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Ordered Recruitment of Chromatin Modifying and General Transcription Factors to the IFN-β Promoter

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Summary

Here, we show that the IFN-β enhanceosome activates transcription by directing the ordered recruitment of chromatin modifying and general transcription factors to the IFN-β promoter. The enhanceosome is assembled in the nucleosome-free enhancer region of the IFN-β gene, leading to the modification and remodeling of a strategically positioned nucleosome that masks the TATA box and the start site of transcription. Initially, the GCN5 complex is recruited, which acetylates the nucleosome, and this is followed by recruitment of the CBP-PolII holoenzyme complex. Nucleosome acetylation in turn facilitates SWI/SNF recruitment by CBP, resulting in chromatin remodeling. This program of recruitment culminates in the binding of TFIID to the promoter and the activation of transcription.

Introduction

A central problem in eukaryotic gene regulation is understanding how the basal transcriptional machinery is targeted to specific sets of genes packaged in chromatin in response to environmental cues. The inability of the basal transcriptional machinery to bind nucleosomal DNA implies that at least one of the activators' functions could be to alter chromatin structure, permitting binding of other transcription factors and subsequent assembly of functional pre-initiation complexes (Struhl, 1999). Thus, activators function not only by inducing localization of the basal machinery to the promoter but also by recruiting chromatin modifying activities.

Two general classes of chromatin modifying activities have been implicated in controlling transcription: ATP-dependent nucleosome remodeling and histone acetylation (reviewed in Kornberg and Lorch, 1999). Many different ATP-dependent chromatin-remodeling complexes have been identified including the SWI/SNF, the ISWI, and the Mi-2 families (Kingston and Narlikar, 1999; Vignali et al., 2000). In humans, the SWI/SNF complexes are multi-subunit assemblies of eight or more polypeptides in which the DNA-dependent ATPase is either the BRG1 or the BRM1 proteins. Chromatin remodeling complexes function by facilitating an exchange between a normal and an altered, more accessible, nucleosome conformation, which could involve histone octamer transfer and/or nucleosome sliding (Schnitzler et al., 1998; Hamiche et al., 1999; Whitehouse et al., 1999). The SWI/SNF complexes lack sequence specific DNA binding, and are therefore thought to be recruited to specific promoters via interactions with DNA binding proteins (Vignali et al., 2000).

By contrast to the remodeling complexes, acetylation complexes covalently modify the amino terminal tails of nucleosomal histones (Strahl and Allis, 2000). The mammalian GCN5/PCAF and the homologous yeast SAGA complex, together with the CBP/p300 proteins, bear intrinsic histone acetyltransferase activities and function as coactivators of transcription after their recruitment to promoters via their interaction with numerous DNA binding proteins, and in several cases, the histone acetyltransferase activity of these complexes is critical for activation of transcription (Berger, 1999; Struhl, 1999). In the case of the yeast HO promoter, activator-dependent recruitment of SWI/SNF is required for subsequent recruitment of SAGA followed by histone acetylation and DNA binding of the other activator named SBF (Cosma et al., 1999; Krebs et al., 1999). In contrast, in the case of a synthetic reporter bearing a GCN4 binding site placed upstream of the PHO5 promoter, SAGA-dependent histone acetylation facilitates SWI/SNF induced chromatin remodeling and transcriptional activation (Syntichaki et al., 2000). However, the functional interplay between histone acetylases and chromatin remodeling factors in complex mammalian eukaryotic promoters is unknown.

A well-characterized example of mammalian enhancers is provided by the virus-inducible enhancer of the IFN-β gene. Virus infection results in the coordinate activation of three distinct sets of transcription factors (NF-κB, IRFs and ATF-2/c-Jun heterodimer), which together with the architectural HMG I(Y) protein bind cooperatively on the enhancer to form the IFN-β enhanceosome (Maniatis et al., 1998; Munshi et al., 1999). Assembly of the enhanceosome results in the formation of a novel-activating surface that optimally interacts with and recruits the transcriptional apparatus (Kim and Maniatis, 1997; Merika et al., 1998). The primary target of this surface is the CBP-PolII holoenzyme whose recruitment is critical for the rapid assembly of functional pre-initiation complexes at the IFN-β promoter (Merika et al., 1998; Kim et al., 1998; Yie et al., 1999). Virus infection leads to localized histone hyperacetylation at the IFN-β promoter in vivo (Parekh and Maniatis, 1999), an observation consistent with the requirement of the histone acetyltransferase activities of CBP and P/CAF for IFN-β gene activation (Munshi et al., 1998).

In this paper, we examined the linkage between histone acetylation and chromatin remodeling at the IFN-β promoter in vivo and in vitro. Our mapping of the chro-

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matin structure at the IFN-β transcriptional regulatory locus revealed that the enhancer is nucleosome-free but flanked by two nucleosomes, one of which is positioned immediately downstream of the TATA box (nucleosome II). We show that the enhanceosome activates transcription in vivo or in vitro by directing ordered recruitment of GCN5 and CBP/PolII holoenzyme complexes followed by recruitment of SWI/SNF. We also demonstrate that SWI/SNF recruitment and subsequent remodeling of nucleosome II are greatly enhanced by histone acetylation, indicating that this modification is a prerequisite for the remodeling function of the SWI/SNF complex. Finally, when nucleosome II has been remodeled, TFIID is recruited to the promoter triggering initiation of transcription.

Results

The IFN-β Enhancer Is Nucleosome-Free In Vivo and In Vitro

The positions of the nucleosomes at the IFN-β promoter in vivo were identified by ligation-mediated PCR of cross-linked chromatin (Fragoso et al., 1995). To accomplish this task, we treated HeLa cells with formaldehyde to fix histone-DNA interactions followed by complete micrococcal nuclease treatment of isolated chromatin. DNA extracted from the resulting mononucleosomes was used as a substrate for ligation-mediated PCR using primers encompassing the IFN-β promoter locus. The primers were annealed with the DNA followed by primer extension, linker ligation, and PCR amplification. As seen in Figure 1A, primer α produces a fragment of 110 bp (lane 1), whereas primer β generates a fragment of 125 bp (lane 2), indicating the presence of a nucleosome (nucleosome II) with boundaries at −15 and +132, relative to the IFN-β transcription start site. Thus, nucleosome II begins 5 bp, only, downstream from the IFN-β TATA box and extends over the start site of transcription (Figure 1A). Primers γ and δ generate fragments of 109 (lane 3) and 98 (lane 4) bp, respectively, designating nucleosome I with boundaries at −268 and −118 (Figure 1A). Previous in vivo DNase I footprinting experiments revealed the existence of a 10 bp periodicity pattern, a hallmark of positioned nucleosomes, in the area we mapped nucleosome I (Zinn and Maniatis, 1986). Importantly, primers complementary with the IFN-β enhancer region failed to produce a PCR product after micrococcal nuclease treatment indicating that the enhancer is nucleosome-free in vivo (data not shown).

Because the bulk of DNA sequences lack an inherent ability to position nucleosomes in vitro, reconstitution of histones to DNA fragments greater than nucleosome length (146 bp) produces a heterogeneous population of core particles with distinct mobilities, because they are positioned at multiple sites on DNA (Lowary and Widom, 1997). An IFN-β promoter fragment (326 bp) spanning the region from −143 to +183 and a CMV promoter fragment (−222 to +113) of equal size were reconstituted into nucleosome core particles and analyzed by EMSA. Figure 1B shows that the IFN-β promoter-containing fragment is reconstituted into a distinct nucleosome complex (lane 3). In sharp contrast, the CMV promoter fragment produced a smear (lane 1), consistent with the presence of a nucleosome at multiple sites along the DNA fragment. The difference in the mobility of naked DNA probes between the CMV and IFN-β promoters is due to an intrinsic bend in the IFN-β promoter DNA sequence (Falvo et al., 1995). Both fragments display identical mobilities on a denaturing polyacrylamide gel (not shown). The boundaries of the in vitro reconstituted nucleosome at the IFN-β promoter were identified after gel purification and exonuclease III digestion. Figure 1C shows that digestion from the 3′ or 5′ ends produces 275 and 198 bp resistant fragments, respectively (lanes 2, 3 and 8, 9), thus indicating that the in vitro assembled nucleosome is positioned from −15 to +132 relative to the start site of transcription. In addition, hydroxyl radical cleavage analysis and DNaseI footprinting revealed that nucleosome II is both translationally and rotationally positioned at the IFN-β core promoter (data not shown). Thus, both in vivo and in vitro, the IFN-β enhancer is nucleosome free.

