

Acetylation of HMG I(Y) by CBP Turns off IFN β Expression by Disrupting the Enhanceosome

Nikhil Munshi, Menie Merika,
Junming Yie, Kate Senger,
Guoying Chen, and Dimitris Thanos*
Department of Biochemistry
and Molecular Biophysics
Columbia University
New York, New York 10032

Summary

The transcriptional coactivators CBP and P/CAF are required for activation of transcription from the IFN β enhanceosome. We show that CBP and P/CAF acetylate HMG I(Y), the essential architectural component required for enhanceosome assembly, at distinct lysine residues, causing distinct effects on transcription. Thus, in the context of the enhanceosome, acetylation of HMG I by CBP, but not by P/CAF, leads to enhanceosome destabilization and disassembly. We demonstrate that acetylation of HMG I(Y) by CBP is essential for turning off IFN β gene expression. Finally, we show that the acetyltransferase activities of CBP and P/CAF modulate both the strength of the transcriptional response and the kinetics of virus-dependent activation of the IFN β gene.

Introduction

In multicellular eukaryotic organisms, dynamic control of gene activity in response to external signals depends upon the execution of a complex hierarchy of differential gene expression in a precise spatial and temporal manner. In most cases, signals received by cells are interpreted as a modulation of the transcriptional activity of specific sets of genes. Amazingly, this enormous diversity of gene activity is achieved by only a relatively small number of transcription factors, many of which can be activated by more than one extracellular signal. The solution to this paradox lies, at least in part, in the phenomena of cooperativity and synergism exerted by transcriptional regulatory proteins (reviewed in Carey, 1998).

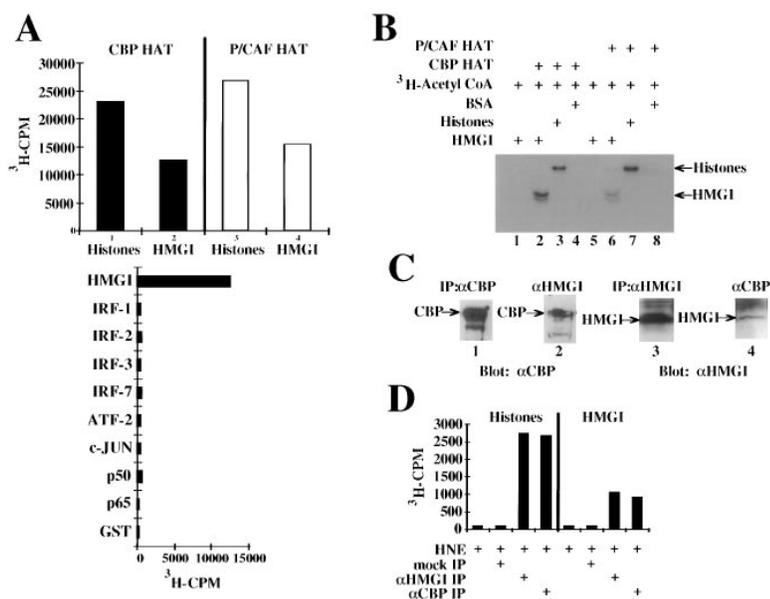
One of the best characterized examples of synergistic interactions between transcription factors is provided by the virus-inducible enhancer of the interferon- β (IFN β) gene (reviewed in Thanos et al., 1993). Transcripts of IFN β mRNA are not detected in uninfected cells, but after virus infection the gene is activated to very high levels and then undergoes a rapid postinduction turnover. Detailed analysis of the IFN β enhancer has revealed a highly compact and complex organization of *cis*-acting regulatory elements (PRDI through PRDIV). PRDII, PRDIV, and PRDIII-I are recognized by NF- κ B, ATF-2/c-Jun heterodimer, and several members of the IRF family, respectively. The high mobility group protein HMG I(Y)

plays a key role in the activation of this gene by functioning as the essential architectural component for the assembly and stability of the IFN β gene enhanceosome (Thanos and Maniatis, 1992, 1995a; Du et al., 1993). Two molecules of HMG I(Y) bind to four sites within the enhancer by employing both intra- and intermolecular cooperativity (Yie et al., 1997). Binding of HMG I(Y) to the enhancer alters the structure of the DNA, allowing cooperative recruitment of the IFN β gene activators that, together with HMG I(Y), assemble into a remarkably stable higher order nucleoprotein complex termed the IFN β enhanceosome (Falvo et al., 1995; Thanos and Maniatis, 1995a). Mutations that decrease HMG I(Y) binding to the enhancer, that alter the positioning of HMG I(Y) and transcription factors on the DNA helix, or that decrease the protein-protein interactions between any of these components decrease enhanceosome stability and transcriptional potency (Thanos and Maniatis, 1995a; Kim and Maniatis, 1997; Yie et al., 1997). Thus, the assembly and function of the IFN β enhanceosome requires a complex network of protein-DNA and protein-protein interactions orchestrated by the HMG I(Y) protein.

Recent studies have established that the IFN β gene enhanceosome displays a second level of spatial specificity necessary for gene activation. The formation of the enhanceosome creates a specific activating surface that recruits the CBP/p300 (CREB-binding protein) coactivator (Merika et al., 1998). CBP makes multiple, contiguous contacts with this novel activating surface that ensure its highly specific and efficient recruitment into the enhanceosome. In fact, deletions, substitutions, or rearrangements of the activation domains in the context of the enhanceosome decrease both recruitment of CBP in vitro and transcription in vivo (Merika et al., 1998). However, the mechanism by which CBP potentiates IFN β gene transcriptional activity remains unknown.

CBP is a huge protein (2441 amino acids [aa]) bearing multiple regions engaged in protein-protein interactions with different transcription factors, viral activators, components of the basal transcriptional apparatus, and other coactivator proteins (reviewed in Shikama et al., 1997). Interestingly, CBP and CBP-associated cofactors (e.g., P/CAF, P/CIP-ACTR, and SRC1) contain an intrinsic histone acetyltransferase (HAT) activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996; Chen et al., 1997; Spencer et al., 1997; Torchia et al., 1997). The current view postulates that recruitment of coactivators bearing HAT activity by promoter-bound transcription factors results in histone acetylation of nearby nucleosomes, thus enhancing access of the transcriptional machinery to the DNA (Grunstein, 1997; Mizzen and Allis, 1998; Struhl, 1998). Furthermore, different transcription factors exhibit distinct requirements for these coactivators and their acetyltransferase functions (Puri et al., 1997; Korzus et al., 1998; Kurokawa et al., 1998). Conversely, some transcriptional repressors can recruit histone deacetylases that inhibit transcription by deacetylating chromatin (Pazin and Kadonaga, 1997; Struhl, 1998). Recent studies have also shown that histones are not the only substrates of HAT proteins. For

*To whom correspondence should be addressed (e-mail: dt73@columbia.edu).



same antibodies. Lane 1, αCBP antibody precipitates CBP. Lane 2, αHMG I antibody coprecipitates CBP. Lane 3, αHMG I antibody precipitates HMG I. Lane 4, αCBP antibody coprecipitates HMG I.

