Mechanism by which the IFN-β enhanceosome activates transcription

Junming Yie, Kate Senger, and Dimitris Thanos*

Department of Biochemistry and Molecular Biophysics, Columbia University, 630 West 168th Street, New York, NY 10032

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We demonstrate that in contrast to previous findings by using simple synthetic promoters or activators, the natural IFN-β enhanceosome activates transcription by causing a dramatic increase of the rate by which preinitiation complexes assemble at the promoter. This effect totally depends on the recruitment of the CBP-PolII holoenzyme by the enhanceosome, because its depletion from the extract decelerates the rate of transcription. However, addition of the CBP-PolII holoenzyme back to these extracts fully restores the speed by which the enhanceosome activates transcription. Strikingly, preincubation of the enhanceosome with the CBP-RNA PolII holoenzyme complex results in instant assembly of preinitiation complexes. In contrast, individual IFN-β gene activators function solely by increasing the number of functional preinitiation complexes and not the rate of their assembly. Thus, fast recruitment of the CBP-RNA PolII holoenzyme complex is critical for the rapid activation of IFN-β gene expression by virus infection.

Enhanceosomes are higher-order multicomponent transcription factor–enhancer complexes whose signal-dependent assembly provides the molecular basis for the gathering, integration, and interpretation of the environmental changes detected by the cells as a modulation in gene activity. The distinct and complex architecture of enhancers and promoters provides virtually unlimited possibilities for the assembly of unique enhanceosomes in response to distinct extracellular signals. Our current view postulates that cells accomplish this by employing the principles of cooperativity and transcriptional synergy (reviewed in ref. 1). The assembly and disassembly of the IFN-β enhanceosome in response to virus infection is one of the best-characterized examples of such combinatorial strategies in switching on and off gene expression in mammals (reviewed in refs. 2 and 3).

The IFN-β gene enhancer is recognized by three separate activators, NF-κB, interferon regulatory factors (IRFs), and ATF-2/c-Jun, which, together with the architectural protein HMG I(Y), bind DNA cooperatively to form the enhanceosome (2, 3). The first step in enhanceosome assembly is the HMG I(Y)-dependent recruitment of the activators to the DNA followed by the establishment of multiple protein–protein interactions occurring between the activators and HMG I(Y) (4–10). The inherent cooperativity of enhanceosome assembly not only facilitates activator DNA binding but also positions them to create a novel activating surface, the enhanceosome "pocket," that optimally interacts with and recruits the basal machinery via a precise alignment of contacts (11). The primary target of this surface is the transcriptional coactivator CBP/p300, along with its associated proteins (11). The enhanceosome also interacts with TFIID, TFIIA, and the USA cofactor in a way that permits synergistic recruitment of TFIIB to the promoter (12). Access of the basal machinery to the DNA may be facilitated by the histone acetyltransferase activities of CREB-binding protein (CBP) and p300/CBP-associated factor (P/CAF) via acetylation of histones in nearby nucleosomes (13, 14). However, both CBP and P/CAF can also acetylate HMG I(Y) at distinct lysine residues, causing opposite effects on IFN-β gene expression. Acetylation of HMG I(Y) by CBP, but not by P/CAF, results in a decrease in its DNA-binding activity and subsequent detachment from the enhanceosome, thus causing enhanceosome disruption and termination of IFN-β gene transcription (13).

The mechanisms by which activator proteins stimulate transcription have been studied almost exclusively by using simple synthetic promoters and hybrid activators. Taken together, these studies led to the realization that activators work by recruitment, that is, by helping the basal transcriptional machinery bind stably to a nearby promoter (reviewed in ref. 15). Synergizing activators must act in the same pathways or in pathways that merge to stimulate the rate of preinitiation complex (PIC) assembly, thus leading to an increased production of transcripts from a single DNA template (reviewed in ref. 16). However, a series of kinetic *in vitro* transcription experiments by using several activators and nuclear extracts have failed to demonstrate an increase in the rate of PIC formation (17–25). Instead, these studies showed that the activators increased the number of DNA templates on which functional PICs are assembled. This observation, taken together with *in vivo* experiments in which single-cell expression was analyzed, led to the alternative hypothesis that enhancers work by increasing the probability of expression and not the rate of transcription from a given template (26). In this paper, we examined the mechanism by which the natural IFN-β enhanceosome activates transcription *in vitro*. We find that in contrast to all previous experiments in which simple synthetic promoters or activators were used, the enhanceosome stimulates transcription by increasing the rate of PIC formation. This kinetic synergism depends on recruitment of the CBP-PolII holoenzyme to the promoter by the enhanceosome, because its removal from the extract decelerates the rate of transcription. Addition of a CBP-PolII holoenzyme complex back to these extracts fully restores the speed by which the enhanceosome activates transcription. In addition, we demonstrate that preincubation of the enhanceosome with the CBP-RNA PolII holoenzyme complex dramatically accelerates the rate of PIC formation. In contrast, we show that each of the IFN-β gene activators alone functions solely by increasing the number of functional PICs and not the rate of PIC assembly. In this case, CBP functions as a classical coactivator of transcription. Finally, we show that the role of CBP in enhanceosome-dependent transcription *in vitro* is to serve as a scaffold on which the PolII holoenzyme is anchored.