As the IFN-β enhancer is nucleosome-free, we hypothesized that the activators and HMG I(Y) would have full access to their binding sites leading to enhanceosome assembly. We carried out EMSA experiments using the nucleosome reconstituted −143/+183 region of the IFN-β gene as a probe. Figure 1D (lanes 1–4) displays the mobilities of activator-DNA complexes containing each of the IFN-β gene activators. Simultaneous addition of all the activators without HMG I(Y) did not lead to enhanceosome assembly (lane 5). However, incubation of the activators with a fixed amount of HMG I(Y) (lane 1) resulted in enhanceosome assembly (compare lane 5 with 6). The specificity of enhanceosome assembly was demonstrated by the inability of HMG I(Y) to promote enhanceosome assembly on a template bearing mutations in all four HMG I(Y) binding sites (Figure 1D, lanes 8–14). Similar results were obtained when an IFN-β promoter fragment bearing both nucleosomes (I and II) was used as a template (data not shown).

Histone Acetylation Facilitates Enhanceosome-Mediated Recruitment of the SWI/SNF Complex and Subsequent Recruitment of TFIID to the IFN-β Promoter

The chromatin structure at the IFN-β promoter locus suggests that transcriptional activation could be associated with remodeling of the strategically positioned nucleosome II. To test this idea, we measured the sensitivity of DNA residing in isolated nuclei to cleavage with the restriction endonucleases AvaII and NcoI (centered at −42 and −10, respectively). Isolated nuclei prepared from mock- or virus-infected HeLa cells were incubated with AvaII or NcoI; DNA was extracted, digested with PvuII, and analyzed by Southern blotting. Figure 2A shows that in uninfected cells the AvaII site is fully accessible and its accessibility is not altered by virus infection (compare lanes 4 and 5), a result consistent with our in vivo and in vitro mapping experiments indicating that this region of the promoter is nucleosome free. In contrast, the NcoI site, which lies within nucleosome II, is inaccessible in uninfected cells, but NcoI cleavage was greatly increased following virus infection (compare lanes 2 and 3). Thus, the change in accessibility to NcoI suggests nucleosome remodeling.

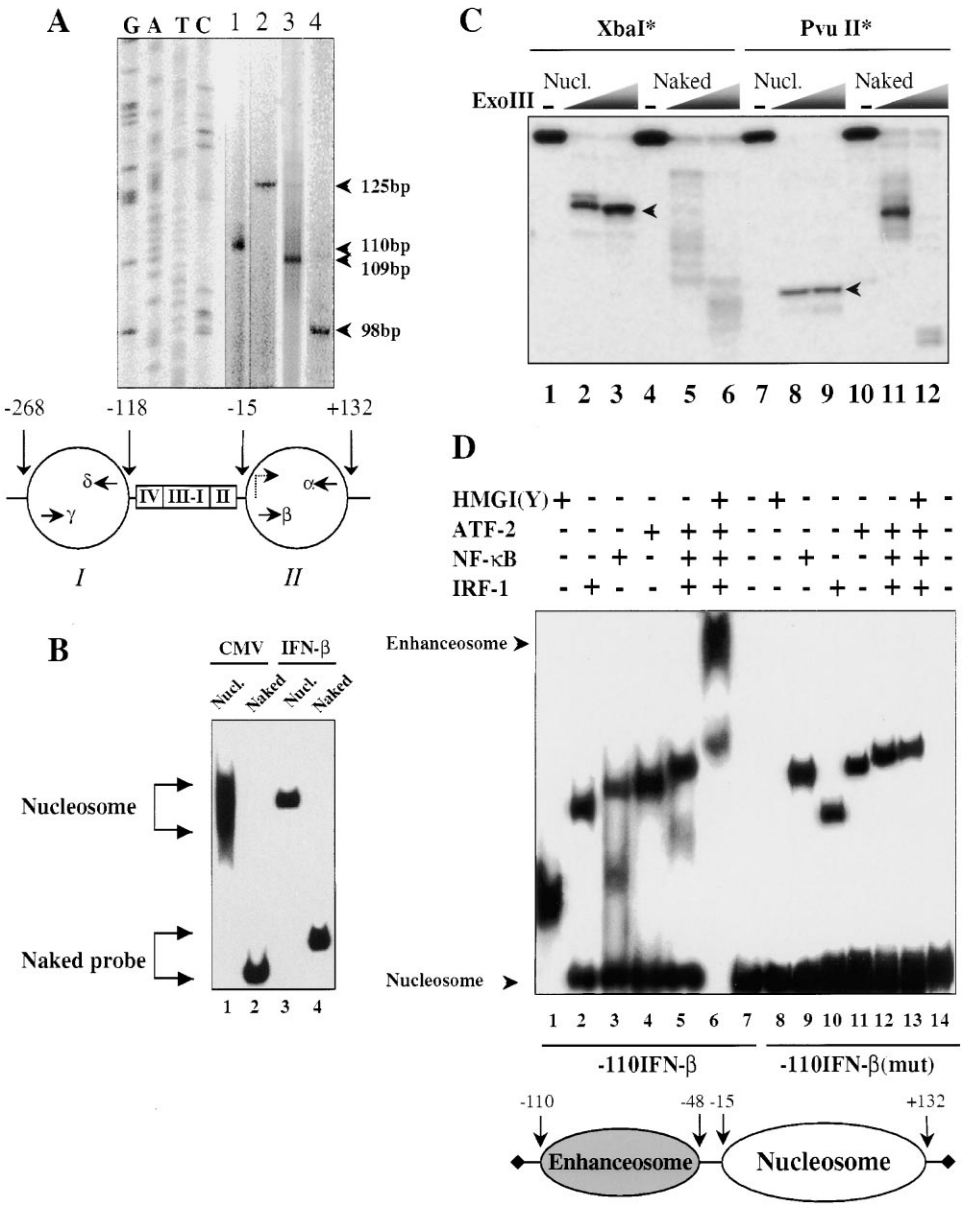

Figure 1. The IFN-β Enhancer Is Nucleosome-Free In Vivo and In Vitro

(A) The positions of the nucleosomes at the IFN-β promoter were mapped by ligation-mediated PCR using the indicated IFN-β primers: Lane 1 corresponds to the boundary of nucleosome II at −15 using primer α; lane 2 corresponds to the boundary of nucleosome II at +132 using primer β; lane 3 corresponds to the boundary of nucleosome I at −118 using primer γ; lane 4 corresponds to the boundary of nucleosome I at −268 using primer δ. The bottom part of the Figure shows a diagrammatic illustration of nucleosome organization at the IFN-β promoter region and the relative location of the primers.

(B) Nucleosome reconstitution was carried out using a restriction fragment spanning the −143 to +183 region of the IFN-β gene (lanes 3 and 4) and the −222 to +113 region from the CMV promoter (lanes 1 and 2). The products were analyzed by EMSA.

(C) IFN-β promoter nucleosome complexes (Figure 1B) labeled at either end were gel purified and incubated with increasing amounts of ExoIII to identify the nucleosome boundaries. The arrowheads depict ExoIII protected nucleosomal DNA.