(D) Shown are acetyltransferase filter-binding assays using core histones (left) or HMG I (right) as substrates and the immunoprecipitates from (C) as enzymes.

example, CBP/p300 can acetylate p53, resulting in an enhancement of its DNA-binding activity, as well as basal transcription factors such as TFII E and TFII F, although the role of this latter acetylation remains unknown (Gu and Roeder, 1997; Imhof et al., 1997).

Interestingly, the IFNβ enhancer, which is sufficient for activation of transcription, is also sufficient for post-induction turnover, although the mechanism of this phenomenon remains unknown (reviewed in Maniatis et al., 1992). Here, we show that CBP and the CBP-associated factor P/CAF acetylate HMG I(Y) either in solution or in the context of the enhanceosome. Acetylation of HMG I(Y) by CBP, but not by P/CAF, decreases its DNA-binding activity and results in enhanceosome destabilization and disassembly. We show that enhanceosome destabilization is due to the inability of CBP-acetylated HMG I(Y) to maintain NF-κB in the enhanceosome. We also find that the different effects of CBP and P/CAF on enhanceosome stability are due to the fact that distinct lysine residues in the HMG I(Y) protein are acetylated by each of these factors. Furthermore, recruitment into the enhanceosome of a CBP molecule bearing mutations in its HAT domain or an HMG I molecule that cannot be acetylated by CBP prevents postinduction turnover of IFNβ gene expression *in vivo*. Finally, we demonstrate that CBP and P/CAF HAT activities are both required for activation, whereas only CBP HAT activity is required for postinduction turnover of IFNβ expression.

Results

CBP and P/CAF Acetylate HMG I(Y)

To investigate whether recruitment of CBP and P/CAF into the enhanceosome could lead to specific acetylation of any of its components, we carried out *in vitro*

Figure 1. CBP and P/CAF Acetylate HMG I(Y)

(A) Shown on the top are the results of *in vitro* acetylation experiments using recombinant 6HisCBP HAT (300 ng) or P/CAF HAT (100 ng) enzymes incubated with HMG I or core histones (2 μg each) in the presence of 26 pmol of ³H-Acetyl-CoA. Acetylation was monitored by filter-binding assays. The bottom of the figure shows a similar assay (using the same amounts of enzyme and substrates), demonstrating that the only component of the enhanceosome that can be acetylated by CBP is HMG I. Evidence supporting the involvement of IRF-3 and IRF-7 in IFNβ expression has been previously published (Lin et al., 1998; Wathlet et al., 1998; Yoneyama et al., 1998).

(B) Same as in (A) except that the *in vitro* acetylation reactions were analyzed by SDS PAGE followed by autoradiography. The (+) sign indicates the materials added to each reaction.

(C) CBP and HMG I form complexes *in vivo*. HeLa nuclear extracts were immunoprecipitated with either CBP or HMG I antibodies followed by Western blot analysis using the

protein acetyltransferase assays using highly purified recombinant proteins. The HAT domains of CBP (aa 1098–1758) and P/CAF (aa 396–718), as well as all of the known components of the IFNβ enhanceosome, were expressed in bacteria and purified to near homogeneity. Figure 1A (lanes 1 and 3) shows that both CBP and P/CAF HAT domains acetylate core histones, in agreement with previous experiments (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996). Remarkably, both HAT proteins can also specifically acetylate recombinant HMG I nearly as efficiently as histones (lanes 2 and 4). Interestingly, none of the other components of the IFNβ enhanceosome can be acetylated by either the CBP or P/CAF HAT domains (Figure 1A, bottom). In addition, HMG I-C can also be acetylated by CBP and P/CAF (data not shown). The specificity of the reaction was demonstrated by the fact that labeling of HMG I depends on the presence of both radioactive Acetyl-CoA (³H) and CBP or P/CAF in the reaction (Figure 1B, lanes 1 and 2 and 5 and 6, respectively), excluding the possibility of either HMG I autoacetylation or non-specific binding of labeled acetyl-CoA to HMG I. Thus, the HMG I protein family [HMG I(Y) and HMG I-C] is a bonafide substrate for CBP and P/CAF acetyltransferase activities. Importantly, this observation is consistent with the fact that acetylation represents one of the prominent posttranslational modifications of HMG I proteins in living cells (Bustin and Reeves, 1996).

To test whether HMG I and CBP interact *in vivo*, we carried out immunoprecipitation experiments using HeLa nuclear extracts treated with HMG I- or CBP-specific antibodies followed by Western blot analysis. Figure 1C shows that the HMG I antibody coprecipitates CBP (lane 2) and that the CBP antibody coprecipitates HMG I (lane 4). Most importantly, the HMG I immunoprecipitate contains acetyltransferase activity as it acetylates both core

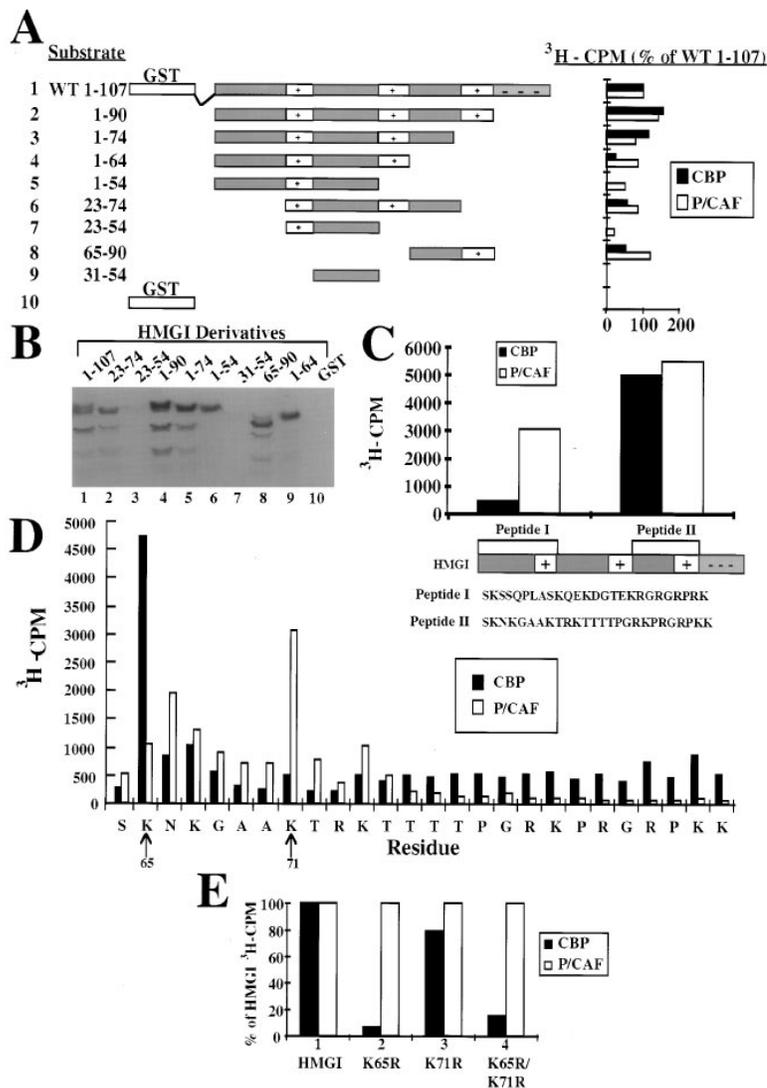


Figure 2. Identification of the Sites in HMG I Acetylated by CBP and P/CAF Domains (A) The indicated GST fused deletions of HMG I (2 μ g) were incubated with 300 ng CBP HAT or 100 ng P/CAF HAT proteins in the presence of 26 pmol ³H Acetyl-CoA. 100% acetylation of full-length HMG I (1-107) corresponds to 12,000 cpm for CBP HAT and 14,000 cpm for P/CAF HAT.