Materials and Methods

CBP/p300 Immunoprecipitation and Western Blot Analysis. We incubated 600 μl (6 mg/ml) HeLa nuclear extract (HNE) with 10 μg CBP and 10 μg p300 antibodies (rabbit polyclonal; Santa Cruz Biotechnology) for 1 hr at 4°C. Protein A agarose beads (Boehringer Mannheim) were washed three times in BC100 followed by incubation in BC100 containing 5% BSA and 0.01% Triton for 1 hr at 4°C. Next, the protein A agarose was added to the

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Abbreviations: PIC, preinitiation complex; HNE, HeLa nuclear extract CBP, CREB-binding protein; IRF, interferon regulatory factor.

*To whom reprint requests should be addressed. E-mail: dt73@columbia.edu.

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antibody–HNE mixture and incubated overnight at 4°C. The agarose was precipitated and washed three times with BC100. Western blots were performed as described previously by using commercially available antibodies (Santa Cruz Biotechnology). The Srb7 antibody has been described previously (27).

Immobilization of the IFN-β Enhancer to Dynabeads. Two picomoles of biotinylated IFN-β enhancer DNA (−105 to −40) was coupled to 100 μg Dynabeads M-280 streptavidin (Dynal) according to the manufacturer's protocol. A magnetic particle concentrator (MPC) removed uncoupled DNA. The DNA-harboring beads were blocked to avoid nonspecific binding in BC100 buffer containing 5% BSA and 0.01% Triton for 1 hr at 4°C. Ten picomoles of each activator (NF-κB, ATF-2/c-Jun, and IRF-1) and 20 picomoles of HMG I were incubated with the conjugated DNA for 2 hr at 4°C. Unbound proteins were removed by using MPC. Then we added 500 μg HNE in 400 μl of blocking buffer, and the mixture was incubated for 3 hr at 4°C. The Dynabeads were washed three times and the bound proteins were detected by Western blot.

***In Vitro* Transcription.** *In vitro* transcription was carried out as described before (8). The transcription signal for each time point (after subtracting the background signal obtained at time 0) was plotted as a function of time. The half-time of PIC assembly then was calculated by using GraphPad PRISM software (GraphPad, San Diego). For the experiment shown in Fig. 3, the biotinylated IFN-β CAT template was generated by PCR by using the −110 CAT plasmid along with biotinylated T7 primer and a CAT primer. Forty nanograms of the PCR fragment was immobilized to Dynabeads and used for enhanceosome assembly (100 ng of NF-κB, ATF-2/c-Jun, and IRF-1 and 400 ng HMG I) as described before (8). Next, 10 μl of 8 mg/ml HNE in 7.5 mM $MgCl_2$ was added and the mixture was incubated for 30 min at 30°C. The pellet was collected by using the magnetic particle concentrator and washed three times in 100 μl transcription buffer (12 mM Hepes, pH 8.0/12% glycerol/60 mM KCl/0.12 mM EDTA/7.5 mM $MgCl_2$/0.5 mM DTT/0.5 mM PMSF). This pellet was resuspended in 40 μl of transcription buffer plus 1 μl 25 mM NTPs and incubated for 30 min at 30°C with occasional agitation. Reactions were terminated as described before (8).

Results

The IFN-β Enhanceosome Stimulates the Rate of Preinitiation Complex Formation. To examine the effect of the IFN-β enhanceosome on PIC assembly, we carried out *in vitro* transcription experiments in which we monitored the rate of functional PIC assembly formed at the core promoter either in the absence or presence of the enhanceosome. The IFN-β enhanceosome was incubated with a HeLa nuclear extract for varying lengths of time to allow PIC formation followed by the addition of NTPs to initiate transcription. Thirty seconds after the addition of NTPs, sarcosyl (0.05%) was added to prevent formation of new PICs and reinitiation of transcription. In separate titration experiments we showed that this amount of sarcosyl completely inhibited PIC formation (data not shown). Thus, under these conditions (single-round transcription experiments), the amount of transcription observed corresponds to the number of functional PICs formed during the incubation time. Fig. 1*A* shows that under basal conditions, PICs are formed at the IFN-β promoter with a half-time of 25 min. This value is similar to those reported for other *in vitro* transcription systems (28–30). Remarkably, the half-time of PIC assembly in the presence of the IFN-β enhanceosome is only 7 min (Fig. 1*A*). Thus, in contrast to all previous experiments in which artificial activators and/or templates were used to assess their effects on transcription *in vitro*, the natural transcriptional activating complex assembled on the IFN-β gene enhancer increases the rate of PIC formation.