(D) Assembly of the IFN-β enhanceosome on nucleosomal IFN-β promoter fragments. An IFN-β promoter fragment (−143 to +183) (lanes 1–7) or an identical-sized fragment bearing mutations in all HMG I(Y) binding sites (lanes 8–14) were reconstituted into a nucleosome, gel purified, and used in EMSA experiments along with recombinant IFN-β activators in the presence or in the absence of HMG I(Y). The following amounts of recombinant proteins were used: HMG I(Y) 10 ng, IRF-1 30 ng, NF-κB 20 ng, ATF-2/c-Jun 50 ng. The bottom part of the Figure depicts a diagrammatic illustration of the enhanceosome bound to the IFN-β nucleosomal promoter fragment.

Because the chromatin surrounding the IFN-β promoter is acetylated in vivo (Parekh and Maniatis, 1999) and because the HAT activities of CBP and P/CAF are required for IFN-β transcription (Munshi et al., 1998), we investigated whether enhanceosome-dependent transcriptional activation is directly stimulated by histone acetylation. First, we tested whether acetylation per se is sufficient to remodel nucleosome II. A biotinylated

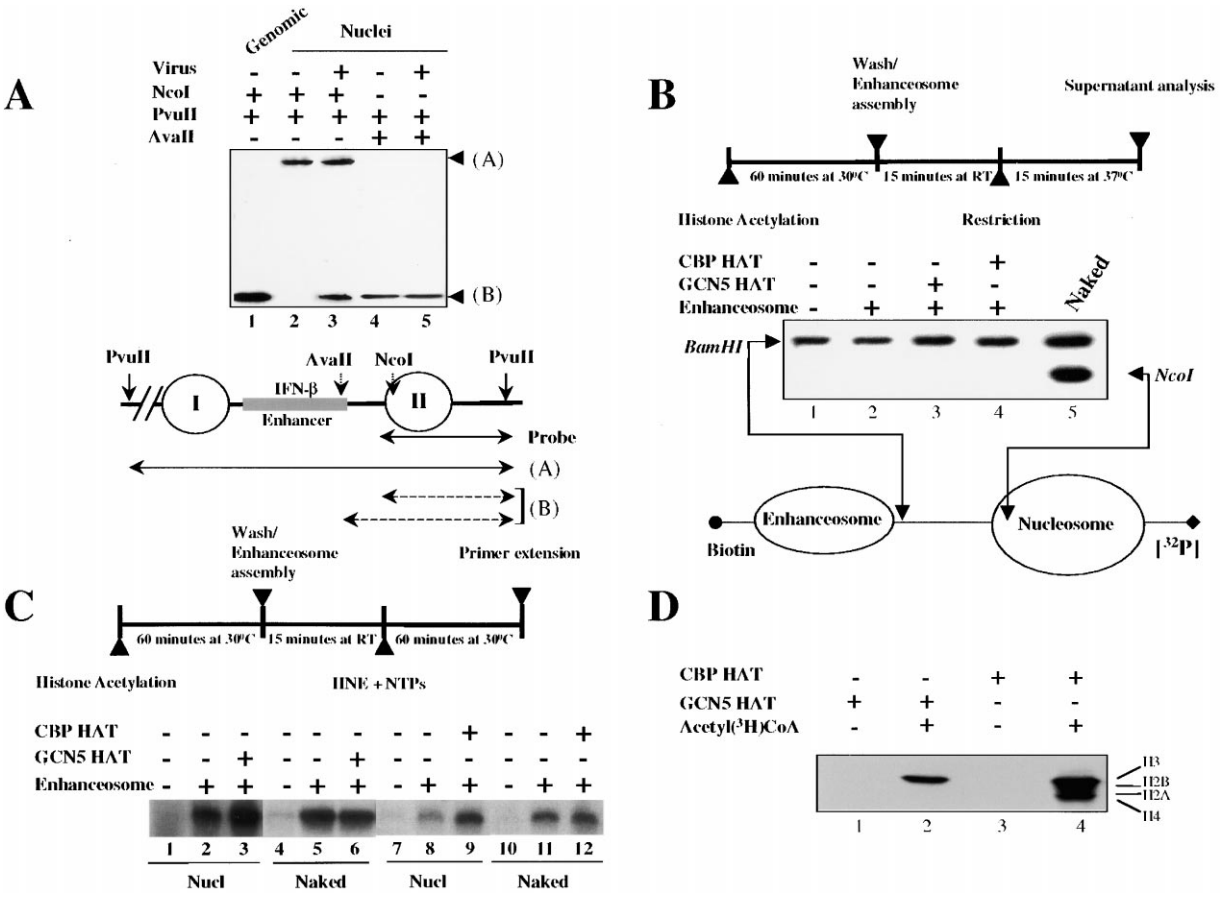

Figure 2. Histone Acetylation Does Not Suffice for Nucleosome Remodeling at the IFN-β Promoter

(A) Virus infection induces nucleosome II remodeling. For determination of nucleosome II remodeling, nuclei isolated from mock-(lanes 2 and 4) or virus-infected cells (lanes 3 and 5) were digested with NcoI or AvaII. The DNA was isolated and cleaved with PvuII before agarose electrophoresis and Southern blotting. The lower part of the Figure, which is not drawn to scale, shows a restriction map of the IFN-β gene locus and the probe used.

(B) The nucleosomal IFN-β promoter fragment (−143 to +183) was radiolabeled at +183 and incubated with either NcoI or BamHI after enhanceosome assembly. After incubation with the restriction enzymes, the beads were concentrated and the DNA in the supernatant was analyzed by PAGE and detected by autoradiography.

(C) HATs facilitate enhanceosome-dependent transcription from chromatin templates. A biotinylated IFN-β promoter fragment (−143 to +183), with or without nucleosome II, was incubated with the indicated HAT proteins in the presence or the absence of Acetyl-CoA. The samples were washed four times to remove the HATs and Acetyl-CoA, and then used as templates for enhanceosome assembly before they were assayed for transcription, as indicated in the schematic representation shown at the top of the Figure.

(D) Same as in (C), but the acetylation reaction was supplemented with ^{3}H labeled Acetyl-CoA before PAGE analysis and detection by autoradiography.

fragment spanning the region from −143 to +183 of the IFN-β gene was radiolabeled at +183 and used as a template for nucleosome reconstitution followed by coupling to Dyna-beads. Next, the nucleosomal template was incubated with GCN5 or CBP HAT domains and Acetyl-CoA, and after washing, it was reacted with NcoI or BamHI. The beads were concentrated and the supernatant was analyzed by PAGE. Figure 2B shows that acetylation per se is not sufficient to alter nucleosome II structure at the IFN-β promoter, as revealed by the inability of NcoI to access its site (compare lanes 2 with 3 and 4). As a control, we show that the BamHI site located between the enhanceosome and the nucleosome is accessible. That histones in nucleosome II are indeed acetylated by the CBP and GCN5 HAT domains is shown in Figure 2D using ^{3}H-Acetyl CoA. Next, we carried out in vitro transcription experiments in which nucleosomal IFN-β templates were either mock or acetylated in vitro by the CBP or GCN5 HAT domains, followed by extensive washes to remove the HATs. Figure 2C (compare lanes 2 and 3) demonstrates that the GCN5 acetylated chromatin templates supported ~5-fold higher amount of transcription when compared to mock-acetylated chromatin, whereas CBP's HAT effect was 2.5-fold (compare lanes 8 and 9). Significantly, no acetylation-dependent increase in transcription was observed from naked DNA templates (lanes 4–6 and 10–12), indicating that the HAT-mediated transcriptional augmentation is nucleosomal template specific.