(B) The same derivatives shown in (A) were acetylated by P/CAF HAT, separated by SDS PAGE, and detected by autoradiography. Some of the HMG I derivatives display anomalous electrophoretic mobility as previously noted (Yie et al., 1997).

(C) Comparative acetylation of peptides I and II (2 μ g each) by CBP HAT and P/CAF HAT as determined by filter-binding assays. P/CAF HAT acetylates both peptides I and II, whereas CBP HAT only acetylates peptide II. The position and sequence of the peptides are also indicated.

(D) Peptide II (6 μ g) was incubated with CBP HAT (900 ng) or P/CAF HAT (300 ng) proteins in the presence of 78 pmol of ³H Acetyl-CoA for 1 hr at 30°C. The peptide was purified and 50% was subjected to N-terminal sequencing. 20% of each cycle was counted for ³H incorporation. The arrows indicate Lys-65 and -71 acetylated by CBP and P/CAF HAT domains, respectively.

(E) Acetylation assays were performed as in Figure 2A using the indicated mutant HMG I derivatives (1 μ g) and either CBP (300 ng) or P/CAF HAT (100 ng) proteins.

histones and HMG I (Figure 1D). Identical results were also obtained after cotransfecting both CBP- and HMG I-expressing plasmids into *Drosophila* Schneider cells (data not shown). Finally, in vitro GST protein-protein interaction experiments demonstrated that the association of CBP and HMG I is direct and that multiple regions of CBP (including the HAT domain) can independently contact HMG I (data not shown).

CBP and P/CAF Acetylate HMG I at Distinct Sites

To map the CBP- and P/CAF-dependent HMG I acetylation sites, we used a series of amino and carboxyl terminal deletions (Yie et al., 1997) of the protein that were expressed and purified to near homogeneity either as GST or His-6 fusions. These derivatives were used in in vitro acetylation experiments and the ³H-labeled proteins were visualized by autoradiography after SDS PAGE electrophoresis or were quantitated by filter-binding assays. Figure 2A shows that deletion of the carboxyl terminal acidic tail and the last basic repeat did not affect either CBP- or P/CAF-dependent acetylation of HMG I (lines 1-3). However, deletion to aa 65, which

removes the region between the middle and the last basic repeats, significantly reduced acetylation by CBP, whereas it did not significantly affect P/CAF-dependent acetylation (Figure 2A, line 4; Figure 2B, lane 9). Comparison of the relative acetylation efficiencies of CBP and P/CAF revealed that the minimal region of HMG I that can be efficiently acetylated by both HAT proteins spans aa 65-90, whereas the amino terminal 34 aa of HMG I are acetylated by P/CAF only (Figures 2A and 2B). Figures 2A and 2B also show that P/CAF can acetylate HMG I with lower efficiency at several additional sites spanning the center of the molecule. Thus, CBP and P/CAF exhibit site specificity in acetyating HMG I.

To verify the acetylation site preference between CBP and P/CAF in HMG I, we synthesized two peptides spanning the P/CAF-specific amino terminal region (aa 6-31) and the CBP-P/CAF common acetylation sites in HMG I (aa 64-89) (Figure 2C, peptides I and II, respectively). Figure 2C demonstrates that peptide I is preferentially acetylated by P/CAF and not by CBP, whereas peptide II is acetylated by both CBP and P/CAF, as predicted from our deletion analysis. To determine whether CBP

and P/CAF acetylate peptide II at the same lysine residues, the ^3H acetylated peptide was purified and subjected to amino terminal sequencing. Figure 2D shows that the ^3H incorporation levels for different positions varies between CBP and P/CAF acetylated peptide II. Specifically, CBP preferentially acetylates Lys-65 whereas P/CAF preferentially acetylates Lys-71 (Figure 2D). Furthermore, mutation of Lys-65 to Arg, in the context of the intact HMG I protein, abolishes CBP-dependent acetylation (Figure 2E) verifying the specificity of this acetylation site. However, mutation of Lys-71 to Arg did not affect P/CAF's ability to acetylate HMG I, which is consistent with the presence of multiple P/CAF-dependent acetylation sites as determined by our deletion analysis (Figure 2A). Taken together, these results indicate that the acetyltransferase activities of CBP and P/CAF show substrate specificity in acetylating HMG I.

Acetylation of HMG I by CBP but Not by P/CAF Decreases Its Sequence-Specific DNA Binding and Disrupts the HMG I/NF- κB Complex

The HMG I protein contains three basic repeats involved in DNA binding from the minor groove. We have previously shown that specific high-affinity DNA binding is mediated by the middle repeat in cooperation with either the first or last repeat depending on the nature of the binding site (Yie et al., 1997). The recent three-dimensional structure of HMG I bound to PRDII has revealed that K65, but not K71, makes backbone contacts with the DNA (Huth et al., 1997). This interaction plays a key role in the high-affinity binding of the middle repeat with the DNA. Therefore, acetylation of K65 might interfere with the DNA-binding affinity of HMG I by neutralizing the positive charge on K65's ϵ -amino group. To test this possibility, we carried out electrophoretic mobility shift assays (EMSAs) using mock or CBP or P/CAF acetylated HMG I and the composite PRDII site as a probe. Figure 3A demonstrates that CBP-acetylated HMG I binds DNA with lower affinity compared to mock or P/CAF-acetylated HMG I. Addition of CBP HAT in the absence of Acetyl-CoA had no effect on HMG I DNA binding (data not shown; see also below). Thus, the acetylation site preference between CBP and P/CAF in HMG I(Y) has different effects on its DNA-binding properties.

One role of HMG I is to promote cooperative binding of the IFN β gene activators on the enhancer. To investigate the consequences of HMG I acetylation on its ability to bind cooperatively with NF- κB , we carried out EMSA experiments using the PRDII/NRDI composite site as a probe under conditions where HMG I and NF- κB bind cooperatively to the DNA. Figure 3B (lane 2) shows that when low concentrations of NF- κB were used, no significant binding was observed. However, addition of HMG I to the reaction resulted in the formation of a high-affinity cooperative complex (lane 3), a result that is consistent with previous studies (Thanos and Maniatis, 1992). Incubation of this complex with either the CBP HAT domain alone or with Acetyl-CoA alone had no effect on its stability (lanes 4 and 5). Remarkably, the HMG I/NF- κB cooperative complex was disrupted when both CBP HAT and Acetyl-CoA were added to the reaction (lane 6). This disruption can be attributed to CBP's acetylation site preference for K65 in HMG I because