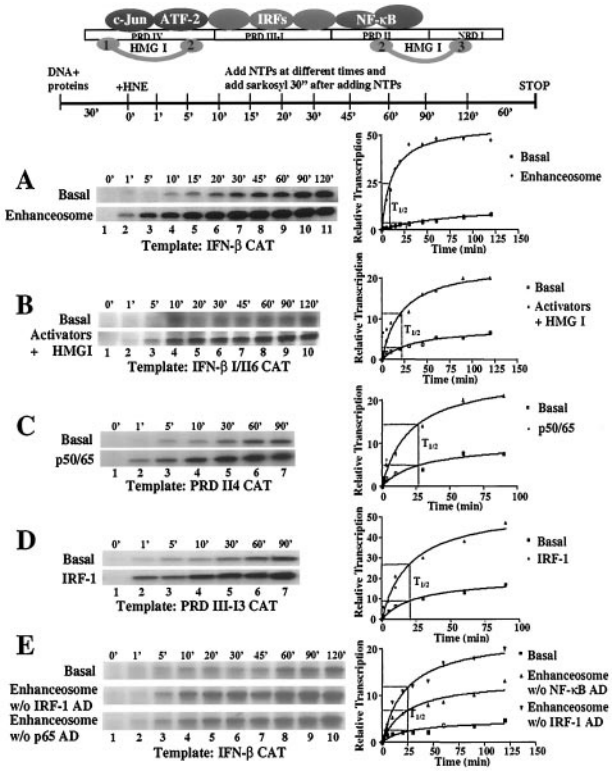

Fig. 1. The IFN-β enhanceosome stimulates the rate of PIC assembly. (*A*) The IFN-β enhanceosome was assembled on the wild-type enhancer (see *Materials and Methods*) and was incubated with a HNE for the indicated amounts of time shown at the top part of the figure. Sarcosyl and NTPs were added, and the incubations were continued for an additional 60 min. Transcript levels were detected by primer extension, quantitated by PhosphorImager, and used as a measure of PIC formation. The right part of the figure shows the relative levels of transcription plotted as a function of time along with a best-fit curve as described in *Materials and Methods*. Shown is one of six independent experiments. The variability from experiment to experiment for the half-time of PIC formation was <8% for the basal level and <10% for activated transcription. (*B*) Same as in *A* but the template contains half-helical DNA insertion between PRDI and PRDII (IFN-β I/II6 CAT). Shown is one of three independent experiments. The variability from experiment to experiment was <12% for the basal and activated transcription. (*C*) Same as in *A* but the template contains four copies of the PRDII element cloned upstream of the IFN-β TATA box. The activator used was NF-κB. Shown is one of three independent experiments, and the variability was <10% and <15% for basal and activated transcription, respectively. (*D*) Same as in *A* but the template contains four copies of the PRDIII-I element cloned upstream of the IFN-β TATA box. The activator used was IRF-1. Shown is one of two independent experiments, and the variability was <7% and 9% for basal and activated transcription, respectively. (*E*) Same as in *A* but the enhanceosome used at *Middle* lacks IRF-1's activation domain, whereas that used at *Bottom* lacks p65's activation domain. Shown is one of two independent experiments, and the variability was <8%, <10%, and 7%, respectively.

To investigate whether this effect is due to the ability of the enhanceosome to function as a unit or could be attributed to any of its components working independently, we carried out similar kinetic experiments by using an IFN-β enhancer in which a half-helical turn was inserted between PRDI and PRDII. This mutation was shown previously to significantly decrease transcription from the IFN-β promoter because it prevents enhanceosome assembly (8–10). Fig. 1*B* shows that when the same IFN-β gene activators are allowed to bind to this mutant enhancer, they promote PIC assembly as slowly as the basal promoter does. Thus, the specific, three-dimensional structure of the enhanceosome is critical for establishing the speed of PIC formation at the IFN-β promoter. Additional evidence for the

unique role of the enhanceosome on the rate of functional PIC assembly was provided in similar experiments in which we used artificial reporters bearing multiple NF-κB- and IRF-1-binding sites. As seen in Fig. 1*C*, NF-κB or IRF-1 alone did not enhance the rate of PIC formation from these artificial promoters. Furthermore, fusion of either NF-κB or IRF-1 activation domains to the DNA-binding domain (amino acids 1–94) of the yeast protein GAL4 also did not enhance the rate of PIC formation (data not shown). The final evidence for the unique role of the activating surface presented by the enhanceosome in determining the rate of PIC formation is shown in Fig. 1*E*. Deletion of either the IRF-1 (Fig. 1*E Middle*) or p65 (Fig. 1*E Bottom*) activation domains in the context of an otherwise intact enhanceosome slows down the rate of PIC formation.