The fact that histone acetylation per se does not alter nucleosome structure yet facilitates transcription, taken together with our observation that chromatin remodeling takes place during IFN-β gene induction, led us to examine the role of histone acetylation in the recruitment of

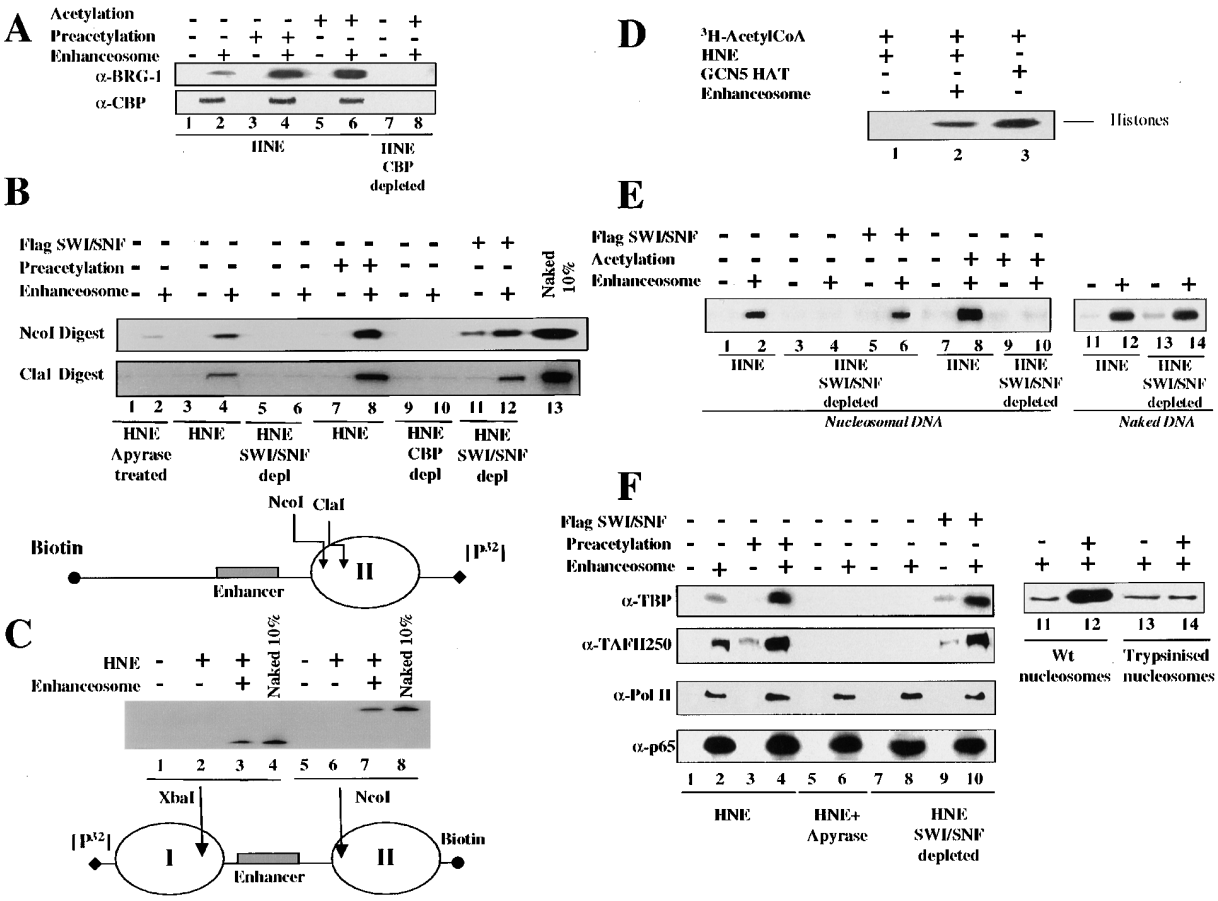

Figure 3. Histone Acetylation-Dependent Recruitment of SWI/SNF and TFIID to the IFN-β Promoter

(A) The biotinylated IFN-β promoter fragment bearing nucleosome II was attached to Dyna-beads and either mock- or GCN5-acetylated. The chromatin template was washed and used for enhanceosome assembly followed by incubation with complete (lanes 1–6) or CBP-depleted (lanes 7 and 8) HeLa nuclear extracts. The reactions were washed four times, and bound proteins were detected by Western blotting using BRG1 and CBP specific antibodies.

(B) Same as in (A) except that a radiolabeled IFN-β promoter fragment was used. Following incubation with complete (lanes 3, 4, 7, and 8), apyrase-treated (lanes 1, 2), SWI/SNF depleted (lanes 5, 6, 11, and 12), or CBP depleted (lanes 11 and 12) HeLa nuclear extracts, the templates were washed and incubated with NcoI or ClaI, followed by PAGE and detection of radioactive released DNA by autoradiography. In lanes 11 and 12, the SWI/SNF depleted extracts were supplemented with Flag-tagged purified SWI/SNF complex. Lane 13 corresponds to digestion with NcoI or ClaI of 10% input naked DNA attached to the beads.

(C) Same as in (B), but the template used for nucleosome assembly and remodeling bears both nucleosomes I and II. Nucleosome I remodeling was assessed by XbaI digestion.

(D) The biotinylated IFN-β nucleosomal template with (lane 2) or without the enhanceosome (lane 1) was incubated with HNE in the presence of 3H-acetyl-CoA followed by extensive washes, PAGE analysis and detection of labeled histones by autoradiography. Lane 3 corresponds to acetylation of nucleosome II by recombinant GCN5.

(E) Same as in (A), except that in vitro transcription was carried out instead of Western blot analysis. In lanes 11–14, the IFN-β promoter fragment used was devoid of nucleosomes (naked).

(F) Same as in (A) but the antibodies used were against TBP, TAFII250, PolII, and p65. In lanes 13 and 14, the nucleosomal template used had been partially proteolyzed with trypsin to remove the histone tails.

remodeling activities at the IFN-β promoter. The biotinylated IFN-β promoter fragment (−143 to +183) bearing nucleosome II with or without the enhanceosome was incubated with the nuclear extract, washed extensively, and Western blotting, using specific antibodies, detected the bound proteins. Figure 3A (lane 2) shows that the enhanceosome recruits the BRG1 component of the SWI/SNF complex or the hBRM protein (not shown). Remarkably, enhanceosomes assembled on nucleosomal templates that had been acetylated by GCN5 (or CBP, data not shown) recruit ~5 times more BRG1, when compared to the non-acetylated counterparts (compare lanes 2 and 4). Enhanced BRG1 recruitment was also observed when acetyl-CoA was added during the incubation period (compare lane 2 with 6). However, recruitment of CBP by the enhanceosome is not affected by histone acetylation (lanes 1–6), because CBP's recruitment mainly depends on the enhanceosome's activating surface (Merika et al., 1998). The enhanceosome's ability to recruit HATs that acetylate nucleosome II was demonstrated by scaling-up (5-fold) the recruitment reaction and supplementing with radioactive Acetyl-CoA (Figure 3D, compare lanes 1 and 2).

To test whether recruitment of SWI/SNF to the promoter is mediated via its association with the enhanceosome or with the CBP-PolII holoenzyme, we used nu-

clear extracts lacking the CBP-PolII holoenzyme in recruitment experiments (Yie et al., 1999). Figure 3A (lanes 7 and 8) demonstrates that the enhanceosome cannot recruit BRG1 in the CBP depleted nuclear extract, indicating that SWI/SNF recruitment requires CBP.

To investigate whether enhanceosome-dependent recruitment of the SWI/SNF complex to the promoter leads to nucleosome remodeling, we repeated the recruitment experiment of Figure 3A using a radiolabeled template followed by NcoI (−10) and ClaI (+20) digestion. The beads were concentrated, and the released fragments were detected by PAGE and autoradiography. As seen in Figure 3B (lane 4), the enhanceosome induces remodeling of nucleosome II in vitro. This remodeling activity depends on ATP (compare lanes 2 and 4), on the SWI/SNF complex (compare lanes 4 and 6) and on CBP (compare lanes 4 and 10). Addition of epitope tagged purified SWI/SNF complex back to the SWI/SNF depleted extracts restored nucleosome remodeling (lanes 11, 12). Interestingly, histone acetylation by GCN5, greatly facilitates enhanceosome-dependent nucleosome remodeling (compare lanes 4 and 8), a result that correlates with enhanced recruitment of SWI/SNF on acetylated nucleosomal templates (Figure 3A). Interestingly, Figure 3C shows that the enhanceosome can direct remodeling of nucleosome I simultaneously to remodeling of nucleosome II. Figure 3E shows that the SWI/SNF complex is critical for enhanceosome-dependent activation of transcription in vitro from chromatin but not from naked DNA templates (compare lanes 2 and 4 with 12 and 14). Taken together, these results demonstrate that there is a perfect correlation between enhanced recruitment of SWI/SNF by histone acetylation, nucleosome remodeling, and strength of transcriptional activation by the IFN-β enhanceosome.