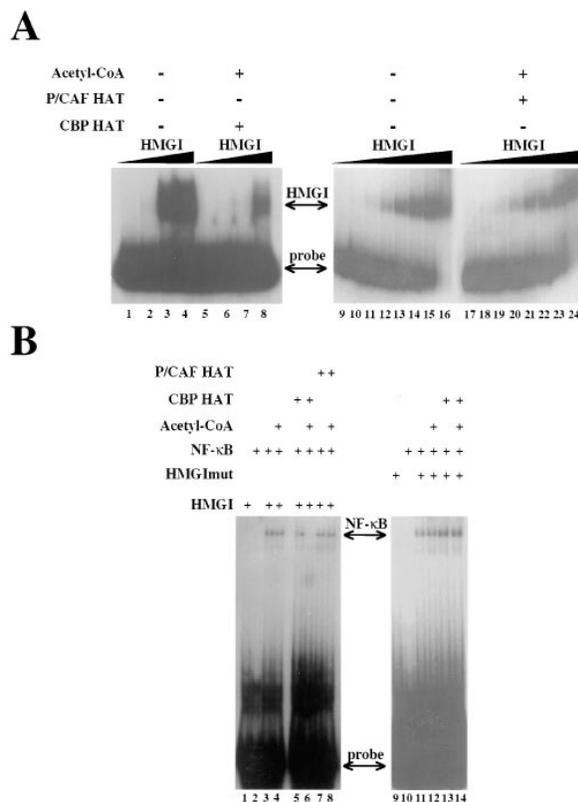


Figure 3. Acetylation of HMG I by CBP but Not by P/CAF HAT Domain Decreases Sequence-Specific DNA Binding and Disrupts the HMG I/NF- κB Complex

(A) HMG I (1 μg) was either mock or acetylated by CBP HAT or P/CAF HAT and then used in EMSA experiments with the IRE fragment (-77 to -37) of the IFN β promoter as a probe. The amounts of HMG I used were 1.7, 2.5, 5, and 8.3 ng for lanes 1-8 and 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, and 17 ng for lanes 9-24.

(B) Shown is an EMSA experiment using recombinant NF- κB along with either the wild-type (lanes 1-8) or the acetylation-defective HMG I (lanes 9-14). Lane 1, HMG I alone; lane 2, NF- κB alone; lane 3, NF- κB +HMG I; lane 4, NF- κB +HMG I+CBP HAT; lane 5, NF- κB +HMG I+Acetyl-CoA; lane 6, NF- κB +HMG I+CBP HAT+Acetyl-CoA; lane 7, NF- κB +HMG I+P/CAF HAT; lane 8, NF- κB +HMG I+P/CAF HAT+Acetyl-CoA; lanes 9-14, same as lanes 1-6 except that HMG Imut was used instead of wild-type HMG I. The amounts of proteins used were as follows: 300 μg of NF- κB , 1.25 ng of HMG I, 5 ng of HMG Imut, 300 ng of CBP HAT, and 100 ng P/CAF HAT. Acetyl-CoA was used at 25 nmoles.

the HAT domain of P/CAF does not affect the HMG I/NF- κB cooperative complex (lanes 7 and 8). The specificity of this phenomenon was further investigated by using an HMG I mutant (HMG Imut) bearing substitutions in the lysine residues acetylated by these HAT proteins. As shown in Figure 3B, HMG Imut forms a cooperative complex with NF- κB (Lane 11; Yie et al., unpublished data) that cannot be disrupted by CBP HAT activity (lane 14). Therefore, acetylation of Lys-65 in HMG I by CBP correlates with the disruption of the HMG I/NF- κB complexes. Finally, the inability of P/CAF's HAT activity to affect complex formation is consistent with the fact that P/CAF acetylates Lys-71, which is not involved in protein-DNA or protein-protein interactions with NF- κB (Yie et al., unpublished data). Thus, the HAT activity of CBP

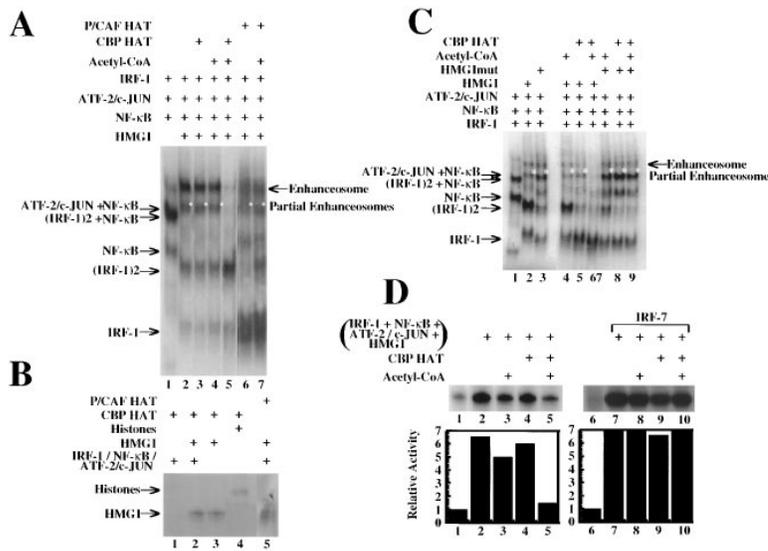


Figure 4. Acetylation of HMG I by CBP Disrupts the IFN β Enhanceosome

(A) Shown is an EMSA experiment using in vitro assembled IFN β enhanceosomes incubated in the presence or the absence of CBP or P/CAF HAT proteins as indicated at the top of the gel. Lane 1, binding of the IFN β gene activators to the enhancer in the absence of HMG I does not suffice for enhanceosome formation; lane 2, addition of HMG I promotes enhanceosome assembly (arrow); lane 3, enhanceosomes incubated with CBP HAT; lane 4, enhanceosomes incubated with Acetyl-CoA; lane 5, addition of both CBP HAT and Acetyl-CoA disrupts the enhanceosome and all of the NF- κ B containing complexes; lane 6, enhanceosomes incubated with P/CAF HAT domain; lane 7, enhanceosomes incubated with P/CAF HAT + Acetyl-CoA. The asterisk indicates partial enhanceosomes containing HMG I and either ATF-2/cJun + NF- κ B or two molecules of IRF-1 + NF- κ B. The identity of these complexes was determined in separate experiments. The

probe was run out of the gel to facilitate separation of the complexes. The amounts of proteins used were 6 ng NF- κ B, 80 ng ATF-2/cJun, 250 ng IRF-1, 25 ng HMG I, 300 ng CBP HAT, and 100 ng P/CAF HAT. Acetyl-CoA was used at 25 nmoles.

(B) CBP and P/CAF HAT domains acetylate HMG I in the context of the enhanceosome. The enhanceosome assembly reaction was scaled up 10-fold, proteins were purified by precipitation with 25% TCA, and they were analyzed by SDS PAGE followed by autoradiography. As seen in lanes 2 and 5, CBP and P/CAF HAT domains, respectively, acetylate HMG I in the context of the enhanceosome. The gel was exposed for 3 weeks.

(C) CBP does not disrupt enhanceosomes containing HMG Imut. The proteins used in each reaction are indicated at the top of the gel. The amounts are as in (A), except that 100 ng of HMG Imut were used in the reaction, since HMG Imut has lower DNA-binding affinity compared to wild-type HMG I (Yie et al., unpublished data).