Importantly, the early saturation of transcription observed in the presence of the enhanceosome (Fig. 1*A*) is not due to titration of general transcription factors but to the efficient recruitment of the low-abundance CBP-PolII holoenzyme complex to the promoter (see below). We showed that addition of fresh HNE depleted of CBP-PolII holoenzyme to the transcription reactions at the saturation points did not further increase activated transcription but did increase basal transcription (data not shown). However, addition of fresh, nondepleted HNE at the same time points increased both activated and basal transcription (data not shown).

Recruitment of a CBP-PolII Holoenzyme Complex by the Enhanceosome Suffices for the Increased Rate of PIC Assembly at the IFN-β Promoter. We have shown previously that assembly of the enhanceosome creates a specific activating surface that recruits the CBP/p300 coactivator (11). Previous studies also have demonstrated that CBP is an integral part of the mammalian RNA PolII holoenzyme (27, 31). Therefore, we tested whether the highly specific interaction of CBP with the IFN-β enhanceosome results in simultaneous recruitment of the PolII holoenzyme by virtue of their association. The enhanceosome was assembled on a biotinylated IFN-β enhancer oligonucleotide (−105 to −40) that lacks core promoter sequences and was immobilized on streptavidin agarose beads. The immobilized template with or without the enhanceosome then was incubated with the nuclear extract and washed extensively, and the bound proteins were detected by Western blotting by using specific antibodies. Fig. 2*A* (lane 3) shows that CBP can be recruited efficiently by the enhanceosome. Surprisingly, the IFN-β enhanceosome can also recruit PolII, TFIIE, and SRB7 in the absence of a core promoter. Importantly, the enhanceosome recruits only the nonphosphorylated, initiation-competent form of RNA polymerase II (Fig. 2*A*, lane 3). In contrast, we found that neither TBP nor TFIIB can be recruited by the enhanceosome in the absence of core promoter sequences (Fig. 2*A*). In addition, we found that mutant enhanceosome lacking either the NF-κB or IRF-1 activation domain did not recruit CBP and components of the RNA PolII holoenzyme (data not shown).

To investigate whether recruitment of PolII by the enhanceosome is mediated via direct interactions between enhanceosome components and PolII or via PolII's interaction with CBP, we repeated the previous experiment by using a CBP-depleted nuclear extract. Fig. 2*B* (compare lanes 2 and 3) shows that more than 95% of CBP/p300 can be depleted from the nuclear extract. Importantly, we find that the CBP/p300 pellet also contains PolII (predominantly the nonphosphorylated form), SRB7, TFIIE, and traces of TFIIB, but is devoid of TBP (Fig. 2*B*, compare lanes 2 and 3). These observations suggest that the CBP/p300 immunoprecipitate contains, among others, the CBP-PolII holoenzyme complex (reviewed in ref. 32). Fig. 2*C* (compare lanes 2 and 3) demonstrates that the enhanceosome does not recruit the PolII holoenzyme in the CBP-depleted nuclear extract. Therefore, we conclude that the PolII holoenzyme complex is recruited to the enhanceosome by means of its association with CBP.

