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The Mechanism of Transcriptional Synergy of an In Vitro Assembled Interferon-β Enhanceosome

Tae Kook Kim and Tom Maniatis*
Harvard University
Department of Molecular and Cellular Biology
Cambridge, Massachusetts 02138

Summary

A functional interferon-β gene enhanceosome was assembled in vitro using the purified recombinant transcriptional activator proteins ATF2/c-JUN, IRF1, and p50/p65 of NF-κB. Maximal levels of transcriptional synergy between these activators required the specific interactions with the architectural protein HMG I(Y) and the correct helical phasing of the binding sites of these proteins on the DNA helix. Analyses of the in vitro assembled enhanceosome revealed that the transcriptional synergy is due, at least in part, to the cooperative assembly and stability of the complex. Reconstitution experiments showed that the formation of a stable enhanceosome-dependent preinitiation complex requires cooperative interactions between the enhanceosome; the general transcription factors TFIID, TFIIA, and TFIIB; and the cofactor USA. These studies provide a direct biochemical demonstration of the importance of the structure and function of natural multicomponent transcriptional enhancer complexes in gene regulation.

Introduction

Gene activation in response to extracellular signals, infection by pathogens, or environmental stresses requires highly integrated signal transduction pathways that direct the transcriptional machinery to the appropriate set of genes. A key issue in understanding inducible gene regulation is how a relatively small number of different transcription factors is used to achieve the high level of specificity required to control complex patterns of gene expression (reviewed by Maniatis et al., 1987; McKnight and Yamamoto, 1992; Tjian and Maniatis, 1994). The answer to this question lies, in part, in a combinatorial mechanism of gene activation. Most genes are regulated by multiple transcriptional activator proteins, each of which plays a role in controlling the transcription of a variety of genes with different expression patterns. The expression of a given gene depends, therefore, on the simultaneous interaction of a specific combination of regulatory proteins with the control DNA elements. Indeed, most transcription enhancers contain distinct sets of transcription factor-binding sites, and variations in the arrangement of binding sites provide the potential to create unique nucleoprotein complexes. Cooperative interactions between the proteins in these complexes can lead to a high level of specificity in gene activation and to a high level of transcriptional synergy.

Activation of the interferon-β (IFNβ) gene provides one of the best-characterized examples of combinatorial interactions among distinct regulatory elements (reviewed by Maniatis et al., 1992; Tjian and Maniatis, 1994). The IFNβ gene is activated in response to virus infection, and the transcription factors required for activating the IFNβ gene have been identified (summarized in Figure 1A). Detailed studies of this promoter revealed a highly compact and remarkably complex organization of regulatory sequences containing four positive regulatory domains (PRDs). None of these domains function on their own, but two or more copies of any one of them can act as a virus-inducible enhancer. However, the synthetic enhancers display varying levels of basal activity, are less inducible than a natural enhancer, and can respond to inducers other than virus infection. Thus, the specificity and activity of the natural intact enhancer are distinct from those of the individual enhancer elements in isolation. The activation of at least some natural enhancers appears to result from the precise arrangement of transcription factors on DNA, resulting in the formation of a highly specific three-dimensional nucleoprotein complex (stereospecific enhancer complex or enhanceosome; reviewed by Grosschedl, 1995). This model for transcriptional specificity is consistent with in vivo studies of IFNβ gene expression, but direct biochemical proof for this model has not been obtained.

Transcriptional activator proteins have been shown to synergize with each other in vitro using synthetic enhancers containing multiple activator binding sites (reviewed by McKnight and Yamamoto, 1992; Tjian and Maniatis, 1994). In some cases, the observed transcriptional synergy could be explained, at least in part, by cooperative binding of the activators to their sites. However, in other cases, the transcriptional synergy could be observed at concentrations of activator in which the binding sites are fully occupied (Carey et al., 1990; Lin et al., 1990). The most straightforward explanation for this synergy is that activators recruit the general transcription apparatus to nearby promoters, and the transcriptional synergy is a consequence of multiple interactions between the activators and components of the transcription apparatus (reviewed by Ptashne and Gann, 1997). This recruitment could involve the stepwise association of general transcription factors with the promotor (reviewed by Orphanides et al., 1996; Roeder, 1996), interactions between activators and specific TATA box binding protein (TBP) associated factors (or TAFs) in the TFIID complex (Sauer et al., 1995), or interactions between activators and components of the RNA polymerase II holoenzyme (reviewed by Koleske and Young, 1995). Strikingly, none of the previous in vitro studies of transcriptional synergy were carried out with complex natural enhancer elements, and none provided evidence for a role of three-dimensional structure in transcriptional synergy. In this paper, we report the in vitro assembly of a functional human IFNβ enhanceosome and investigate the mechanisms of the enhanceosome-dependent transcriptional synergy.

*To whom correspondence should be addressed.

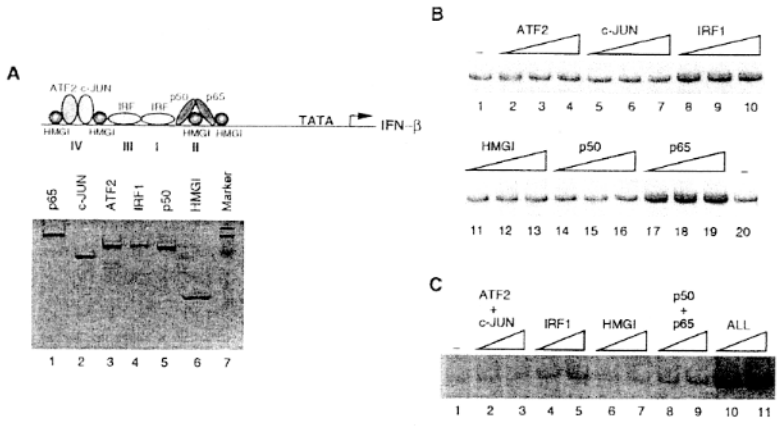

Figure 1. Establishment of an In Vitro Transcription System for Synergistic Activation of the IFNβ Promoter

(A) Human IFNβ gene promoter. The enhancer region contains four positive regulatory domains designated PRDI, II, III, and IV. Transcription factors that bind to each of the elements are shown: the ATF2/c-JUN heterodimer binds to PRDIV, an IRF family member protein binds to both PRDI and PRDIII, the p50/p65 heterodimer of NF-κB binds to PRDII, and HMG I(Y) binds to the three AT-rich sequences within the enhancer (reviewed by Maniatis et al., 1992) and to one site between PRDII and the TATA box (Maher and Nathans, 1996; Yie et al., 1997). We note that recent studies suggest that a protein yet to be identified (rather than IRF1) may be required for virus induction of the IFNβ gene in vivo (Matsuyama et al., 1993; Reis et al., 1994; M. Wathelet and T. M., unpublished data). However, based on the observation that IRF1 can synergize with other components of the IFNβ enhanceosome in transfection experiments (Thanos and Maniatis, 1995b), we have used recombinant IRF1 in our in vitro studies of the enhanceosome-dependent transcriptional synergy. All of the PRD-binding factors were purified by nickel affinity chromatography. The purified hexahistidine-tagged proteins were separated by SDS-PAGE and detected by staining with Coomassie blue.