(D) Shown are the results of in vitro transcription experiments with HeLa nuclear extracts using the IFN β CAT template either alone (basal level, lane 1) or in the presence of the enhanceosome (lane 2). In lanes 3 and 4, the enhanceosome was incubated with Acetyl-CoA alone or with CBP HAT alone followed by the addition of extract and NTPs. Lane 5, CBP HAT and Acetyl-CoA were added together followed by the addition of the extract and NTPs. Lanes 6–10 are the same as in lanes 1–5 except that the activator used was IRF-7 alone. Correctly initiated transcripts were identified and quantitated by primer extension using a CAT primer.

destabilizes the HMG I/NF- κ B complexes formed at PRDII.

Acetylation of HMG I by CBP Disrupts the IFN β Enhanceosome

To investigate the consequences of HMG I acetylation by CBP and P/CAF HAT domains in the context of the natural IFN β enhanceosome, we carried out EMSA experiments where enhanceosomes containing either wild-type or mutant HMG I protein were challenged with the CBP or P/CAF HAT activities. Figure 4A demonstrates that addition of HMG I to a low amount of IFN β activators promoted the assembly of the enhanceosome (compare lanes 1 and 2). The amount of HMG I used was determined in separate experiments to ensure that there was no free HMG I protein in the reaction (data not shown). Incubation of these enhanceosomes with either CBP HAT or Acetyl-CoA separately did not have any effect on their stability (lanes 3 and 4). However, enzyme and Acetyl-CoA added together significantly decreased enhanceosome stability (lane 5). Interestingly, along with the fully assembled enhanceosome, all partial enhanceosomes containing NF- κ B were also destabilized. In sharp contrast, the P/CAF HAT activity did not affect enhanceosome stability (lanes 6 and 7).

To directly demonstrate that both CBP and P/CAF HAT domains can acetylate HMG I in the context of the enhanceosome, we scaled up the assembly reaction 10-fold and replaced cold Acetyl-CoA with 3 H Acetyl-CoA.

Following incubation with the enzymes, the enhanceosome components were purified and visualized by autoradiography after SDS PAGE. Figure 4B demonstrates that both CBP and P/CAF can acetylate only HMG I in the context of the enhanceosome (lanes 2 and 5). Thus, the different effects of CBP and P/CAF HAT activities on enhanceosome stability are likely due to the differential lysine residues acetylated in HMG I. Figure 4C demonstrates that destabilization of the enhanceosome correlates with acetylation of HMG I since enhanceosomes containing the HMG Imut protein, which cannot be acetylated (data not shown), are not affected by the CBP HAT activity (compare lane 3 with 9).

To test whether the CBP HAT activity can disrupt a transcriptionally active enhanceosome, we carried out in vitro transcription experiments. Under these conditions (low activator concentration), addition of HMG I is absolutely required for enhanceosome assembly and activation of transcription (Kim and Maniatis, 1997; Yie et al., unpublished data). Figure 4D shows that assembly of the enhanceosome in vitro leads to high levels of activated transcription (compare lanes 1 and 2). Incubation of these enhanceosomes with CBP HAT or Acetyl-CoA alone did not have a significant effect on the amount of transcription (lanes 3 and 4). However, addition of both CBP HAT and Acetyl-CoA to the reaction strongly inhibited transcription (lane 5). This effect is specific to the enhanceosome since neither the activation of the IFN β promoter by IRF-7 alone (Figure 4D, lanes 6–10) nor the

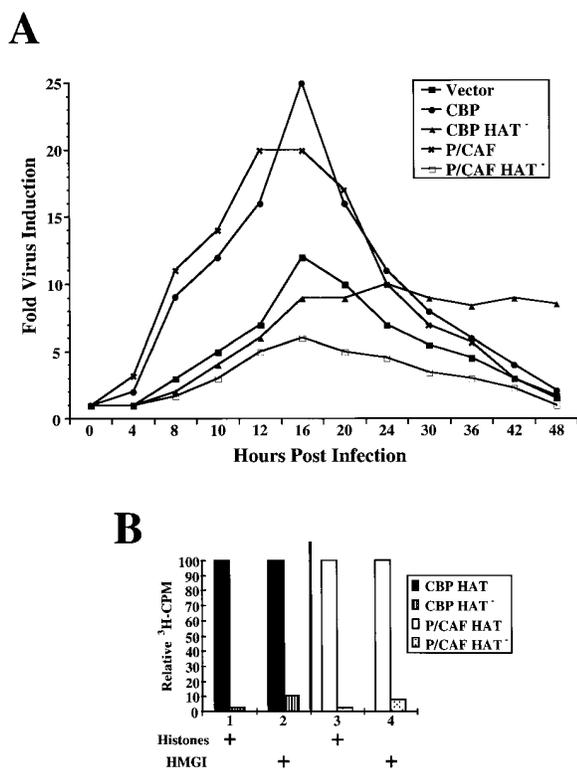


Figure 5. The HAT Activity of CBP Is Required for Both Activation and Postinduction Turnoff of IFN β Gene Expression

(A) COS cells were cotransfected with the -110 IFN β CAT reporter (1 μ g), along with mammalian expression vectors (3 μ g) encoding wild-type CBP and P/CAF or CBP and P/CAF bearing amino acid substitutions in their HAT domains (HAT⁻ constructs) (Korzus et al., 1998). The cells were induced for the indicated time, and the CAT activity was determined and plotted as fold virus induction. Shown is the average of ten independent experiments.

(B) In vitro acetylation assays were performed as in Figure 2A using wild-type or mutant CBP or P/CAF HAT proteins.

basal levels of transcription is affected (data not shown). Thus, acetylation of HMG I by CBP specifically disrupts transcriptionally active enhanceosomes.

The CBP HAT Activity Is Required for Postinduction Turnoff of IFN β Expression

We have previously shown that recruitment of CBP into the enhanceosome is required for virus-induced transcription from the IFN β promoter (Merika et al., 1998). However, here we show that when CBP is recruited into the enhanceosome, it also can acetylate HMG I, resulting in enhanceosome destabilization. These contradictory observations led us to the hypothesis that CBP's HAT activity may be involved in turning off IFN β gene expression. To address this, we carried out transfection experiments in COS cells where the transcriptional activity of the IFN β promoter was monitored at different times following virus infection. Figure 5A demonstrates that the transfected IFN β promoter is transiently activated by virus infection, mimicking the overall pattern of activation of the endogenous gene (Maniatis et al., 1992). As seen in the figure, expression levels peak at 16 hr post infection. After that time, transcription

gradually decreases and finally reaches the basal uninduced level at 48 hr post infection. Cotransfection of an expression vector encoding wild-type CBP or P/CAF proteins further stimulated the transcriptional activity of the enhanceosome, with no effect on the kinetics of virus-induced transcription. Thus, in both cases, the virus-inducible expression reached the basal (uninduced) level by the end of the time course (Figure 5A). To investigate the role of the HAT activities, we transfected derivatives containing two amino acid substitutions in the Acetyl-CoA-binding site of either CBP or P/CAF. It has been previously demonstrated that these derivatives (CBP HAT⁻ and P/CAF HAT⁻) lack HAT activity (Korzus et al., 1998; Figure 5B). We demonstrate here that these mutant HAT proteins also lack the ability to acetylate HMG I in vitro (Figure 5B). Remarkably, transfection of the CBP HAT⁻ expression vector altered the kinetics of virus-induced transcription from the IFN β enhanceosome by preventing postinduction turnoff. Importantly, the levels of activated transcription obtained with CBP HAT⁻ were lower compared to wild-type CBP (Figure 5A). Thus, the HAT activity of CBP is required for both optimum activation and postinduction turnoff of IFN β gene expression. In contrast, the P/CAF HAT activity is only required for optimum activation of transcription with no effect on postinduction turnoff (Figure 5A).