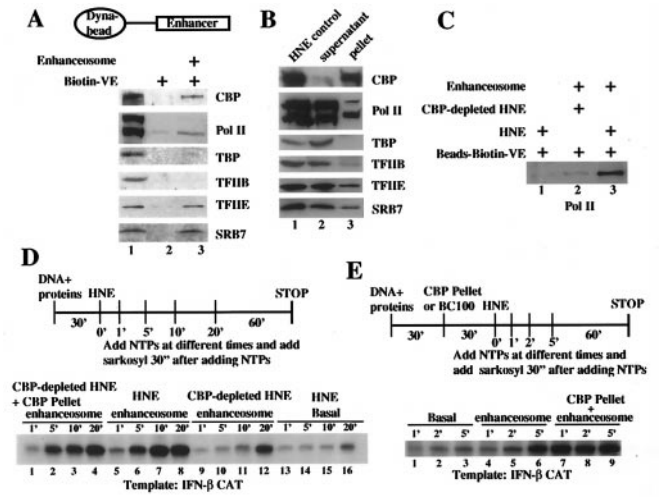

Fig. 2. Recruitment of the CBP-RNA PolII complex by the enhanceosome is the rate-limiting step that determines the speed of PIC assembly at the IFN-β promoter. (*A*) The IFN-β enhanceosome recruits CBP and the PolII holoenzyme. A biotinylated IFN-β enhancer oligonucleotide (−105 to −40) with or without the enhanceosome was coupled to magnetic Dynabeads and was incubated with 500 μg of HeLa nuclear extract. Western blotting by using CBP, PolII, TBP, TFIIB, TFIIEβ, and SRB7 specific antibodies detected bound proteins. Lane 1 detects the indicated proteins in the HNE, lane 2 corresponds to the precipitated proteins in the absence of the enhanceosome, whereas lane 3 depicts the proteins precipitated by the enhanceosome. (*B*) Antibodies against CBP coprecipitate components of the RNA PolII holoenzyme. HNE (3.6 mg) was incubated with CBP and p300 specific antibodies (10 μg each), and 1/100 of the pellet or supernatant was analyzed by Western blotting by using antibodies against the depicted. (*C*) CBP mediates PolII recruitment by the enhanceosome; same as in *A* except that a CBP-depleted HNE (lane 2) was used in parallel with a complete extract (lane 3). (*D*) Removal of CBP-RNA PolII holoenzyme from the extract decelerates PIC formation at the IFN-β promoter. Shown is an *in vitro* transcription experiment performed as detailed in Fig. 1 except that complete (lanes 5–8), CBP-depleted (lanes 9–12), or reconstituted nuclear extracts (lanes 1–4) were used. (*E*) Same as in *D* except that the CBP pellet was preincubated with the enhanceosome (lanes 7–9) before the addition of the extract for the indicated amounts of time.

To test whether recruitment of the CBP-PolII holoenzyme complex is one of the rate-limiting steps that determine the speed of PIC formation, we carried out *in vitro* transcription experiments by using the CBP-depleted nuclear extracts either alone or supplemented with the pellet containing the CBP-PolII holoenzyme complex. Fig. 2*D* (compare lanes 5–8 with 13–16) demonstrates that the enhanceosome increases the rate of PIC formation, a result that is consistent with the data shown in Fig. 1*A*. However, depletion of the CBP-PolII holoenzyme complex significantly decreased the rate of PIC formation, thus resembling that of basal transcription (Fig. 2*D*, compare lanes 9–12 with 13–16). For example, the number of PICs formed after 20-min incubation of the enhanceosome with the CBP-depleted extract is similar to that formed after 5-min incubation with the complete nuclear extract (compare lanes 6 and 12). Remarkably, however, addition of the immunoprecipitate containing the CBP-PolII holoenzyme complex back to the depleted extract almost completely restored the speed of PIC formation (compare lanes 1–4 with 5–8). As a control, we showed that mock-depleted HNE or mock pellets did not influence the rate of PIC assembly (data not shown). To demonstrate directly that recruitment of components present in the CBP pellet is indeed the rate-limiting factor that determines the speed of PIC assembly by the enhanceosome, we preincubated the enhanceosome with the

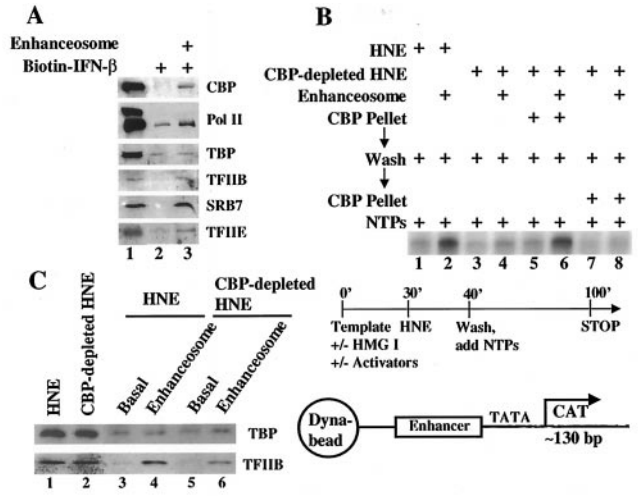

Fig. 3. Enhanceosome–CBP RNA PolII holoenzyme interactions are required at the early steps of PIC assembly. (*A*) Immobilized IFN-β enhancer–promoter DNA with (lane 3) or without (lane 2) the enhanceosome was preincubated with HNE followed by washes and Western blot analysis by using antibodies against the depicted proteins. (*B*) An immobilized IFN-β enhancer–promoter DNA with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) the enhanceosome was preincubated with a complete nuclear extract (lanes 1 and 2) or with the CBP/p300-depleted extract (lanes 3–8), followed by wash and addition of NTPs to initiate transcription. In lanes 5 and 6, the CBP/p300 immunoprecipitate was added before wash whereas in lanes 7 and 8, the immunoprecipitate was added after washing the beads. (*C*) Same as in *A* except a CBP-depleted HNE (lanes 2, 5, and 6) was used in parallel with the complete HNE (lanes 1, 3, and 4).