The experiment of Figure 3F demonstrates that the enhanceosome, in contrast to its activity on naked DNA templates (Kim et al., 1998; Yie et al., 1999), actively recruits TBP and $TAF_{II}250$ on nucleosomal templates (compare lanes 1 and 2). Recruitment of TBP/ $TAF_{II}250$ depends on ATP (compare lanes 2 and 6) and on SWI/SNF (compare lane 2 with 8 and 10). Remarkably, histone acetylation further increased enhanceosome-dependent recruitment of TBP/$TAF_{II}250$ (compare lanes 2 and 4). Proteolytic removal of the histone amino-terminal tails abolished acetylation-dependent enhanced TBP recruitment by the enhanceosome (lanes 11–14). Interestingly, recruitment of PolII to the IFN-β enhancer is unaffected by either histone acetylation or nucleosome remodeling (Figure 3F). This result is consistent with our previous demonstration that PolII is recruited to the natural IFN-β promoter primarily via its association with CBP (Yie et al., 1999; see also below).

The Mechanism of Enhanceosome-Induced PIC Assembly at the IFN-β Promoter

The experiments described above suggest that PolII is recruited to the IFN-β promoter independently of TBP, a result that contrasts previous observations where synthetic enhancers and/or activators were used (Ranish et al., 1999). To address this point, we compared recruitment of basal factors to wild-type and mutant TATA box (TGTA) IFN-β promoters. Figure 4A shows that the enhanceosome recruits TBP to the WT (compare lanes 1 and 2) but not to the TGTA promoter (compare lanes 2 and 3), indicating that the observed recruitment of TBP to the IFN-β promoter is sequence-specific. Remarkably, we found that both PolII and TFIIB along with CBP and SWI/SNF are recruited to the TGTA promoter in the absence of stable TBP binding (lanes 1–3). In addition, other known components of the PolII holoenzyme (TFIIE and SRB7) can be efficiently recruited to the TGTA promoter (data not shown). None of these factors, including TBP, can be recruited in the absence of the CBP/PolII holoenzyme (lanes 4 and 5). Thus, the CBP-PolII holoenzyme and the SWI/SNF complexes are recruited to the promoter independently of TBP. That recruitment of PolII is required for subsequent recruitment of TBP was demonstrated in extracts lacking PolII. Figure 4A shows that recruitment of TBP is greatly reduced in the absence of PolII (compare lanes 7 and 9). The fact that BRG1 is efficiently recruited by the enhanceosome in the absence of PolII (lanes 8 and 9), but not in the absence of CBP (lanes 4 and 5), suggests that recruitment of BRG1 is mediated via its interactions with CBP and not with the PolII holoenzyme complex. The NcoI accessibility assay of Figure 4B shows that the SWI/SNF complex that is recruited to the TGTA promoter template can remodel nucleosome II. However, a remodeled nucleosome and the CBP-PolII holoenzyme do not suffice for activation of transcription in the absence of TBP binding (Figure 4C).

Our results suggest that TBP is recruited to the IFN-β promoter subsequent to the CBP-PolII holoenzyme and SWI/SNF complexes. The latter is recruited via its association with CBP and functions by remodeling nucleosome II, thus permitting TBP DNA binding. To investigate whether the order of recruitment of basal factors to the IFN-β promoter is property of the natural enhanceosome as opposed to artificial enhancers, we carried out side by side recruitment experiments using the natural IFN-β enhanceosome and synthetic NF-κB (4 tandem sites) bearing promoters as templates. Figure 4D shows that recruitment of PolII or SWI/SNF by the enhanceosome is mediated via its association with CBP (compare lanes 2 and 4). In sharp contrast, PolII and SWI/SNF are efficiently recruited by the multiple NF-κB molecules independently of CBP (compare lane 6 with 8). Furthermore, although the enhanceosome recruits PolII independently of TBP (Figure 4D, lanes 9–12), the synthetic NF-κB enhancer recruits PolII in a TBP-dependent manner (lanes 13–16). Finally, the observation that enhanceosome-dependent recruitment of TBP and $TAF_{II}250$ depends on PolII, contrasts with the PolII-independent recruitment of TFIID by the synthetic NF-κB complexes (Figure 4D, lanes 17–24). Importantly, a requirement for PolII for stable TBP recruitment has been also demonstrated in endogenous yeast promoters (Kuras and Struhl, 1999; Li et al., 1999). Thus, the mechanism of PIC assembly is distinct between natural and artificial enhancer complexes.

Ordered Recruitment of Histone Acetyltransferases, SWI/SNF and Basal Transcription Factors to the IFN-β Promoter In Vivo

To investigate recruitment of histone acetyltransferases, SWI/SNF and basal transcription factors to the IFN-β

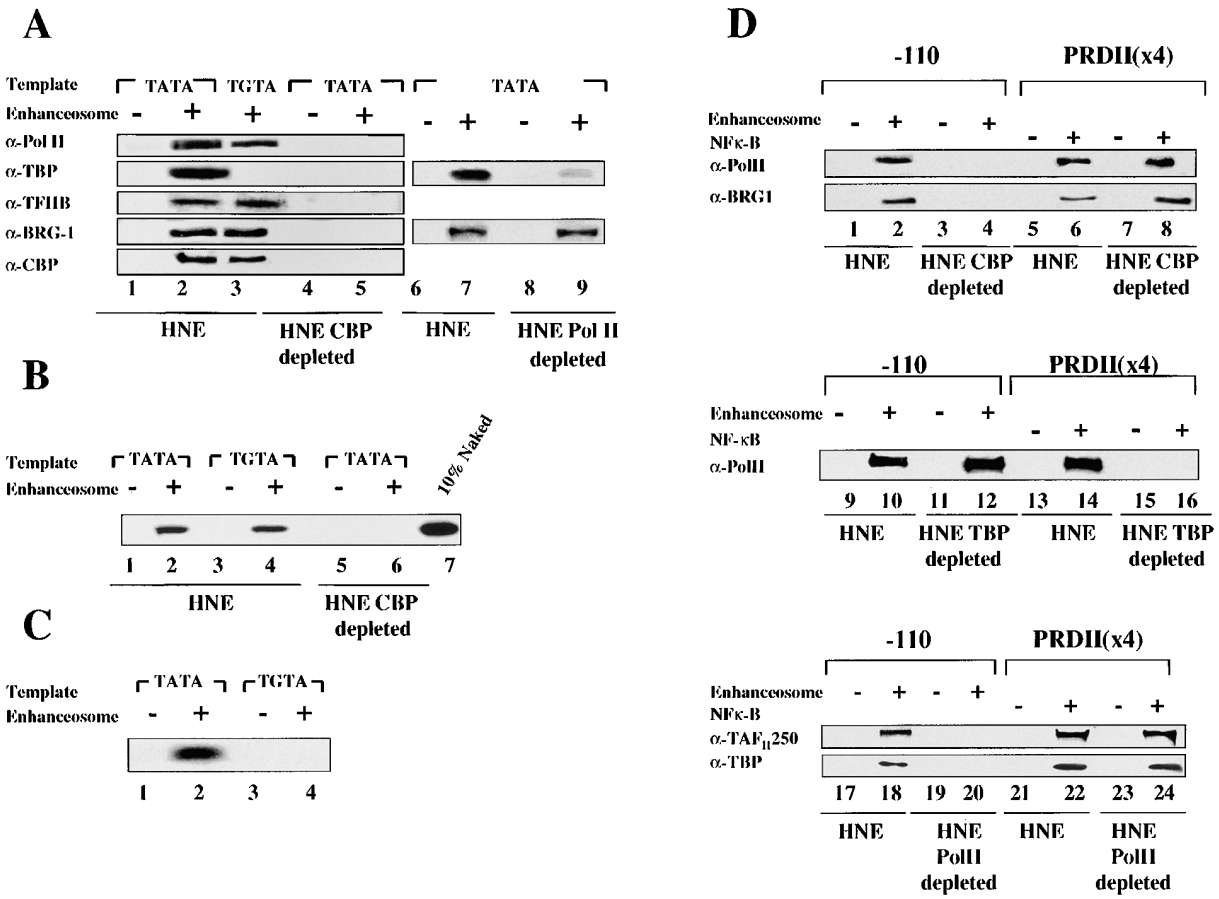

Figure 4. Distinct Mechanisms of PIC Assembly Induced by Natural versus Synthetic Enhancer Complexes