To investigate whether these effects are due to specific acetylation of HMG I by CBP in the context of the enhanceosome, we carried out transfection experiments in COS cells using expression vectors encoding either the wild-type or the HMG I mutant proteins. Figure 6A demonstrates that transfection of wild-type HMG I affected neither the magnitude (Thanos and Maniatis, 1992) nor the kinetics of virus-induced IFN β transcription. Interestingly, overexpression of the HMG I mutant protein prevented postinduction turnoff of IFN β expression. Thus, although enhanceosomes assembled in vivo with HMG I mutant are transcriptionally active, they are resistant to the postinduction turnoff mechanisms. The role of CBP's HAT activity in this process was revealed by showing that enhanceosomes bearing either wild-type HMG I and CBP HAT⁻ or wild-type CBP and HMG I mutant are incapable of postinduction shutoff of IFN β transcription (Figure 6A). Taken together, these experiments strongly support the notion that there is a direct correlation between the ability of CBP to specifically acetylate HMG I and postinduction turnoff of IFN β gene expression.

Additional evidence for the functional interplay between CBP HAT activity and HMG I in the context of the IFN β enhanceosome was provided by transfection experiments in *Drosophila* Schneider cells. Figure 6B demonstrates that transfection of wild-type HMG I potentiates enhanceosome formation and transcription from the IFN β promoter (compare lanes 5 and 6), and these levels were further enhanced (~2.5-fold) by cotransfecting CBP (lane 7). Figure 6B (lane 8) demonstrates that transfection of HMG I mutant also facilitated enhanceosome assembly, albeit to a lower extent. Interestingly, the HMG I mutant-containing enhanceosomes are coactivated by CBP (~8-fold) to significantly higher levels compared to enhanceosomes bearing wild-type HMG I (compare lanes 6 and 7 with 8 and 9). Since HMG I mutant containing enhanceosomes cannot be destabilized by CBP HAT activity (Figures 4C and 6A), we conclude

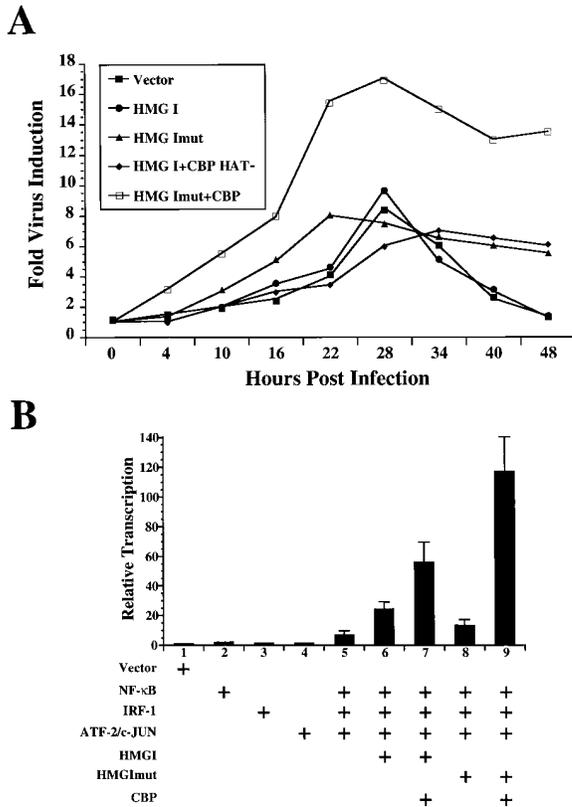


Figure 6. Acetylation of HMG I by CBP Is Required for Postinduction Shutoff of IFN β Expression

(A) COS cells were transfected with the IFN β CAT reporter (Figure 5) along with expression vectors encoding wild-type HMG I (1 μ g) or HMG Imut (1 μ g) in the absence or the presence of the indicated CBP derivatives (2 μ g). The cells were induced for the indicated time, and the CAT activity was determined and plotted as fold virus induction.

(B) *Drosophila* Schneider cells were cotransfected with a CAT reporter (200 ng) bearing the IFN β gene enhancer cloned upstream of the ADH TATA box, along with expression vectors encoding the indicated proteins. The amounts of expression vectors used were 100 ng of an equimolar mixture of pPAC p50 and pPAC p65, 800 ng of pPAC IRF-1, 600 ng of an equimolar mixture of pPAC ATF-2 and pPAC c-JUN, 600 ng of pPAC HMG I or pPAC HMG Imut, and 1 μ g of pPAC CBP. The total amount of transfected DNA was brought to 4 μ g by adding pPAC vector as necessary.

that the higher activity of these enhanceosomes in vivo is due to their increased stability. These results, taken together with our other experiments, strongly suggest that the HAT activity of CBP potentiates transcription, perhaps by acetylation of histones, but equally importantly, acetylation of HMG I by CBP results in enhanceosome destabilization and therefore termination of transcription.

Further evidence for the distinct in vivo roles of CBP and P/CAF HAT activities was provided by the use of chimeric CBP and P/CAF proteins containing their HAT domains interchanged. Figure 7A shows that substitution of CBP's HAT domain with P/CAF's HAT domain completely prevented transcriptional postinduction turnoff (CBP^{P/CAF HAT} construct). On the other hand, replacement of P/CAF's HAT domain with that of CBP (P/CAF^{CBP HAT} construct) did not affect postinduction turnoff (Figure

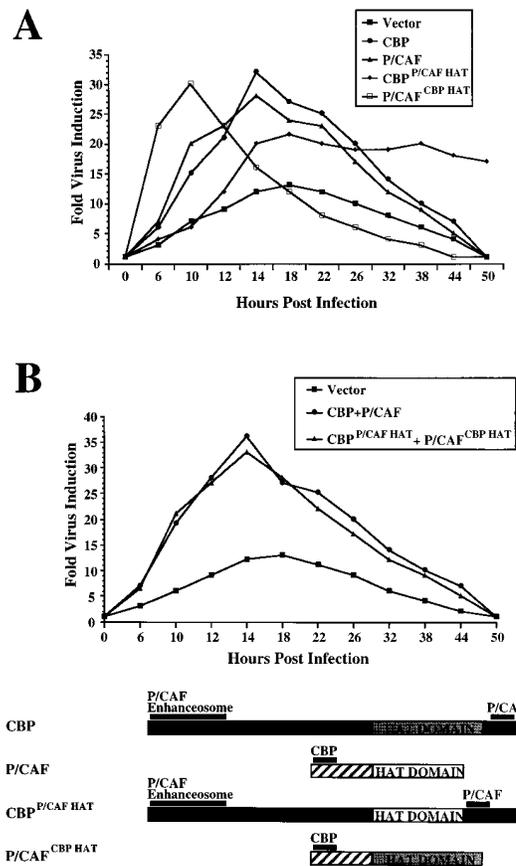


Figure 7. The HAT Domains of Both CBP and P/CAF Are Required for Proper Kinetics of IFN β Virus Induction

(A) COS cells were transfected as in Figure 5 with the indicated expression vectors. CBP^{P/CAF HAT} refers to a CBP expression vector bearing the P/CAF HAT domain in place of that of CBP. Accordingly, P/CAF^{CBP HAT} refers to a P/CAF expression vector bearing the CBP HAT domain in place of that of P/CAF.