(B) Titration of PRD-binding factors. Increasing amounts of each purified PRD-binding factor (ATF2, c-JUN, IRF1, HMG I[Y], p50, and p65) were assayed for in vitro transcription. The amounts of each recombinant PRD-binding factor added were as follows: lanes 2, 5, 8, 11, 14, and 17, 200 fmol; lanes 3, 6, 9, 12, 15, and 18, 400 fmol; and lanes 4, 7, 10, 13, 16, and 19, 800 fmol.

(C) Titration of PRD-binding units. In vitro transcription was performed in the absence (lane 1) or presence (lanes 2–9) of each PRD-binding unit (ATF2/c-JUN, IRF1, HMG I[Y], and p50/p65) or all of the PRD-binding units (lanes 10 and 11) as indicated. The amounts of each recombinant PRD-binding factor were as follows: lanes 2, 4, 6, 8, and 10, 200 fmol and lanes 3, 5, 7, 9, and 11, 800 fmol.

Results

In Vitro Assembly of a Functional IFNβ Enhanceosome

To analyze the activation of the intact IFNβ promoter in vitro, each of the PRD-binding factors were purified as recombinant proteins from bacteria (Figure 1A). The transcription reactions were carried out in HeLa cell nuclear extracts depleted of endogenous PRD-binding proteins by sequence-specific DNA and antibody affinity chromatographies (data not shown).

A low level of accurately initiated RNA was observed in the absence of added factors (basal transcription), and little or no increase in transcription was observed with increasing amounts of each PRD-binding protein alone (ATF2, c-JUN, IRF1, HMG I[Y], p50, and p65; Figure 1B). These titration experiments were also carried out with preassembled heterodimers of ATF2/c-JUN and p50/p65, which bind to PRDIV and PRDII, respectively (Du et al., 1993; Thanos and Maniatis, 1995a). Increasing the amounts of either heterodimer (PRD-binding unit) failed to stimulate transcription significantly over the levels observed with each of the individual factors (Figure 1C). Increasing the amounts of the individual factors or the heterodimers above those tested in Figures 1B and 1C did not lead to a further significant increase in transcription (data not shown). In sharp contrast, when all of the PRD-binding factors were included in the reaction mixture, a 27.1-fold increase in the level of transcription was observed (Figure 1C, lanes 10 and 11). Thus, a high level of transcriptional synergy was observed when all of the PRD-binding proteins were present.

Synergistic Activation of the IFNβ Gene Promoter In Vitro Requires HMG I(Y) When the Activator Proteins Are Limiting

To examine the role of HMG I(Y) in the in vitro activation of the IFNβ promoter, we compared the levels of transcriptional activation by ATF2/c-JUN, IRF1, and p50/p65 in the absence and presence of HMG I(Y) (Figure 2A, lanes 1–6 and 7–12, respectively). HMG I(Y) did not affect the basal level of transcription (compare lanes 1 and 7). However, HMG I(Y) significantly increased the level of transcription at lower concentrations of the transcriptional activator proteins (e.g., compare lanes 3 and 9). At these concentrations, each PRD-binding unit alone did not stimulate transcription significantly (Figure 2B, lanes 2–5), and addition of all of the PRD-binding factors except HMG I(Y) resulted in a 7.1-fold increase in the level of activation (lane 6). In contrast, the presence of HMG I(Y) under the same conditions resulted in a 31.8-fold activation (lane 8). We conclude that HMG I(Y) is required for the high level of transcriptional synergy in vitro. The observation that the greatest effects of HMG I(Y) are observed at lower concentrations of PRD-binding proteins is consistent with in vitro binding studies showing that HMG I(Y) promotes cooperative DNA binding of PRD-binding factors at low concentrations of these factors (Du et al., 1993). Taken together, these observations are consistent with the hypothesis that HMG I(Y) promotes the assembly and possibly the stability of the IFNβ enhanceosome.

A Specific Combination of All of the PRD-Binding Proteins Is Required for Synergistic Activation of an IFNβ Promoter In Vitro

One prediction of the enhanceosome model is that when each of the PRD-binding factors is limiting, the omission of any one of them will result in a decrease in the level of transcription. As shown in Figure 2C, maximal levels of transcription were observed when all of the PRD-binding factors were present in the reaction mixture (lane 2). However, the level of activation significantly decreased in the absence of any one of the PRD-binding factors (lanes 3–8). Thus, a combination of all of the PRD-binding factors (ATF2/c-JUN, IRF1, HMG I[Y], and p50/p65)

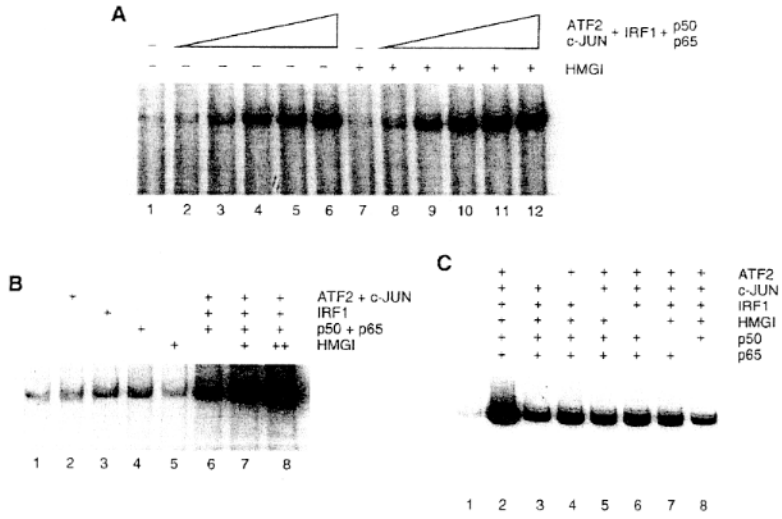

Figure 2. HMG I(Y) and Specific Dimeric bZIP/Rel Complexes Are Required for Transcriptional Synergy of the IFNβ Promoter When the Activator Proteins Are Limiting

(A) Effect of HMG I(Y) on activation of the IFNβ promoter. Increasing amounts of ATF2/c-JUN, IRF1, and p50/p65 were added to in vitro transcription reactions in the absence (lanes 2–6) or presence (lanes 8–12) of HMG I(Y) (200 fmol). The amounts of each recombinant PRD-binding factor were as follows: lanes 2 and 8, 50 fmol; lanes 3 and 9, 100 fmol; lanes 4 and 10, 200 fmol; lanes 5 and 11, 400 fmol; and lanes 6 and 12, 800 fmol.