(A) The biotinylated IFN-β nucleosomal promoter fragment bearing a wild-type TATA box or the mutant TGTA sequence with or without the enhanceosome was incubated with either complete HNE or HNEs depleted for the indicated proteins, followed by Western blotting, using the depicted antibodies.

(B) Same as in (A) except that a radioactive template was used, which after extensive washes was incubated with NcoI to assess nucleosome remodeling.

(C) Shown is an in vitro transcription experiment using nucleosomal wild-type or mutant TATA box IFN-β promoter fragments.

(D) The biotinylated nucleosomal IFN-β promoter fragment was used side by side in recruitment experiments with a similar biotinylated fragment containing four tandem NF-κB sites cloned upstream from the IFN-β TATA box.

promoter in vivo, we carried out chromatin immunoprecipitation experiments. HeLa cells were infected with Sendai virus for different amounts of time followed by formaldehyde treatment to cross-link protein-protein and protein-DNA complexes, followed by precipitation using specific antibodies against p65, GCN5, TBP, acetyl Histone 4, BRG1, PolII, TBP, and $TAF_{II}250$. The IFN-β promoter fragment in the immunoprecipitates was quantified using PCR. The time course of p65's association with the IFN-β enhancer in vivo shows that p65 is recruited to the enhancer at 2 hr following virus infection and remains relatively stably bound to the enhancer until 19–24 hr post infection where it declines (Figure 5A, top panel). The bottom panel of Figure 5A depicts the abundance of IFN-β mRNA during the time course of virus infection. IFN-β mRNA is first detected at 6 hr post infection, peaks at 9–19 hr and then is down regulated. Comparison of p65 recruitment with IFN-β mRNA abundance suggests that post-induction repression of IFN-β transcription correlates with detachment of enhanceosome components from the enhancer (enhanceosome disassembly), thus verifying our previous observations (Munshi et al., 1998).

Figure 5B shows that the enhanceosome recruits the GCN5 acetyltransferase in vivo, a result consistent with in vitro recruitment experiments (Munshi et al., personal communication). Our results also show that GCN5 recruitment is first seen at 3 hr post infection, peaks at 4–5 hr and then rapidly declines. This transient recruitment is not due to epitope masking of the antibody used, because similar patterns of GCN5 recruitment were obtained using two different GCN5-specific antibodies (data not shown). Figure 5B (bottom panel) shows that recruitment of GCN5 is followed by an increase in targeted histone acetylation at the IFN-β promoter. Again, histone acetylation is transient and declines after GCN5 departs from the enhanceosome. Interestingly, maximal histone acetylation coincides with the appearance of the first IFN-β transcripts (compare 6 hr time point between Figures 5A and 5B). Importantly, when histone acetylation was examined using antibodies that distinguish between the GCN5 and CBP targeted lysines, we found

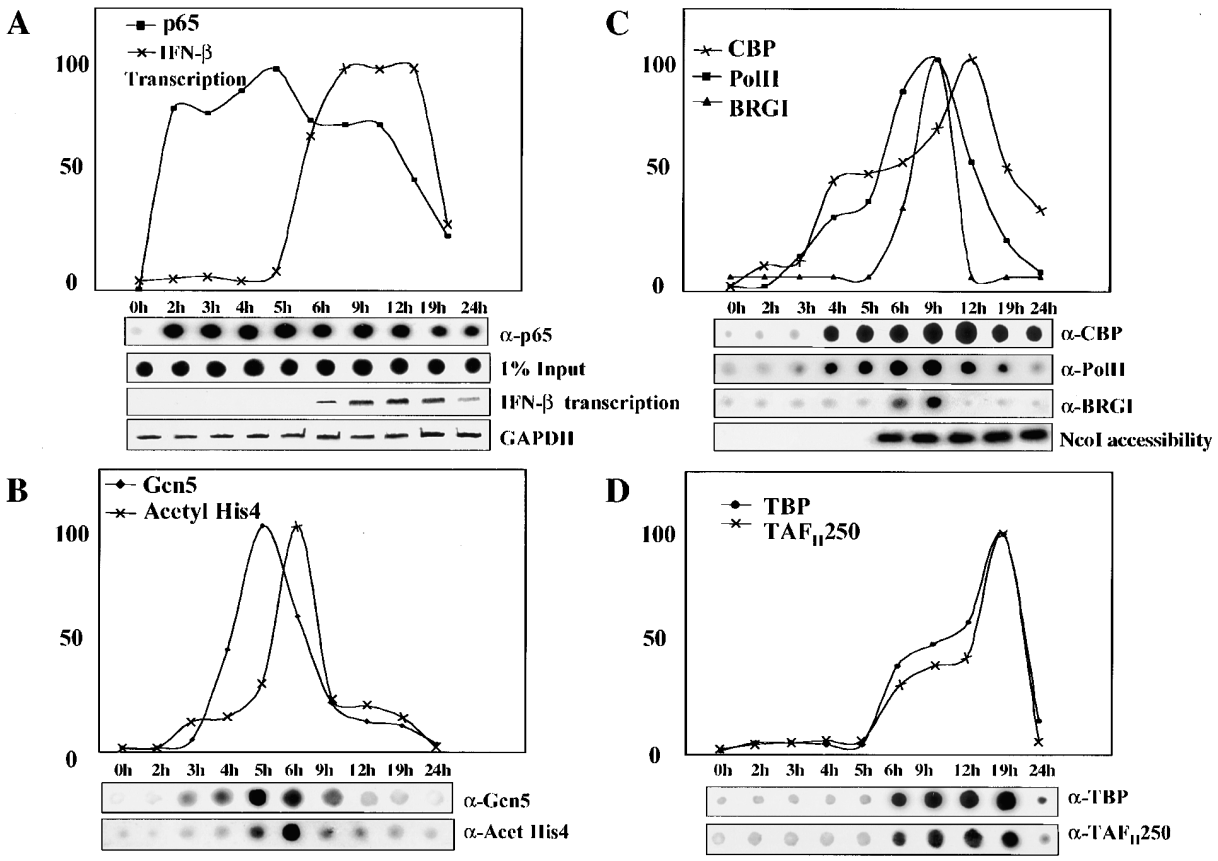

Figure 5. The IFN-β Enhanceosome Directs Ordered Recruitment of Histone acetyltransferases, SWI/SNF, and general transcription factors to the IFN-β promoter in vivo. The immunoprecipitations were repeated two times for p65, three times for hGCN5, three times for acetyl-Histone 4, three times for CBP, two times for PolII. two times for hBRG1, three times for TBP and two times for $TAF_{II}250$. Shown are the results of one immunoprecipitation using all antibodies. The variability from experiment to experiment was small with respect to the kinetics of recruitment of individual factors.