(B) Comparison of the effects of cotransfecting wild-type CBP and P/CAF or HAT domain swap CBP and P/CAF chimeras.

7A). However, recruitment of this chimera into the enhanceosomes resulted in a strikingly earlier onset of transcriptional activation. A dramatic illustration of this is the amount of transcription obtained at 6 hr post infection (Figure 7A). Finally, cotransfection of both chimeric proteins did not affect either the kinetics of expression or the levels of transcription when compared to cotransfection of the wild-type counterparts (Figure 7B). Taken together, these experiments strongly suggest that the P/CAF and CBP HAT activities per se, as well as their geometry within the enhanceosome, are interchangeable for activation of transcription. However, the CBP HAT activity is also required for postinduction turnoff of IFN β transcription by acetylating HMG I. Thus, the timing of virus-dependent IFN β gene expression in vivo is regulated by the distinct functional properties of CBP and P/CAF HAT activities.

Discussion

In this paper, we have examined the functions of the CBP and P/CAF coactivators subsequent to their recruitment

into the IFN β gene enhanceosome. Both CBP and P/CAF acetylate HMG I(Y), the essential architectural component for the assembly and stability of the enhanceosome. Moreover, the lysine residues in HMG I(Y) acetylated by CBP and P/CAF differ, and this difference accounts for their distinct effects on the fate of transcription. More specifically, HMG I K65, which is acetylated by CBP, is critical for high-affinity binding to DNA, whereas K71, which is acetylated by P/CAF, is not. In addition, in the context of the enhanceosome, acetylation of HMG I by CBP, but not by P/CAF, leads to enhanceosome destabilization and disassembly. Furthermore, we have established a biologically relevant function *in vivo* for the acetylation of a non-histone protein by showing that the HAT activity of CBP on HMG I is essential for turning off IFN β gene expression. Finally, the distinct functional properties of CBP and P/CAF HAT activities fine tune the timing of virus-dependent activation of the IFN β gene.

HMG I Proteins Are Bonafide Substrates for CBP and P/CAF Acetyltransferase Activity

A large body of previous studies have established that the HMG I proteins function as pleiotropic regulators in the cell. For example, HMG I proteins regulate the expression of a large number of genes such as cytokines, cell adhesion molecules, growth factors, transcription factors, and viral gene products (Bustin and Reeves, 1996). The commonality of these diverse regulatory functions lies in the HMG I family's remarkable properties of interacting with many different transcription factors and binding and altering the structure of DNA in a way that facilitates assembly of functional higher order nucleoprotein complexes (Thanos and Maniatis, 1995a). Thus, it is not surprising that disruption of the HMG I genes directly correlates with tumorigenesis and that a null mutation of HMG I-C in mice decreases the rate of cell proliferation, resulting in the pygmy phenotype (Ashar et al., 1995; Zhou et al., 1995). Similar to HMG I, CBP/p300 is also a pleiotropic regulator. CBP/p300 interacts with a diverse collection of transcription factors and participates in a broad spectrum of biological activities. However, the mode of CBP's action differs from that of HMG I. Most likely, CBP is recruited to already preformed enhanceosomes and thereafter modulates their transcriptional properties (Merika et al., 1998). Again, disruption of the CBP/p300 genes causes severe global developmental abnormalities (Petrij et al., 1995; Yao et al., 1998). Therefore, our demonstration that CBP alters the DNA-binding affinity of HMG I by acetylation provides an important means for the integration and interpretation of multiple signal transduction pathways. For genes whose expression is positively affected by HMG I proteins, for example, CBP-dependent acetylation of HMG I could lead to termination of transcription, as is the case for IFN β . By contrast, for genes negatively regulated by HMG I, the same acetylation events may facilitate their activation. Thus, the functional linkage between these two global and pleiotropic regulators could generate and fine tune multiple expression and developmental programs.

HMG I is the only known substrate for which there is

a distinction between the HAT activities of CBP and P/CAF. It appears that the substrate specificity between CBP and P/CAF HAT activities in acetylating HMG I provides the molecular basis for their distinct roles in the activity and fate of the IFN β gene enhanceosome. However, it is possible that acetylation of HMG I by P/CAF may affect its DNA-binding activity on other promoters or its association with other transcription factors. In addition, other HAT proteins may also acetylate HMG I and influence its activity. Finally, the *in vivo* association of HMG I with the CBP-P/CAF complex may also influence its interaction or activity with several other cellular or viral (e.g., E1A) regulators.

The IFN β Enhanceosome Is a "Time Bomb"

The discovery of enhanceosomes provided the first compelling mechanistic basis for explaining the phenomenon of transcriptional synergy in natural promoters and enhancers (reviewed in Carey, 1998). Thus, the extraordinary stability of the IFN β enhanceosome, along with its abilities to recruit CBP and to simultaneously contact several components of the basal machinery, ensures high levels of activated transcription. However, the IFN β gene is only transiently activated, implying that transcription driven by the enhanceosome must be terminated. In principle, this can be accomplished by mechanisms that allow transient formation of the enhanceosome followed by recruitment of repressors and corepressors to maintain shutoff of transcription. Previous studies have established that virus infection induces the synthesis of several proteins that can function as inhibitors of IFN β gene expression. Among these are DNA-binding proteins (with no activation potential) that may directly compete for binding to the enhancer (e.g., IRF-2, PRDI-BF1, PRDII-BF1, and p50 homodimers) as well as proteins that inhibit NF- κ B, such as I κ B α (Maniatis et al., 1992; Thanos and Maniatis, 1995b; Tran et al., 1997). However, none of the above mentioned DNA-binding proteins possesses a DNA-binding affinity significant enough to disrupt the enhanceosome by competition, nor can I κ B α remove NF- κ B in the context of the enhanceosome (our unpublished data). Instead, as we demonstrate here, the enhanceosome contains all of the necessary information for self destruction. The driving force for enhanceosome disassembly appears to be the inability of K65-acetylated HMG I to maintain NF- κ B on DNA. Remarkably, this effect is specific for NF- κ B because acetylation of HMG I by CBP does not affect its ability to form cooperative complexes with ATF-2/cJun at PRDIV (data not shown). Thus, perhaps acetylation of HMG I initially destabilizes the enhanceosome at PRDII (NF- κ B and HMG I binding site) and subsequently detachment of NF- κ B and HMG I mediate its disruption. The reason for these differential effects of HMG I at PRDII and PRDIV could be due to the dual role of HMG I at PRDII. Thus, binding of HMG I at PRDII not only counteracts an unfavorable intrinsic DNA bend allowing stable binding of NF- κ B to the site but is also required to relieve the stereospecific clashes between NF- κ B and IRF proteins bound at the nearby PRDI element (Falvo et al., 1995; Thanos and Maniatis, 1995a; Escalante et al., 1998). Furthermore, since the middle basic repeat

of HMG I is its high-affinity DNA-binding domain, acetylation of K65 provides a simple and elegant mechanism to significantly decrease its ability to bind DNA. Thus, a weakening of the HMG I-DNA interactions by acetylation suffices for enhanceosome disruption.