(B) HMG I(Y)-mediated synergistic activation of the IFNβ promoter. Transcription was performed in the presence of indicated PRD-binding factors (ATF2/c-JUN, IRF1, and p50/p65; 100 fmol) and increasing amounts of HMG I(Y) (lane 7, 200 fmol; lane 8, 400 fmol).

(C) Synergistic activation of the IFNβ promoter by PRD-binding factors. The transcription reactions contained all of the PRD-binding factors (lane 2) or all but one of these factors (lanes 3–8).

is required for the high level of synergistic activation of IFNβ promoter. Notably, under these conditions, the level of synergistic activation was reduced by omission of any one protein in the ATF2/c-JUN or p50/p65 heterodimer combinations. Thus, specific dimeric complexes are required for maximal levels of synergistic activation from PRDII and PRDIV.

A Specific Interaction between HMG I(Y) and ATF2 Is Required for Transcriptional Synergy In Vitro

To examine the involvement of specific interactions between PRD-binding factors in the assembly of the enhanceosome, we took advantage of two naturally occurring ATF2 isoforms generated by alternative splicing within the basic region of the bZIP domain (Figure 3A). $ATF2_{195}$ (which is the same as ATF2 in Figure 1A) binds specifically to PRDIV and interacts with HMG I(Y) and p50, and its affinity for PRDIV is enhanced by HMG I(Y). In contrast, $ATF2_{192}$ binds to PRDIV but does not interact with either HMG I(Y) or p50 (Du and Maniatis, 1994). To compare transcriptional activities of these two ATF isoforms in vitro, purified recombinant $ATF2_{195}$ and $ATF2_{192}$ proteins were tested for in vitro transcriptional activation of the IFNβ promoter in the absence or presence of HMG I(Y) (Figure 3A). In the absence of HMG I(Y), both ATF2 isoforms supported similar levels of transcriptional activation in conjunction with all of the other PRD-binding factors (c-JUN, IRF1, and p50/p65; compare lanes 3 and 7). As expected, a high level of synergistic activation was observed with the $ATF2_{195}$ isoform in the presence of all of the other PRD-binding factors, including HMG I(Y) (lane 8). However, $ATF2_{192}$ failed to support the high level of synergistic transcriptional activation under the same conditions (lane 4). Thus, specific protein–protein interactions between ATF2 and the other PRD-binding factors such as HMG I(Y) and p50 are required for the high level of synergistic activation of IFNβ promoter.

HMG I(Y) Binding to the IFNβ Enhancer Is Required for Maximal Levels of Transcriptional Synergy

Given the critical role of HMG I(Y) in the synergistic activation in vitro, we carried out experiments to determine whether mutations that interfere with HMG I(Y) binding to DNA decrease the synergistic activation of IFNβ promoter in vitro. To this end, we compared the levels of transcriptional activation by PRD-binding factors to the mutants in the natural context of the IFNβ promoter (Figure 3B). None of the mutations that influence the affinity of HMG I(Y) for the promoter affected activation mediated by all of the PRD-binding factors in the absence of HMG I(Y) (compare lane 2 with lanes 5, 8, and 11). By contrast, mutations in either the 5′ (lane 6) or 3′ (lane 9) HMG I(Y) binding sites flanking PRDIV significantly reduced the HMG I(Y)-dependent synergistic activation (lane 3). A mutation that inhibits binding of HMG I(Y) to PRDII also decreased the level of synergistic activation mediated by PRD-binding factors, including HMG I(Y) (lane 12). Thus, the function of HMG I(Y) for the maximal level of synergistic activation in vitro requires its interaction with DNA in addition to interaction with activator proteins (Figure 3A) in the IFNβ enhanceosome.

Synergistic Activation of the IFNβ Promoter In Vitro Requires the Stereospecific Alignment of PRD-Binding Sites

To test the stereospecific requirements for enhanceosome function in vitro, we tested IFNβ promoters in which a half- or full-helical turn of DNA was inserted between PRDI and PRDII (Figure 3C). The level of transcription was determined from these phasing mutant IFNβ enhancers in the absence or presence of all of the PRD-binding factors. Wild-type IFNβ promoter showed the high level of synergistic activation by all of the PRD-binding factors (compare lanes 1 and 2). However, when a half-helical turn (6 base pairs [bp]) was introduced

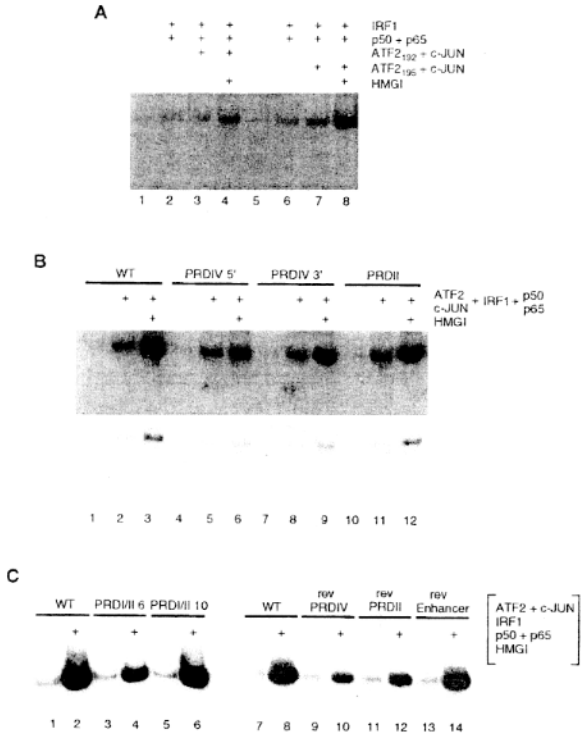

Figure 3. Specific Protein–Protein and DNA–Protein Interactions Are Required for the Synergistic Activation of the IFNβ Promoter In Vitro

(A) Interaction between ATF2 and HMG I(Y) for the IFNβ promoter activation. In vitro transcription experiments were performed with each combination of indicated PRD-binding factors with $ATF2_{192}$ (lanes 2–4) or $ATF2_{195}$ (lanes 6–8).

(B) Interaction of HMG I(Y) with DNA for the IFNβ promoter activation. In vitro transcription reactions were performed in the presence or absence of PRD-binding factors (ATF2/c-JUN, IRF1, and p50/p65) and HMG I(Y) using DNA templates containing mutations of HMG I(Y) binding sites in the 5′ (lanes 4–6) or 3′ (lanes 7–9) sites flanking PRDIV or in a site within PRDII (lanes 10–12). The bottom panel is a shorter exposure of the top panel.