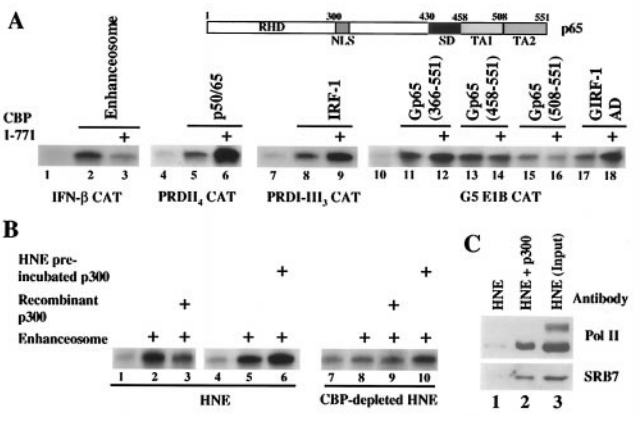

Fig. 4. CBP/p300 that is not complexed with the PolII holoenzyme inhibits enhanceosome-dependent transcription. (*A*) Shown is an *in vitro* transcription experiment by using the IFN-β enhancer–promoter as template and the enhanceosome as an activator (lanes 1–3), PRDII$_4$CAT and NF-κB (lanes 4–6), PRDI-III$_3$CAT and IRF-1 (lanes 7–9), or G5E1BCAT and GAL4 p65 or GAL4 IRF-1 fused proteins (lanes 10–18). In lanes 3, 6, 9, 12, 14, 16, and 18, we added 200 ng of GST-CBP (1–771) protein. The top part of the figure depicts a diagrammatic illustration of the p65 protein. The two independent activation domains (TA1 and TA2) as well as the synergism-specific domain required for interaction with CBP are indicated. (*B*) Shown is an *in vitro* transcription experiment by using the IFN-β enhanceosome along with 100 ng of baculovirus-expressed and purified, full-length p300. In lanes 1–6, a complete HNE was used whereas in lanes 7–9, a CBP/p300-depleted HNE was used. Recombinant p300 was added either with the enhanceosome lanes 3 and 9 or after preincubation with the complete (lane 6) or CBP/p300-depleted (lane 10) HNE. (*C*) Recombinant His-tagged p300 (lane 2) was incubated with a HNE followed by the addition of Ni^{2+}-nitrilotriacetic acid agarose beads. The precipitated complexes were washed extensively and immunoblotted by using antibodies against PolII and SRB7. Lane 1 represents nonspecific binding to the agarose beads.

pellet and then added back the CBP-depleted nuclear extract for different amounts of time followed by NTPs and sarcosyl. Fig. 2*E* shows that preincubation of the enhanceosome with this pellet resulted in instant assembly of functional PICs at the promoter after the addition of the remaining factors present in the extract (compare lanes 1, 4, and 7). These results, taken together with the fact that the CBP immunoprecipitate contains the RNA PolII holoenzyme but lacks TBP and nearly all TFIIB, demonstrate that the CBP-PolII holoenzyme pellet is one of the rate-limiting components whose recruitment is required for the fast rate of enhanceosome-dependent PIC formation.

To identify the step in PIC assembly that requires the pellet containing the CBP-PolII holoenzyme complex, we carried out *in vitro* transcription experiments by using an immobilized IFN-β enhancer–promoter DNA fragment and the CBP-depleted extracts. When the IFN-β template was preincubated with the complete HeLa nuclear extract in the presence of the enhanceosome and washed, and NTPs were added, a high level of transcription was obtained (Fig. 3*B*, compare lanes 1 and 2). The Western blot experiment of Fig. 3*A* demonstrates that several components of the CBP-RNA-PolII holoenzyme are recruited by the enhanceosome. In addition, TFIIB but not TBP recruitment also can be facilitated by the enhanceosome *in vitro*, a result consistent with a previous report (12). Thus, enhanceosome-dependent recruitment of TFIIB requires the core promoter elements, whereas enhanceosome-dependent recruitment of the CBP-PolII holoenzyme complex occurs in the absence of core promoter sequences. When the *in vitro* transcription reactions were carried out with the IFN-β enhanceosome in the presence of CBP-depleted nuclear extracts, the levels of activated transcription were reduced significantly (lanes 3 and 4). However, the high levels of enhanceosome-dependent transcription were reconstituted when the CBP-depleted extract was supplemented with the CBP immunoprecipitate during the initial incubation period (lanes 5 and 6). In contrast, addition of the CBP immunoprecipitate after the promoter complexes were washed on beads did not restore the transcriptional activity (lanes 7 and 8). Thus, the CBP-PolII holoenzyme complex must be present during the early steps of PIC assembly. The Western blot of Fig. 3*C* shows that the enhanceosome still recruits TFIIB in the CBP-depleted extracts albeit with lowered efficiency. Thus, recruitment of TFIIB can take place in the absence of the CBP-PolII holoenzyme, but it does not suffice for quick activation of transcription.