Our experiments, taken together with previous studies, are consistent with the following model for activation and postinduction shutoff of IFN β gene expression. Virus infection causes the coordinate activation of multiple transcriptional activator proteins (NF- κ B, ATF-2/cJun, IRFs) that, in the presence of HMG I, bind cooperatively and form the enhanceosome on the IFN β enhancer (Thanos and Maniatis, 1995a). As a result of enhanceosome assembly, the activation domains of the activators create a novel activating surface that, in turn, recruits CBP and CBP-associated proteins or complexes (e.g., P/CAF, polII holoenzyme, etc.) (Merika et al., 1998). Simultaneously, the activation domains also establish contacts with other components of the basal machinery (e.g., TFIID, TFIIA, TFIIB, USA, etc.) (Kim and Maniatis, 1998). Access of the basal machinery to the promoter may be facilitated by the HAT activities of CBP and P/CAF via acetylation of histones in nearby nucleosomes. In fact, we have shown that the HAT activities of both CBP and P/CAF are required for transcriptional activation from the enhanceosome. Consistent with this, histones H3 and H4 of chromatin associated with the IFN β promoter are acetylated in response to virus infection (B. S. Parekh and T. Maniatis, personal communication).

A striking aspect highlighting the mechanism of action of both coactivators is the relative redundancy in the use of their HAT activities for activation of transcription. From the HAT domain swap experiments, we have demonstrated that enhanceosomes bearing only the CBP HAT or P/CAF HAT domains stimulate transcription at similar levels (Figure 7A). Thus, we imagine that the acetylation targets of these domains might be either the same or related. The difference in the onset of gene activity between these enhanceosomes could be the result of the relative efficiency or rate by which the HAT proteins acetylate their targets. Another important conclusion from these experiments is that the geometry of the HAT domains in the context of the enhanceosome is not critical for their function. However, this latter observation contrasts with the spatial specificity required for recruiting the coactivators into the enhanceosome (Merika et al., 1998).

The demonstration that the CBP HAT activity plays a role in both transcriptional activation and postinduction turnoff is intriguing. For example, what prevents CBP from acetylating HMG I immediately after its recruitment into the enhanceosome, thereby leading to its disruption? We imagine two different models to explain this puzzle. In the first, due to the enhanceosome context, CBP may acetylate HMG I with lower efficiency and rate compared to its other targets (e.g., histones), thus allowing activation of transcription. Alternatively, the substrate specificity of CBP's HAT activity may be regulated by posttranslational modifications or by other cofactors synthesized after virus infection. Therefore, after histones or other targets have been acetylated, CBP acetylates HMG I and disrupts the enhanceosome. In

contrast, the second model predicts that the choice for CBP to acetylate histones or HMG I is stochastic. Thus, a significant number of initially formed enhanceosomes will be disrupted before they activate transcription because CBP may acetylate HMG I first. Consistent with this idea is the observation that only a fraction of virus-infected cells are induced to express IFN β (Maniatis et al., 1992). Thus, it is conceivable to assume that in nonexpressing cells, the enhanceosomes formed at the onset of virus infection have been disrupted because CBP acetylates HMG I first. The rest of the cells transcribe IFN β because CBP acetylates histones first. Activated transcription continues until CBP acetylates HMG I, thus leading to enhanceosome disruption. Perhaps most or all of the disrupted enhanceosomes do not reassemble because of the appearance in the nucleus of newly synthesized repressors (mentioned above) that bind to the IFN β promoter and of the ability of newly synthesized I κ B α to enter the nucleus, to associate with NF- κ B, and to thus prevent its DNA binding (Tran et al., 1997). Therefore, we propose that the acetylation of HMG I by CBP shifts the dynamic equilibrium of IFN β transcription from activation towards repression.

Experimental Procedures

Plasmid Constructions

The HAT domain swap CBP and P/CAF constructs were generated by replacing the HAT domain of CBP (aa 1098–1758) with the HAT domain of P/CAF (aa 352–832) and vice versa, by stepwise subcloning of the appropriate fragments. The 6HisCBP HAT expression vector was generated by cloning the CBP HAT domain into PRSETA (Invitrogen). HMG I mutants K65R, K71R, and K65R/K71R were generated by standard PCR mutagenesis. In the HMG I mut protein aa 64–71 have been mutated to AAIESDPE. The HMG I and HMG I mut mammalian expression vectors were generated by cloning the corresponding open reading frames into the pCDNA3.1 HisC expression vector (Invitrogen).

Recombinant Protein Purification

GST-HMG I fusions were expressed and purified as described previously (Yie et al., 1997). His-6-fused proteins were purified on a nickel column under native conditions as suggested by the manufacturer (Qiagen) except that the binding buffer used contained 10 mM Tris Cl [pH 8.0], 10% glycerol, 500 mM NaCl, 0.1% NP-40, 10 mM β -mercaptoethanol, 1 mM PMSF, 10 mM imidazole, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin.

Acetyltransferase Assays

Acetyltransferase assays were essentially carried out as previously described (Gu and Roeder, 1997). A typical reaction was performed in a volume of 30 μ l containing 1–2 μ g highly purified recombinant protein, 300 ng CBP HAT or 100 ng P/CAF HAT, 26 pmol 4.8 Ci/mmol 3 H-Acetyl-CoA (Amersham), and a standard buffer (10% glycerol, 50 mM HEPES [pH 8.0], 1 mM DTT, 1 mM PMSF, and 10 mM sodium butyrate) at 30°C for 1 hour. Filter-binding assays and gel assays were performed as described previously (Gu and Roeder, 1997).

Mapping of HMG I Acetylation Sites

Peptides corresponding to two portions of HMG I (peptide I, aa 6–31; peptide II, aa 64–89) were synthesized and purified by HPLC to 95% purity. The peptides were used in a standard acetyltransferase reaction and thereafter subjected to N-terminal microsequencing in which 20% of each cycle was used to determine 3 H-Acetyl-CoA incorporation, and the rest was used for amino acid identification.

Electrophoretic Mobility Shift Assays and In Situ Acetylation

DNA-binding reactions with the appropriate amounts of proteins (indicated in figure legends) and DNA probe were mixed and incubated in 15% glycerol, 10 mM HEPES (pH 8.0), 10 mM Tris Cl (pH

8.0), 15 mM NaCl, 10 mM Na butyrate, 2 mM DTT, 2 mM PMSF, 0.05% NP-40, 1 mg/ml BSA, and 50 ng pdG-dC for 15 min at room temperature. For EMSA's involving *in situ* acetylation, DNA-binding reactions were challenged with CBP HAT or P/CAF HAT and cold Acetyl-CoA for 90 min at room temperature. Protein-DNA complexes were subsequently resolved by electrophoresis as previously described (Merika et al., 1998).

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