression of p300 function that was specific for H3 and H4 tails. Similar inhibitory effects were also observed with free histone H3 and H4 tails containing lysine-to-arginine substitutions in major acetylation sites. This suggests that p300 may recognize distinct features of the H3 and H4 tails other than acetylated lysines, as recently reported for GCNS bromodomain and histone H4 tail interaction (27). Therefore, the observed inhibitory effects could reflect masking of substrate recognition sites (e.g., bromodomains) in p300 and/or the induction of conformational changes that decrease p300 acetylation activity. The in vitro binding assays (Fig. 5) further confirm dominant interactions of p300, not only with wild type H3 and H4 tails but also with mutant tails containing lysine-to-arginine substitutions, again suggesting the selective recognition of H3 and H4 tails by p300.

Although structural studies of bromodomain-acetylated peptide complexes support the hypothesis that acetylated tails may directly recruit factors that are functionally associated with chromatin (26, 28–30), our results suggest that p300 alone may associate with chromatin through interactions with unmodified H3 and H4 tails. Indeed, our results are in good agreement with a recent report (38) showing that histone acetylation plays a minor role (at least quantitatively) in the stable bromodomain-dependent association of p300 with chromatin or isolated histones. However, our finding that H3 and H4 interact equally well with p300 differs slightly from the finding (38) that p300 interacts preferentially with H3. These divergent results may reflect differences in the assays employed—namely GST-p300 bromodomain binding with a mixture of all four core histones versus binding of individual histone tail-fused proteins with full-length p300.

It is worth noting that incubation with acetyl-CoA promotes interactions between p300 and mutant H3 and H4 tails containing lysine-to-arginine substitution at the major acetylation sites (Fig. 5A, lanes 15 and 16 versus 19 and 20). Because these mutations do not abrogate acetylation of the free histones (Fig. 2A), secondary acetylation may compensate for reduced interactions that result from the lysine-to-arginine mutations at the major acetylation sites but may involve mainly other residues. In this regard, previous studies have indicated that other residues or regions of the histone tails may provide major sites for acetylation-independent recognition of histone tails by bromodomains (27, 30).

**The Role of Histone Modifications in Gene Regulation**—The finding that free H3 and H4 tails interact strongly with p300, together with the observation that acetylation of major lysine substrates of histone tails seems not to be necessary for this association, raises the possibility that the acetylation of specific lysine residues may mainly be involved in recruiting other regulatory factor(s). Such factors could be associated with subsequent chromatin reorganization events that lead to an active state of transcription. In this respect, our observations support the view that the recognition of modified histone tails by various factors or protein complexes (10, 11, 19, 39, 40) may account for the indispensable features of histone H3 and H4 tails in our studies. In the context of this model, the identification and characterization of potential factors/complexes that associate with modified histone tails will facilitate the elucidation of the mechanistic role of histone modifications. Indeed, recent studies have documented modified histone tails as recognition motifs for highly conserved domains in chromatin-associated factors (10, 11, 19, 39, 40). Moreover, specific tail modifications seem to be primarily, if not solely, responsible for the recruitment and anchoring of various transcriptional regulatory factors in transcription (26, 28–30, 41, 42). In this regard, a recent study (43) has demonstrated that the histone H3 N terminus can bind to the NuRD complex when lysine 9 is methylated, but that methylation at lysine 4 effectively disrupts this interaction. These results suggest specific roles for different tail modifications in regulating the association or recruitment of various factors.

Our findings suggest a novel role(s) for H3 and H4 tails in p300 recruitment and retention that is correlated with essential roles of histone H3 and H4 tails in p300-mediated transcription (31). However, it is not yet clear at which stage(s) the described p300-tail interactions play a role. They could be required before or during activator-targeted recruitment of
p300 to promoters or for continuous and more stable association of p300 with chromatin after recruitment and in fulfillment of the p300 coactivator function. In any case, our results suggest that direct contact between p300 and histone H3 and H4 tails is involved at some stage, in p300 function. Further characterization of the timing and function of histone acetylation events during the course of the overall transcription reaction will facilitate the elucidation of the functional role of histone tails and their modifications.

Acknowledgments—We thank Dr. J. T. Kadonaga for ACF and p300 baculovirus vectors; Drs. K. Luger and T. J. Richmond for histone expression vectors; and Drs. V. B. Palhan and A. E. Wallberg for critical reading of the manuscript.

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