CBP/p300 That Is Not Complexed with the PolII Holoenzyme Inhibits Enhanceosome-Dependent Transcription. The experiments described so far do not exclude the possibility that the requirement for the CBP-PolII holoenzyme complex could be due solely to CBP because it bears two putative transcriptional activation domains, located at the amino and carboxyl termini (reviewed in ref. 33). Thus, in this scenario, CBP may enhance the rate of PIC formation by providing additional activation domains to the enhanceosome. Therefore, to verify that the enhanced rate of PIC formation is due to the CBP-RNA PolII holoenzyme complex and not to CBP itself, we carried out *in vitro* transcription experiments by using recombinant CBP/p300 proteins. We have shown previously that the amino-terminal 771 aa of CBP are responsible for its recruitment into the enhanceosome (11). Fig. 4*A* shows that this fragment of CBP strongly inhibits enhanceosome-dependent transcription (lanes 1–3), a result that is consistent with our previous transfection experiments showing that CBP (1–771) functions in a dominant-negative fashion (11). In contrast, CBP (1–771), which does not interact with the RNA PolII holoenzyme (33), strongly potentiates transcription from NF-κB and IRF-1 when these activators are present alone (lanes

4–9). Thus, this fragment of CBP, which activates transcription on its own, has two opposite effects on transcription depending on the activator context. The specificity of CBP-dependent coactivation of transcription by NF-κB also was investigated by using GAL4-based deletions in the activation domain of p65, which do not bind CBP (11). Fig. 4*A* (lanes 10–12) shows that CBP (1–771) potentiates activation by the full-length p65 activation domain. However, deletion of the synergism-specific domain of p65, which is required for interaction with CBP (11), although it did not affect activation by p65 (lane 13), abolished CBP-dependent coactivation of transcription (lane 14).

Fig. 4*B* demonstrates that preincubation of baculovirus-expressed and purified full-length p300 protein with the IFN-β enhanceosome strongly inhibited activation of transcription (compare lanes 2 and 3). Thus, free CBP/p300 inhibits the enhanceosome activity presumably by competing with the CBP-PolII holoenzyme complex present in the extract for entry into the enhanceosome "pocket." In contrast, however, when CBP/p300 was first incubated with the nuclear extract for 3 hr and then added to the transcription reaction, a strong stimulation of enhanceosome-dependent transcription occurred (lanes 4–6), thus implying that recombinant CBP/p300 is incorporated into PolII holoenzyme complexes. In fact, agarose beads harboring His-tagged p300 specifically associate with the PolII holoenzyme complex in the context of the nuclear extract (Fig. 4*C*). These conclusions were strengthened further by the observation that free p300 did not inhibit enhanceosome-dependent activation in the CBP/p300-depleted extract (compare lanes 8 and 9). However, preincubation of recombinant p300 with the CBP/p300-depleted extract restored enhanceosome-dependent activation of transcription. Thus, at least *in vitro*, one of the CBP/p300 functions is to serve as a bridge that connects the IFN-β enhanceosome with the PolII holoenzyme complex.

Discussion

In this paper, we have examined the mechanisms by which the IFN-β enhanceosome activates transcription *in vitro*. We found that in contrast to all previous studies in which simple, artificial promoters or activators were used, the natural IFN-β enhanceosome activates transcription by stimulating the rate of functional PIC formation at the promoter. Thus, in all previously documented cases, activators work by increasing the proportion of DNA templates on which a functional PIC is assembled. Consistently, we showed that both NF-κB and IRF-1 work similarly on synthetic templates bearing multiple copies of their binding sites or on mutant IFN-β enhancers, which cannot assemble an enhanceosome. These results indicate that the mechanism of activation of transcription from natural enhancer complexes is qualitatively distinct from that of synthetic enhancer elements. It is conceivable that synthetic activating complexes, although capable of establishing multiple interactions with various targets, do not cause an increase in the rate of PIC assembly, because these interactions are of intrinsically low affinity and specificity, and perhaps some of them may be mutually exclusive. We propose that the independent and fortuitous recruitment of targets by each activation domain in the synthetic activation complexes would result in enhancement of the rate of PIC formation, only if these targets are necessary for execution of distinct and rate-limiting steps during PIC assembly. Evidently, the probability of recruiting the appropriate combination of targets on the same template is very low. On the other hand, the outcome of the unusually high affinity and specificity of the natural IFN-β enhanceosome for the CBP-PolII holoenzyme is the increased rate of PIC assembly at the IFN-β promoter.