(C) Correct alignment of PRD-binding sites for the IFNβ promoter activation. Mutant IFNβ DNA templates contained either a 6 bp (a half-helical turn; PRDI/II 6) and a 10 bp (a full-helical turn; PRDI/II 10) insertion between PRDI and II (lanes 3–6) or a reverse orientation of PRDIV (rev PRDIV), PRDII (rev PRDII), and entire PRDs (rev Enhancer; lanes 9–14) in the context of enhancer.

between PRDI and PRDII, the level of activation decreased significantly (lane 4). Remarkably, insertion of a full-helical turn (10 bp), which reestablishes the relative positions of PRD-binding sites on the face of the DNA helix, almost fully restored the activation of IFNβ promoter (lane 6). These results strongly support the conclusion that maximal levels of synergistic activation require the correct helical phasing of transcription factor-binding sites in the IFNβ enhancer. Consistent with this enhanceosome model, inversion of PRDIV (lane 10) or PRDII (lane 12) dramatically reduced the level of synergistic activation by specifically affecting HMG I(Y)-mediated protein–protein interactions (data not shown). However, the construct in which the entire IFNβ enhancer was reversed relative to the TATA box was transcribed at nearly wild-type levels (compare lanes 8 and 14).

Synergistic Activation of the IFNβ Promoter Correlates with an Increase in Stability of the Functional Enhanceosome

To gain insights into the mechanisms of transcriptional synergy in vitro, we compared the stabilities of distinct complexes assembled under conditions that can or cannot support a high level of synergistic activation (Figure 4). The IFNβ enhancer complexes were assembled on the wild-type or mutant (PRDI/II 6) enhancer DNA and then challenged with an IFNβ enhancer oligonucleotide for different times of incubation before the start of the transcription reaction (Figure 4A). The wild-type enhanceosome, which can support high levels of synergistic activation, was resistant to the competitor DNA. In sharp contrast, activated transcription (which was lower prior to addition of competitor) rapidly decreased to the basal level of transcription during the first 10 min of incubation in the enhancer complexes either containing no HMG I(Y) or assembled on the PRDI/II 6 mutant enhancer. These correlations between stability and transcriptional activity show that assembly of a stable complex is required for the high level of synergistic activation by the IFNβ enhanceosome in vitro.

To further test this idea, competition assays for enhancer complexes were performed with increasing amounts of an IFNβ enhancer oligonucleotide (Figure 4B). Consistent with data in Figure 4A, at a 60-fold excess concentration, this oligonucleotide almost completely dissociated the IFNβ enhancer complexes assembled in the absence of HMG I(Y) or assembled on the helical phasing mutant enhancer DNA, thus reducing transcription to the basal level. By comparison, the IFNβ enhanceosome assembled on the wild-type promoter in the presence of HMG I(Y) was significantly more resistant to oligonucleotide challenge. Under these conditions, transcriptional activation, but not basal transcription, from the IFNβ promoter was specifically inhibited by addition of increasing amounts of an oligonucleotide containing the PRD-binding sites, and addition of a large molar excess of an oligonucleotide lacking PRD-binding sites did not inhibit IFNβ-activated transcription with any of the complexes (data not shown). Thus, transcriptional synergy of the IFNβ promoter is due, at least in part, to the stable assembly of the enhanceosome, which requires HMG I(Y) as well as stereospecific interactions between transcription factors in the complex.

The Assembly of an Enhanceosome Capable of Transcriptional Synergy Is Highly Cooperative

We next investigated the possibility that the cooperative assembly of the enhanceosome plays a role in transcriptional synergy. As demonstrated above, HMG I(Y) is required for high levels of transcriptional synergy. We therefore analyzed the effects of HMG I(Y) on the cooperative assembly of the enhanceosome in vitro (Figure 5).

Each of the PRD binding units (ATF2/c-JUN, IRF1 and p50/p65) was tested alone or in combination in the presence or absence of HMG I(Y) (Figure 5A). Low levels of transcriptional activation were observed with each PRD-binding unit (lanes 4, 7, and 10), and this activated transcription was only slightly (1.4- to 1.8-fold) stimulated

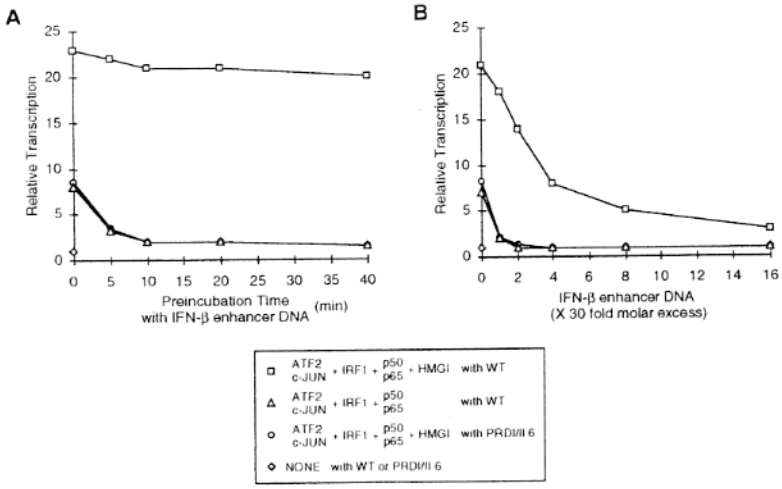

Figure 4. Synergistic Activation of the IFNβ Promoter Requires a Stable Enhanceosome

The IFNβ enhancer complexes were preassembled for 30 min with wild-type or PRDI/II 6 mutant enhancer DNA and challenged with a competitor oligonucleotide for the indicated time (10 min in [B]) before the start of the transcription reaction.

(A) Relative levels of transcription plotted against increasing time (min) of incubation with an IFNβ enhancer oligonucleotide (a 30-fold molar excess).

(B) Relative levels of transcription plotted against increasing amounts (multiples of a 30-fold molar excess) of an IFNβ enhancer oligonucleotide.

by the addition of increasing amounts of HMG I(Y) (lanes 5, 6, 8, 9, 11, and 12). However, under these conditions HMG I(Y) significantly stimulated the level of transcription induced by combinations of each of the PRD-binding units (lanes 16, 18, 20, and 22). The effects of HMG I(Y) on transcriptional activation were maximal when all of the PRD-binding units were present (compare lanes 21 and 22; 4.0-fold; Figure 5A). Thus, HMG I(Y) promotes transcriptional synergy by facilitating multiple functional interactions between PRD-binding units in the IFNβ promoter. These observations are consistent with previous studies showing that HMG I(Y) can interact with both ATF2 and p50 in vitro and could therefore promote interactions between proteins bound to PRDIV and PRDII (Du et al., 1993). In addition, IRF1 was shown to interact directly with p50 and HMG I(Y) and could therefore promote interactions between PRDIII-I and PRDII (Neish et al., 1995).

To further address the cooperative assembly of a functional enhanceosome, we carried out titration experiments with increasing amounts of ATF2/c-JUN, IRF1, and p50/p65 in the absence or presence of HMG I(Y) under the limiting conditions (Figure 5B). In the presence of HMG I(Y), a sigmoidal increase in transcription was observed as the amounts of PRD-activator proteins were increased (e.g., compare lanes 10 and 11). By contrast, in the absence of HMG I(Y), only a gradual increase in transcription was observed under the same conditions (lanes 1–6). Thus, HMG I(Y) promotes the highly cooperative assembly of the enhanceosome. These data are consistent with results (Figure 5A) showing that HMG I(Y) facilitates multiple protein–protein interactions in the cooperative assembly of the enhanceosome. Taken together, these observations show that the synergistic activation of the IFNβ enhancer involves the cooperative assembly and increased stability of the enhanceosome.