(A) HeLa cells were either mock- or virus-infected with Sendai virus for the indicated amounts of time. Cross-linked chromatin was immunoprecipitated with a p65 antibody and the IFN-β promoter was detected by dot blot hybridization of partially amplified PCR products. The bottom part of the Figure shows the abundance of the IFN-β mRNA as detected by RT-PCR, along with the abundance of GAPDH mRNA as a control. The radioactive bands were quantitated with a phosphorimager and, after subtracting the background the net values, were plotted as percentage of factor recruitment or transcription and correspond to the highest amount of recruitment or transcription obtained at one of the time points. For example, in the case of p65, 100% recruitment is the amount of IFN-β promoter recovered at the 6 hr time point.

(B) Same as in (A), but the antibodies used were against hGCN5 or acetyl-Histone 4.

(C) Same as in (A), but the antibodies were against the large subunit of PolII, CBP or hBRG1. The bottom part of the Figure depicts nucleosome II remodeling as judged by NcoI accessibility (see Figure 2A).

(D) Same as in (A) but the antibodies used were against TBP or $TAF_{II}250$.

that the overall pattern of acetylation correlates, with GCN5 being the primary histone acetyltransferase (data not shown). The latter point was also illustrated when we measured CBP recruitment to the IFN-β promoter. As seen in Figure 5C, shortly after virus infection, the association of CBP with the IFN-β enhanceosome is gradually increased, reaching a maximum at 9–12 hr post infection. However, CBP's presence to the promoter is decreased toward the end of the time course. Furthermore, we found that there is little correlation between the peaks of histone acetylation and CBP recruitment. Maximum CBP recruitment occurs at time points (9–12 hr) where the overall histone acetylation at the IFN-β promoter declines. Thus, CBP might contribute to the residual histone acetylation observed at these time points (our unpublished results).

We then examined the pattern of PolII and BRG1 recruitment to the IFN-β promoter. As seen in Figure 5C, the time course of PolII recruitment correlates with CBP's recruitment, a result consistent with our in vitro experiments, suggesting that the enhanceosome recruits the CBP-PolII holoenzyme complex as a unit. Remarkably, BRG1 is recruited to the enhancer only for a very short period of time (6–9 hr time points), and its recruitment perfectly correlates with remodeling of nucleosome II (Figure 5C, bottom panel). Importantly, the nucleosome remains in the remodeled state, even after BRG1 leaves the promoter, a result consistent with the dispensable role of SWI/SNF in maintaining a stable remodeled state of chromatin in vitro (Imbalzano et al., 1996). Taken together, these experiments demonstrate that CBP, PolII and SWI/SNF are coordinately recruited to the IFN-β enhancer in vivo, thus verifying our in vitro experiments.

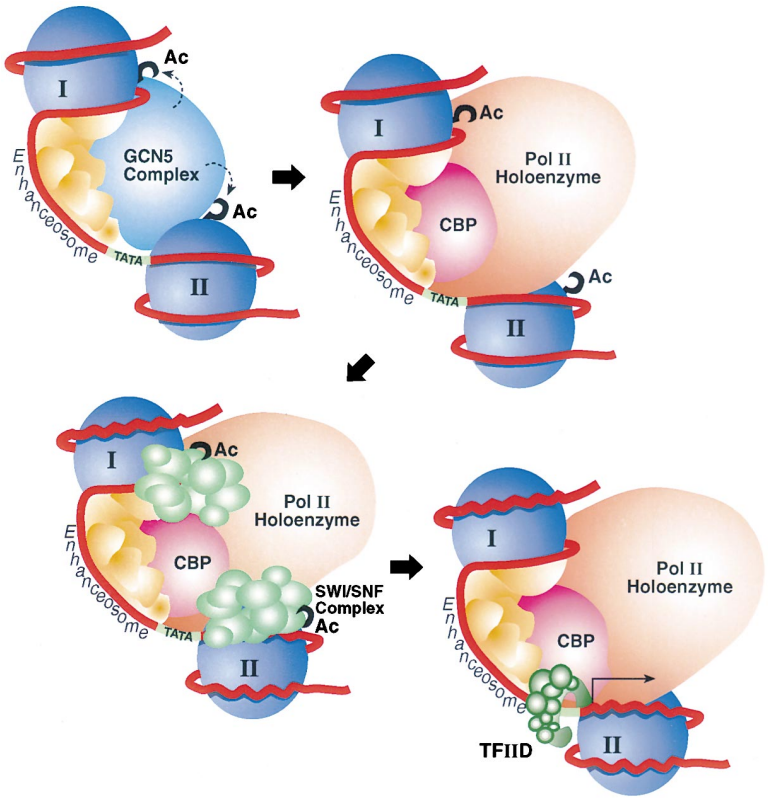

Figure 6. Model Depicting the Ordered Recruitment of Chromatin-Modifying and Basal Factors to the IFN-β Promoter

Shortly after virus infection, the enhanceosome assembles on the nucleosome-free IFN-β enhancer and recruits the GCN5 complex, which acetylates nucleosomes I and II (acetylated histone N-termini are shown as hooks). GCN5 departs from the promoter and the CBP-PolII holoenzyme complex is recruited by the enhanceosome. Next, SWI/SNF associates with the promoter via its interactions with CBP. This recruitment is stabilized by the acetylated histone N-termini, which presumably interact with the bromodomain of BRG1/BRM proteins present in the SWI/SNF complex. SWI/SNF remodels the nucleosomes (DNA shown as ruffled lines), thus allowing recruitment of TFIID, completion of preinitiation complex assembly at the core promoter and initiation of transcription (arrow).

The profile of TBP and $TAF_{II}250$ recruitment to the IFN-β promoter is shown in Figure 5D. We found that TBP and $TAF_{II}250$ (TFIID) are not recruited to the promoter during the first 5 hr of virus infection, despite the presence of CBP, PolII, GCN5 and acetylated chromatin. TFIID is first seen at 6 hr post infection, that is at the time where nucleosome II has been remodeled, with maximal association occurring at 9–19 hr. Taken together, our chromatin immunoprecipitation experiments suggest that recruitment of TBP to the IFN-β promoter by the enhanceosome occurs at a step following the engagement of histone acetyltransferases, PolII and SWI/SNF to the promoter.

Discussion

We have examined the order of events occurring on a natural enhancer/promoter in vivo and in vitro during signal-dependent transcriptional activation. Following virus infection, the IFN-β enhanceosome is assembled on the nucleosome-free enhancer region of the IFN-β gene and functions by targeting a specifically positioned nucleosome (nucleosome II) that masks the TATA box and the start site of transcription. We revealed an enhanceosome-dependent cascade of recruitment events (Figure 6) that begin with the recruitment of the GCN5 complex, which acetylates nucleosome II, followed immediately by recruitment of the CBP/PolII holoenzyme complex. Next, the SWI/SNF complex, which functions by remodeling nucleosome II, is recruited by CBP. The nucleosome remodeling appears to require prior histone acetylation of the nucleosome. This cascade of enhanceosome-dependent recruitment events culminates with the entrance of TFIID to the complex, and the activation of transcription.