The increased assembly rate of functional PICs at the IFN-β promoter correlates with the ability of the enhanceosome "pocket" to interact with the CBP-RNA PolII holoenzyme complex. On the other hand, the slow rate by which each activator separately recruits the basal machinery correlates with the lower affinity by which they individually recruit CBP when compared with the enhanceosome (11). Thus, the strength of CBP–activator interactions establishes the pace of transcriptional activation. As predicted from the kinetic experiments, we showed that preincubation of the enhanceosome with the CBP immunoprecipitate that contains the CBP-RNA PolII holoenzyme dramatically stimulated the already high speed of PIC assembly, thus bypassing the need for additional incubation with the nuclear extract. Finally, as revealed by the immobilized DNA template experiments, the immunoprecipitate containing the CBP-RNA PolII holoenzyme complex must be present at the early steps of PIC assembly. Based on these observations, we propose that the highly efficient and specific recruitment of the CBP-PolII holoenzyme by the enhanceosome results in the formation of a platform on which the other basal factors rapidly assemble to initiate transcription.

The natural IFN-β enhanceosome is the only activating complex we know of that can enhance the rate of PIC formation at a promoter in the absence of chromatin. Previous studies have demonstrated a critical role for CBP in synergistic activation of transcription from artificial estrogen receptor-responsive promoters, or from the low density lipoprotein receptor promoter, only in the context of chromatin DNA templates (34, 35). Thus, we anticipate that the enhanced rate of enhanceosome-dependent PIC assembly would be significantly greater on chromatin templates, because the core promoter elements are not readily accessible to TFIID and the rest of the basal machinery. The experiments described here together with previous studies (4–13) imply that the high levels of transcriptional synergy exerted by the enhanceosome are due to a complex network of multiple, direct and indirect interactions between and within three distinct subcomplexes: the enhanceosome, the CBP-PolII holoenzyme, and the other basal factors. Thus, of a plethora of targets, which potentially could be selected in the nuclear extract, the enhanceosome specifically picks the CBP-RNA PolII complex, despite its low abundance. CBP is recruited preferentially because it recognizes with high affinity a contiguous surface, the enhanceosome "pocket," consisting of a specific arrangement of the activation domains in three-dimensional space (11). Simultaneously, however, the enhanceosome can recruit independently TFIIB into a template-committed TFIID-TFIIA-USA complex (12). We propose that protein–protein interactions between specific components present in the two different subcomplexes could stabilize their overall interaction with the DNA and promote quick initiation of transcription. This idea is consistent with recent experiments demonstrating that assembly of functional PICs *in vivo* requires the concerted action of TFIID and PolII holoenzyme (36, 37).

That the enhanceosome is capable of activating transcription, albeit with a slower rate in the absence of CBP, is intriguing. It implies that a new network of protein–protein interactions between the activation domains and the basal apparatus is "rewired" in the absence of CBP. This notion is consistent with the fact that, in general, activation domains represent marginally selective and sticky surfaces that can contact multiple targets within the transcriptional apparatus (38). It is therefore conceivable that in the absence of CBP, the activation domains shift their contacts, thus promoting PIC assembly. However, we showed that there is a quantitative difference between CBP-dependent and CBP-independent modes of transcriptional activation. The former is a fast reaction, whereas the latter is slow. Additional evidence for a noncritical role of CBP in transcriptional activation *per se* stems from the observation that antibodies against CBP only partially inhibited enhanceosome function in reactions containing purified basal factors and the CBP-RNA PolII holoenzyme complex (12). We also provided evidence that

CBP/p300 can function as a scaffold for the formation of functional PICs. We showed that preincubation of the enhanceosome with recombinant p300 inhibited enhanceosome function, but it stimulated activation by NF-κB or IRF-1 alone, thus implying that incorporation of free CBP into the enhanceosome "pocket" competes with the recruitment of the CBP-PolII holoenzyme complex. However, CBP can function as a typical coactivator of transcription when it is recruited to simple promoters containing NF-κB- or IRF-1-binding sites, consistent with its similar effects on Tax-dependent transactivation *in vitro* (39).

The role of CBP in enhanceosome function is not restricted to its ability to mediate recruitment of the RNA PolII holoenzyme complex only. We have shown previously that the acetyltransferase activity of CBP is critical in modulating both the strength of the transcriptional response and the kinetics of virus-dependent activation of the IFN-β gene [Munshi *et al.* (13)]. Thus, CBP plays multiple roles in activation of transcription by the enhanceosome. First, it functions as a chromatin-remodeling factor that could acetylate histones in nearby nucleosomes (14), thus facilitating recruitment of the transcriptional apparatus. Second, it can acetylate HMG I(Y), causing enhanceosome disruption and, therefore, termination of gene activation (13). Finally, aside from its function as an acetyltransferase, we demonstrated here that the unusual strength of CBP–enhanceosome interactions is responsible for the fast recruitment of the RNA PolII holoenzyme, thus ensuring rapid activation of IFN-β gene expression in response to virus infection.

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