The IFNβ Enhanceosome Facilitates Formation of a Template-Committed General Transcription Complex Containing TFIID, USA, TFIIA, and TFIIB

To examine the interaction between the IFNβ enhanceosome and the general transcription apparatus, we carried out experiments to identify components in the general complex required for enhanceosome-mediated activation of the IFNβ promoter. To this end, we used a sarkosyl sensitivity assay to identify stable intermediates in preinitiation complex assembly (Hawley and Roeder, 1985). The stabilities of various preinitiation complexes were compared in the absence or presence of HMG I(Y) and all of the other PRD-binding factors (ATF2/c-JUN, IRF1, and p50/p65; Figure 6).

To assemble the various preinitiation complexes, general transcription factors (TFIIA, TFIIE/F/H, and RNA polymerase II) and the USA cofactor were purified from HeLa nuclear extracts (Meisterernst et al., 1991). Flag-tagged TFIID was highly purified by affinity chromatography, and TFIIB was purified as a hexahistidine-fusion recombinant protein from bacteria (Chiang et al., 1993). All of these factors were required for the high level of synergistic activation by the IFNβ enhanceosome in vitro (data not shown; see below).

As indicated in Figure 6, the general transcription factors were preincubated with or without the assembled IFNβ enhanceosome for 20 min, and then sarkosyl was added to a final concentration of 0.04% along with the remaining factors and nucleotides. Thus, after sarkosyl addition, all of the reaction mixtures contained the same components and were incubated for an additional 40 min. In the absence of sarkosyl, addition of the IFNβ enhanceosome before or after preincubation of general transcription factors resulted in a similar level of activated transcription (lanes 1–3). When the IFNβ enhanceosome was preincubated with the complete set of general transcription factors, followed by the addition of 0.04% sarkosyl at 20 min, the level of activated transcription was similar to that observed for the transcription reaction performed in the absence of sarkosyl (compare lanes 21 and 3). In sharp contrast, when the IFNβ enhanceosome was added immediately after adjustment of the reaction mixture to 0.04% sarkosyl at 20 min, no transcription was detected (lane 20). Comparison of lanes 20 and 21 shows that preincubation with an IFNβ enhanceosome was required for the formation of a sarkosyl-resistant preinitiation complex.

To determine which of the general factors are required for the formation of this IFNβ enhanceosome-dependent, sarkosyl-resistant preinitiation complex, specific

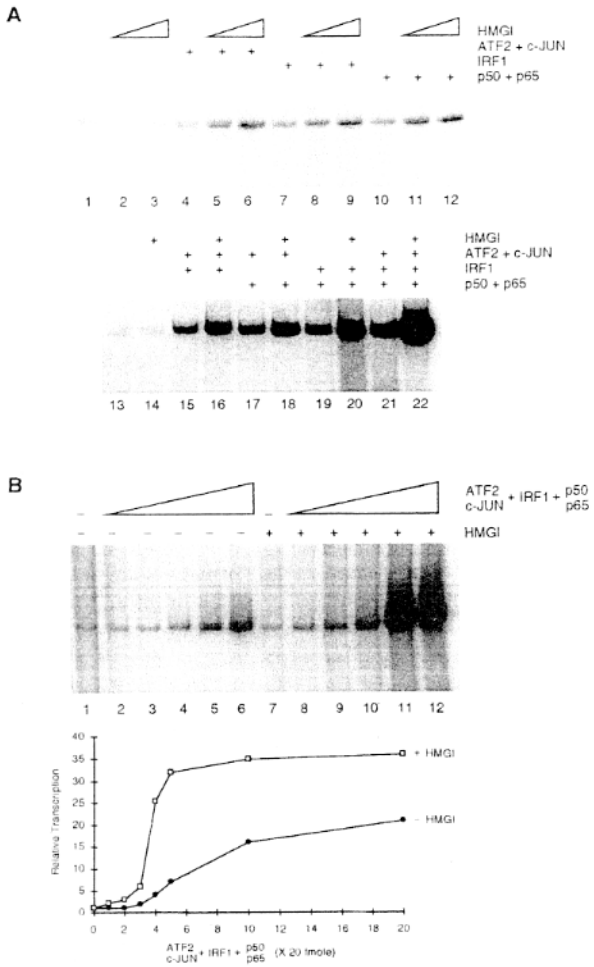

Figure 5. The Assembly of an IFNβ Enhanceosome Capable of Transcriptional Synergy Is Highly Cooperative

(A) Effect of HMG I(Y) on activation by each combination of PRD-binding units. Each of the indicated PRD-binding factors was used for in vitro transcription in the absence (lanes 1, 4, 7, and 10) or presence (lanes 2, 5, 8, and 11, 200 fmol; lanes 3, 6, 9, and 12, 400 fmol) of increasing amounts of HMG I(Y). Transcription reactions were performed in the absence or presence of each indicated combination of PRD-binding units (lanes 13–22).

(B) Effect of HMG I(Y) on the cooperative assembly of the enhanceosome. Increasing amounts of ATF2/c-JUN, IRF1, and p50/p65 were added to in vitro transcription reactions in the absence (lanes 2–6) or presence (lanes 8–12) of HMG I(Y) (400 fmol). The amounts of each recombinant PRD-binding factor were as follows: lanes 2 and 8, 20 fmol; lanes 3 and 9, 40 fmol; lanes 4 and 10, 60 fmol; lanes 5 and 11, 80 fmol; and lanes 6 and 12, 100 fmol. Relative levels of transcription were plotted against increasing amounts (multiples of 20 fmol) of PRD-binding activators in the absence or presence of HMG I(Y). Not shown was the transcription result with 200 or 400 fmol of PRD-binding factors.

sets of general transcription factors were included in the preincubation step. Preincubation of TFIID, USA, TFIIA, and TFIIB resulted in sarkosyl-resistant transcription in the presence of an IFNβ enhanceosome, but not when the IFNβ enhanceosome was added after sarkosyl addition (lanes 18 and 19). However, this sarkosyl-resistant transcription could not be detected when any one of the components tested (TFIID, USA, TFIIA, and TFIIB) was not added to the reaction mixture (lanes 4–17). Thus, at least four separate general factors are required to form a sarkosyl-resistant preinitiation complex in the presence of an IFNβ enhanceosome, consistent with the possibility that the enhanceosome promotes the cooperative assembly of a stable general transcription complex.