A Functional Linkage between Histone Acetylation and Chromatin Remodeling at the IFN-β Promoter

Recently, it was proposed that covalent modification of histones might provide a molecular code for the specific recruitment of transcription factors and coactivators to the promoter (Strahl and Allis, 2000). Indeed, we found that histone acetylation per se does not suffice for chromatin remodeling at the IFN-β promoter but rather is a signal for enhanceosome-dependent recruitment of SWI/SNF by CBP. Thus, recruitment of CBP by the enhanceosome not only installs the PolII holoenzyme complex at the promoter but also leads to recruitment of SWI/SNF. By comparing the timing of recruitment of GCN5, CBP, and SWI/SNF with the overall pattern of histone acetylation and chromatin remodeling we have been able to provide a nearly complete picture of the functional interplay between histone acetylation and chromatin remodeling at the IFN-β promoter (Figure 6). Association of the GCN5 complex with the IFN-β promoter reaches its maximum at 4 and 5 hr post-infection, at which time it dissociates. GCN5 recruitment correlates with an increase in histone acetylation, which precedes optimal CBP recruitment (6 vs. 12 hr, respectively). Importantly, although low levels of CBP has been recruited to the promoter at the time of peak histone acetylation, the overall pattern of histone acetylation correlates better with the recruitment of GCN5 at these

early time points. The early association of GCN5 has an additional consequence on enhanceosome function. GCN5 acetylates HMG I(Y) at lysine 71 resulting in a strengthening of the HMG I(Y)-mediated protein-protein interactions within the enhanceosome (Munshi et al. personal communications). Significantly, GCN5-acetylated HMG I(Y) cannot be acetylated by incoming CBP, and therefore the enhanceosome is protected from the CBP-mediated HMG I(Y) acetylation disassembly (Munshi et al., 1998).

When histone acetylation of nucleosome II reaches its peak, the BRG1 component of the SWI/SNF complex associates with the promoter. In parallel, chromatin remodeling at nucleosome II takes place, and the first transcripts of the IFN-β gene are synthesized. Similar to GCN5, BRG1 associates with the promoter for a limited amount of time, approximately 3 hr. However, despite SWI/SNF's disassociation from the promoter, nucleosome II remains remodeled throughout the time course, even at times when transcription has ceased. Thus, a continuous presence of SWI/SNF is not required for maintenance of nucleosome remodeling.

Our experiments suggest that acetylated chromatin is the preferred substrate for SWI/SNF recruitment, at least at the IFN-β promoter. The fact that BRG1 bears a bromodomain, taken together with the recent demonstration that bromodomains can interact with acetylated histone N-termini (Dhalluin et al. 1999; Jacobson et al., 2000), suggests that histone acetylation provides a new higher affinity surface for interaction with BRG1, thus leading to more stable nucleosomal binding. The interdependence between histone acetylation and SWI/SNF recruitment or chromatin remodeling at the IFN-β promoter is exactly opposite to the order recruitment at the yeast HO promoter (Cosma et al., 1999; Krebs et al., 1999). Thus, the particular order by which these chromatin-modifying activities are recruited and/or function to allow gene activation appears to be gene-specific. That is, it depends on the exact chromatin structure and on the unique combination of transcription factors bound to each promoter.

Mechanism of PIC Assembly by Natural and Artificial Enhancer Complexes

PIC formation requires the assembly of an astonishingly large number of proteins whose function is to ensure accurate initiation of transcription (Orphanides et al., 1996). Our in vitro recruitment experiments using the IFN-β enhanceosome revealed a novel pathway of PIC assembly, as opposed to the pathway operating in cases where synthetic enhancer complexes were tested (Ranish et al., 1999). We showed that the enhanceosome recruits the CBP-PolII holoenzyme complex, TFIIB, and chromatin remodeling activities in vitro in the absence of stable TFIID binding. The biological significance of these in vitro recruitment experiments is underscored by our chromatin immunoprecipitation experiments that showed that maximal TFIID recruitment to the promoter in vivo takes place after recruitment of PolII. In sharp contrast, we demonstrated that artificial promoters composed of multiple NF-κB binding sites do recruit PolII in the absence of CBP, and that in this case recruitment of PolII depends on TFIID, a result consistent with a previous report (Ranish et al., 1999). Another difference between the enhanceosome and synthetic NF-κB enhancer complexes is that the former recruits SWI/SNF via CBP, whereas the later utilize a CBP-independent pathway to recruit SWI/SNF, presumably via direct interactions between NF-κB and SWI/SNF, similar to what has been previously described in other synthetic promoters (Vignali et al., 2000). Strikingly, this unorthodox pathway of enhanceosome-induced PIC assembly in vitro or in vivo agrees with the fact that TBP cannot be recruited in the absence of PolII holoenzyme components on endogenous yeast promoters (Kuras and Struhl, 1999; Li et al., 1999).

An interesting question arising from these studies is what determines the mechanism of PIC assembly on natural versus artificial promoters. We propose here that, at least in the IFN-β enhanceosome case, the difference resides in the properties of the transcriptional activating surface created by enhanceosome assembly. Thus, the enhanceosome presents a unique continuous surface assembled from each one of the activation domains interacting at multiple points with the complementary surface on CBP (Merika et al. 1998). This interaction is of unusually high affinity and specificity, thus enabling the enhanceosome to recruit efficiently the low abundance CBP/PolII holoenzyme complex to the promoter. Maximal recruitment of CBP-PolII coincides with the association of the SWI/SNF complex, which remodels nucleosome II, thus allowing the binding of TFIID at the promoter. However, we do not know whether TFIID is recruited via its direct interactions with the enhanceosome or indirectly via its interactions with some of the already present general transcription factors. As opposed to the IFN-β enhanceosome, the uniform nature of the activating surface presented by synthetic enhancer complexes appears to result in the formation of a complex of intrinsically low-affinity and specificity, a fact consistent with numerous previous studies (Triezemberg, 1995). We speculate that functional PICs form on synthetic promoters only when TFIID is recruited first, because the weak activator-TFIID interactions are stabilized by the subsequent interaction of TFIID with promoter DNA, thus providing the binding energy needed for the subsequent recruitment of the rest of the general transcription factors.

Experimental Procedures

Donor Chromatin Preparation, Nucleosome Reconstitution, and In Vivo and In Vitro Nucleosome Mapping

Nucleosome reconstitutions were carried out as described previously (Utley et al., 1996). The nucleosome borders were determined by limited ExoIII digestion as previously described (Hamiche et al., 1999). LMPCR was performed exactly as described in Fragoso et al., 1995

Immunodepletions and Western Blot Analysis

We incubated 600 μl (6 mg/ml) of HeLa nuclear extract (HNE) with 20 μg of each antibody for 1 hr at 4°C, followed by addition of protein A/G agarose beads (Boehringer) in BC100 containing 5% BSA and 0.01% Triton. The beads were precipitated and washed three times in BC100. Western blots were performed as previously described using commercially available antibodies (Santa Cruz).

Immobilization of the IFN-β Nucleosomal Templates to Dynabeads, Enhanceosome Assembly, and In Vitro Transcription

Biotinylated templates were generated by PCR using the appropriate set of primers. Two pmols of the PCR product were coupled to 100 μg of streptavidin Dyna-beads (Dynal) as previously described (Yie et al. 1999). 0.5μg of donor chromatin/pmol of DNA was used for nucleosome reconstitution. The immobilized nucleosomal DNA was precleared and 10 pmols of NF-κB, ATF-2/c-Jun, and IRF-1 and 5 pmols of HMG I were incubated with the conjugated DNA for 30 min at RT. Unbound proteins were removed, 60 μg of HeLa nuclear extract were added, and the mixture was incubated for 1 hr at 30°C. ATP was added at 4mM as necessary. The Dynabeads were washed three times and the bound proteins were detected by Western blot. When we used preacetylated templates, the nucleosomal DNA was incubated with recombinant CBP or GCN5 for 1hr at 30°C as previously described (Munshi et al., 1998). Restriction site accessibility assays on radiolabeled nucleosomal DNA template were carried out by incubating the complexes with 50 unit/ml of the appropriate restriction enzyme for 15 min at 37°C. The in vivo restriction accessibility experiments were performed as previously described (Archer et al., 1991).

In vitro transcription experiments using immobilized templates were carried out as previously described (Yie et al., 1999). Chromatin immunoprecipitation experiments were carried out as previously described (Parekh and Maniatis, 1999). Partially amplified products were spotted on nylon filters and hybridized with a radiolabeled IFN-β promoter probe. The antibodies used were obtained from Santa Cruz (p65, TBP, $TAF_{II}250$, GCN5, PolII, CBP) and Upstate Biotechnology (anti-Acetyl Histone 4). The Taq polymerase used was obtained from Eppendorf.

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