We have shown that HMG I(Y) is required for the assembly of an IFNβ enhanceosome required for synergistic transcriptional activation in vitro. We therefore examined the effect of HMG I(Y) on the formation of a sarkosyl-resistant preinitiation complex. As shown in Figure 6, when the enhanceosome was assembled in the absence of HMG I(Y) and preincubated with TFIID, USA, TFIIA, and TFIIB, sarkosyl-resistant transcription was observed (lanes 40 and 42). However, the level of transcription was significantly less than that observed when the enhanceosome was assembled in the presence of HMG I(Y) (compare lanes 19 and 21 with 40 and 42, respectively). As with the HMG I(Y)-containing enhanceosome, sarkosyl-resistant transcription with the enhancer complex assembled in the absence of HMG I(Y) required all four of the general transcription factors (TFIID, USA, TFIIA, and TFIIB; lanes 25–38). The increased activation potential of the IFNβ enhanceosome could therefore be due to the increased stability of the general transcription complex (TFIID–USA–TFIIA–TFIIB) in the presence of HMG I(Y).

TFIID, TFIIA, USA, and TFIIB Are Required to Form Oligonucleotide-Resistant Transcription Complexes Capable of Synergistic Activation

To gain further insights into the interactions between the enhanceosome and the general transcription apparatus, an oligonucleotide containing an IFNβ enhancer DNA was used to challenge the assembly of the preinitiation complexes and the IFNβ enhanceosome (Figure 7). Highly purified general transcription factors, as described in Figure 6, were preincubated in the absence or presence of an IFNβ enhanceosome. After 20 min of preincubation, a large molar excess of an IFNβ enhancer oligonucleotide was added to the transcription reaction mixtures along with the remaining factors and nucleotides. As expected, in the absence of an oligonucleotide, the IFNβ enhanceosome (ATF2/c-JUN, IRF1, HMG I[Y], and p50/p65) supported high levels of transcriptional activation (compare lanes 1 and 2). However, no transcription was detected when excess IFNβ enhancer competitor DNA was preincubated with the assembled enhanceosome and all of the general factors (TFIID, USA, TFIIA, TFIIB, TFIIE/F/H, and RNA polymerase II; lane 3). This inhibitory effect was not observed with a nonspecific control oligonucleotide (lane 4). If the IFNβ oligonucleotide was added after preincubation of an IFNβ enhanceosome with all of the general factors, the high level of activated transcription was observed (lane 5). These data show that preincubation of the IFNβ enhanceosome was required for the activated transcription that is resistant to the oligonucleotide competitor.

We then determined which of the general transcription factors are required for the enhanceosome-dependent, oligonucleotide-resistant activated transcription. Preincubation of the enhanceosome with TFIID, USA, TFIIA,

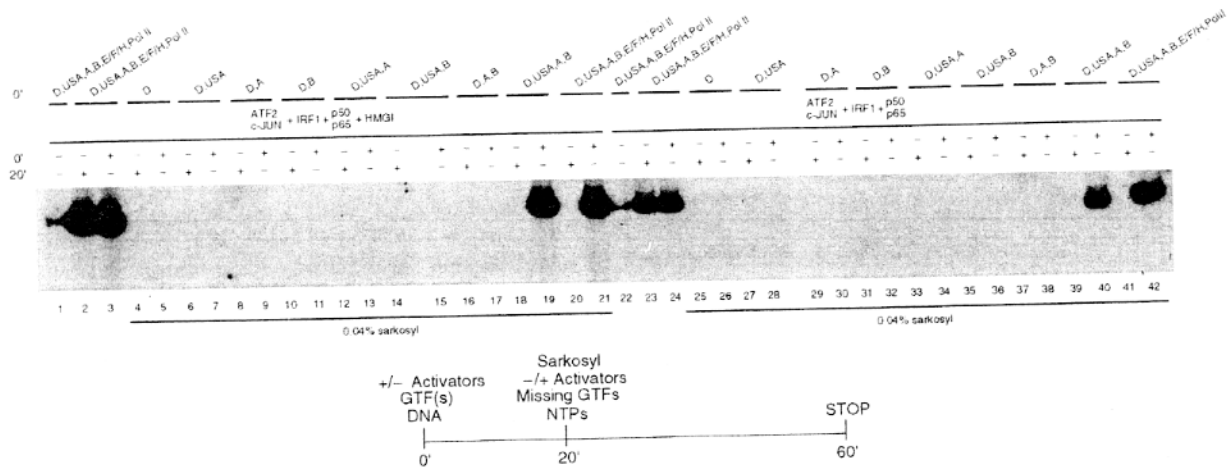

Figure 6. The IFNβ Enhanceosome Facilitates Formation of a Template-Committed General Transcription Complex Containing TFIID, TFIIA, TFIIB, and USA

At time zero (0'), the −110 IFNβ CAT template DNA was preincubated with the indicated general transcription factors in the presence or absence of PRD-binding activators (ATF2/c-JUN, IRF1, and p50/p65) containing HMG I(Y) (lanes 1–21) or no HMG I(Y) (lanes 22–42). At 20 min (20'), sarkosyl was added to a final concentration of 0.04% (lanes 4–21 and 25–42) along with the missing factors and nucleotide triphosphates. Complete sets of general transcription factors were incubated with PRD-binding factors containing HMG I(Y) or no HMG I(Y) in the absence of sarkosyl (lanes 1–3 and 22–24). Transcription reactions were stopped at 60 (60') and analyzed by primer extension. Abbreviations: D, TFIID; USA, Upstream Stimulatory Activity; A, TFIIA; B, TFIIB; E/F/H, TFIIE, TFIIF, and TFIIH; and Pol II, RNA polymerase II.

and TFIIB showed the high level of transcriptional activation resistant to the competitor DNA (lane 7). A somewhat higher level of transcription was observed in the presence of TFIIE/F/H along with these general factors (lane 6). Importantly, under these conditions, elimination of any one of four factors (TFIID, USA, TFIIA, and TFIIB) from the preincubation reaction resulted in complexes that were highly sensitive to oligonucleotide challenge (lanes 8–13). These data, in conjuction with the results of Figure 6, suggest that TFIID, USA, TFIIA, and TFIIB are all required for the cooperative assembly of an enhanceosome-dependent stable general transcription complex. Conversely, these observations are also consistent with the possibility that the general transcription factors TFIID, TFIIA, and TFIIB and the USA cofactor stabilize the enhanceosome against competition by the enhancer-containing oligonucleotide; that is, there can be a mutual stabilization between the two complexes.

Discussion

The Role of HMG I(Y) in Enhanceosome Assembly, Structure, and Function

Previous in vivo studies demonstrated that HMG I(Y) is required for virus induction of the IFNβ gene (Thanos and Maniatis, 1992). In this paper, we show that HMG I(Y) is required for the cooperative in vitro assembly and stability of the enhanceosome and that it is necessary for maximal levels of transcriptional synergy under conditions in which the transcriptional activator proteins are limiting. HMG I(Y) functions as an architectural protein by directly interacting with transcriptional activator proteins and by inducing conformational changes in DNA (reviewed by Bustin and Reeves, 1996). The requirement for specific protein–protein interactions is best illustrated by the observation that an isoform of ATF2 ($ATF2_{192}$), which cannot interact with HMG I(Y), is unable to participate in HMG I(Y)-dependent transcriptional synergy, even though it can bind to PRDIV (Du and Maniatis, 1994) and function as part of the IFNβ enhancer complex (Figure 3A).

HMG I(Y) has also been shown to partially reverse intrinsic bends in the IFNβ enhancer and to facilitate a further reversal of DNA bending by NF-κB and ATF2/c-JUN (Falvo et al., 1995). In vitro DNA binding studies have shown that HMG I(Y) promotes the binding of ATF2 and NF-κB to PRDIV and PRDII, respectively (Thanos and Maniatis, 1992; Du et al., 1993). Remarkably, the HMG I(Y) binding sites in the IFNβ promoter are located on the same face of the DNA helix, leading to intra- and intermolecular cooperative binding of HMG I(Y) (Maher and Nathans, 1996; Yie et al., 1997).

The importance of the positioning of the HMG I(Y) and transcription factor-binding sites on the DNA helix was clearly demonstrated by the analysis of helical phasing mutations in the IFNβ enhancer (Figure 3C). A high level of synergistic activation was not observed when the stereospecific alignment of the PRD elements was altered, or when HMG I(Y) was not added to the transcription reaction. Taken together, these observations reveal a critical and complex role for HMG I(Y) in the assembly and function of the IFNβ enhanceosome, including DNA binding and bending, cooperative binding, and the facilitation of protein–protein interactions.

The agreement between the effects of HMG I(Y) in our in vitro system and those previously observed in vivo is remarkable, as summarized in Table 1. The apparent exception to this is the magnitude of the effect of mutation in the HMG I(Y) DNA binding site within PRDII (Figure 3B). In earlier experiments, mutations in PRDII that decreased the affinity of HMG I(Y) resulted in a 10- to 20-fold decrease in the level of virus induction (Thanos and Maniatis, 1992). More recently, the effect of these mutations on virus induction in vivo was found to vary considerably from experiment to experiment, with an average effect of ~8-fold. On the other hand, we observed a <2-fold decrease in the level of transcription

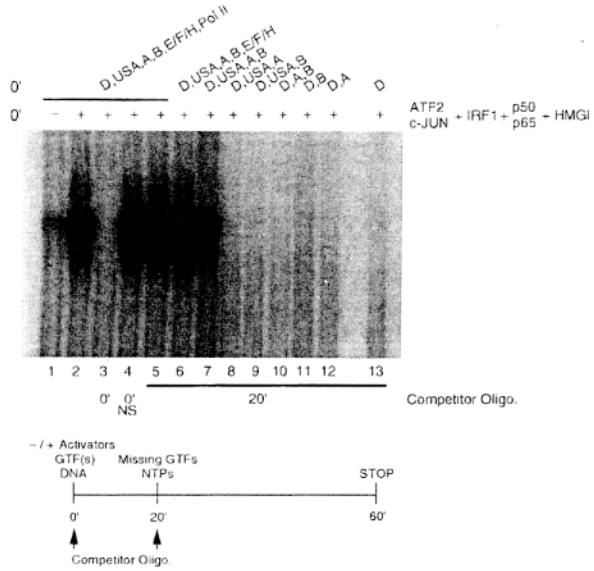

Figure 7. TFIID, TFIIA, TFIIB, and USA Are Required to Form the Oligonucleotide-Resistant General Transcription Complex and/or Enhanceosome

At time zero (0'), the −110 IFNβ CAT template DNA was preincubated with the indicated general transcription factors in the presence or absence of an assembled IFNβ enhanceosome (ATF2/c-JUN, IRF1, HMG I(Y), and p50/p65; lanes 1–13). At 20 min (20'), an oligonucleotide containing an IFNβ enhancer was added (lanes 5–13) along with the missing factors and nucleotide triphosphates. Lane 3 or 4 contained the IFNβ enhancer or nonspecific (GAL4 binding site-containing) competitor DNA at time zero (0'), respectively. Complete sets of general transcription factors were incubated with the IFNβ enhanceosome in the absence of a competitor DNA (lanes 1 and 2). Transcription reactions were stopped at 60 min (60') and analyzed by primer extension. Abbreviations: D, TFIID; USA, Upstream Stimulatory Activity; A, TFIIA; B, TFIIB; E/F/H, TFIIE, TFIIF, and TFIIH; and Pol II, RNA polymerase II.

with the PRDII mutant in vitro. At present, we do not fully understand the variability in vivo or the small effect observed in vitro. However, because of the complex role of HMG I(Y) in enhanceosome function, it is possible that the effects of DNA binding site mutations could vary depending on the relative concentrations of transcription activators and HMG I(Y) both in vivo or in vitro (Du and Maniatis, 1994). For example, higher concentrations of transcription factor could enhance the binding of HMG I(Y) to the mutant site through cooperative binding, thus compensating for the effect of the mutation.

Mechanisms of Transcriptional Synergy

Most previous studies of the mechanism of transcriptional synergy employed simple artificial promoters containing multiple DNA binding sites recognized by the same activator protein (reviewed by Ptashne and Gann, 1997). Recently, in vitro transcriptional synergy was observed between four distinct proteins that bind to the T cell receptor α gene enhancer (Mayall et al., 1997), but the mechanism of the synergy was not addressed. In this paper, we present a detailed in vitro analysis of the mechanism of transcriptional synergy of a complex natural multi-protein enhancer complex. We find that in the presence of HMG I(Y), the assembly of a functional IFNβ enhanceosome is highly cooperative (Figure 5). By contrast, in the absence of HMG I(Y), only a small degree of cooperativity was observed. The cooperativity observed with HMG I(Y) provides a highly sensitive on-off switch for IFNβ gene expression, capable of responding to small differences in the levels of transcriptional activator proteins. Examples in which shallow gradients of activator proteins mediate sharp boundaries of gene activation have been reported in vivo (Driever and Nüsslein-Volhard, 1989; Johnston and Nüsslein-Volhard, 1992) but have not readily been demonstrated in vitro (see Laybourn and Kadonaga, 1992 for a discussion).

Another mechanism for enhanceosome-dependent transcriptional synergy is the formation of a highly stable enhanceosome. Using oligonucleotide competition experiments, we have shown that functional enhanceosomes formed on the intact, wild-type enhancer DNA in the presence of HMG I(Y) are stable (Figure 4). By contrast, enhancer complexes assembled in the absence of HMG I(Y), or on enhancers containing DNA insertions that alter the helical phasing of protein binding sites, are relatively unstable. This lack of stability correlates with low levels of transcriptional synergy. Thus, we conclude that the cooperative assembly and enhanced stability of the enhanceosome contribute significantly to the transcriptional synergy observed with the IFNβ enhanceosome.

Table 1. Summary of the Effects of PRD-Binding Factors on Transcriptional Activation of Wild-Type and Mutant IFNβ Enhancers under In Vitro and In Vivo Conditions

	WT		Mutant	
PRD-binding factors	In Vitro	In Vivo	In Vitro	In Vivo
ATF2 + c-JUN	1.5	1.5	1.6	1.5
IRF1	2.1	2.0	2.1	2.0
p50 + p65	2.4	2.0	2.4	2.0
HMGI	1.0	1.0	0.9	1.0
ATF2 + c-JUN + IRF1 + p50 + p65	7.2	7.0	7.4	6.0
ATF2 + c-JUN + IRF1 + p50 + p65 + HMGI	31.1	41.0	9.5	4.0

The relative activation folds by each PRD-binding factor are presented. In vitro results are from transcriptional analyses of wild-type (WT) and mutant IFNβ enhancers. The mutant contains a 6 bp insertion between PRDI and II under the natural context of IFNβ enhancer. Shown is the average value of two independent experiments. A summary of in vivo results was obtained from transfection experiments (Thanos and Maniatis, 1995b). Increasing amounts of HMG I(Y) could give rise to ~70-fold activation in the presence of all of the other PRD-binding factors from wild-type IFNβ enhancer in vivo.

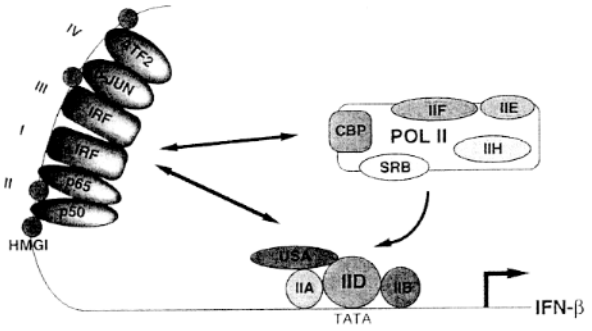

Figure 8. Model for the Enhanceosome-Dependent Transcriptional Synergy

A highly stable IFNβ enhanceosome (ATF2/c-JUN, IRF1, HMG I[Y], and p50/p65) is assembled cooperatively on DNA, resulting in the formation of a surface on which each of the activation domains can optimally interact with the TFIID–USA–TFIIA–TFIIB complex and with specific components of the RNA polymerase II holoenzyme (e.g., CBP). The cooperative interactions among the three complexes would then lead to the specific recruitment of the transcriptional apparatus to the promoter and the formation of a stable preinitiation complex.

Recruitment of General Transcription Factors to the Promoter by the IFNβ Enhanceosome

We have shown that the general transcription factors TFIID, TFIIA, and TFIIB and the cofactor USA are all required for the IFNβ enhanceosome-dependent assembly of a functional preinitiation complex (Figures 6 and 7). The formation of this complex is consistent with previous studies showing that many activators, including those that bind to the IFNβ enhancer, can interact with TFIID, USA, TFIIA, and/or TFIIB (reviewed by Burley and Roeder, 1996; Kaiser and Meisterernst, 1996). Transcriptional activator proteins have been shown to promote the binding of TFIID to the promoter and have been implicated in the stabilization (and/or isomerization) of the TFIID–DNA complex with TFIIA and TFIIB (reviewed by Roeder, 1996). For example, with promoters containing multiple binding sites for the ZEBRA activator, ZEBRA promotes the cooperative assembly of a TFIID–TFIIA complex on DNA. Once formed, the TFIID–TFIIA complex is capable of efficiently recruiting TFIIB to the complex (Chi et al., 1995 and references therein). Importantly, relevance of these activation-specific interactions has been confirmed by mutational analyses of TBP in the TFIID complex (e.g., Kim et al., 1994). The requirement for USA in the formation of a stable preinitiation complex is consistent with previous studies showing its requirement in activator-dependent transcription (Meisterernst et al., 1991). The USA is thought to function by promoting the formation of an active TFIID–TFIIA complex and by promoting interactions during later stages of preinitiation complex assembly, such as TFIIB recruitment (reviewed by Kaiser and Meisterernst, 1996).

The oligonucleotide competition experiments provide interesting insights into the preinitiation complex assembly process. The IFNβ enhanceosome assembled in the absence of the preinitiation complex components TFIID, USA, TFIIA, and TFIIB (target complex) is sensitive to disruption by an enhancer DNA oligonucleotide competitor (Figure 7). By contrast, the enhanceosome formed in the presence of the target complex is significantly more resistant to disruption by the competitor. Thus, the enhanceosome recruits the target complex to the promoter, but once bound the interactions between the two complexes may stabilize the enhanceosome. However, an alternative explanation of the competition experiments is that the enhanceosome is required only transiently in preinitiation complex assembly.

A striking aspect of the transcriptional synergy of the IFNβ enhanceosome is the requirement of a higher-order structure. This requirement could be a part of the mechanism of cooperative assembly and would likely result in a more stable complex. However, it is also possible that the formation of a specific structure creates a three-dimensional surface of the enhanceosome that facilitates optimal interactions between the activation domains of the bound proteins and the target general complexes. Evidence for this possibility is provided by studies of physical and functional interactions between the enhanceosome components c-JUN, IRF1, and p65 and the transcriptional coactivator protein CBP/p300 (Arias et al., 1994; Gerritsen et al., 1997; Merika et al., personal communication; Perkins et al., 1997). Recent transfection studies have implicated a role for CBP/p300 in enhanceosome-dependent transcriptional synergy in vivo (Merika et al., personal communication). In addition, we have directly shown that the IFNβ enhanceosome recruits CBP/p300 for high levels of transcriptional synergy in vitro (unpublished data). Thus, the IFNβ enhanceosome may form a specific activating surface that interacts optimally with general transcription factors and the CBP/p300 coactivator. These specific interactions could promote the formation of the target complex (TFIID–USA–TFIIA–TFIIB; Figures 6 and 7) and the recruitment of the RNA polymerase II holoenzyme (unpublished data), since CBP/p300 is thought to be a component of the mammalian holoenzyme (Nakajima et al., 1997).

Based on these observations, we propose a model for enhanceosome-dependent transcriptional synergy, shown in Figure 8. In this model, a highly stable IFNβ enhanceosome (ATF2/c-JUN, IRF1, HMG I[Y], and p50/p65) is assembled cooperatively on DNA, resulting in the formation of a surface on which each of the activation domains can optimally interact with the TFIID–USA–TFIIA–TFIIB complex and with specific components of the holoenzyme (e.g., CBP). The cooperative interactions among the three complexes would then lead to the highly specific recruitment of the transcriptional apparatus to the promoter and the formation of a stable preinitiation complex for high levels of transcriptional synergy.

Experimental Procedures

Expression in E. coli and Purification of IFNβ Transcription Factors

Bacterial strain BL21(DE3)pLysS was transformed with pET plasmids encoding $ATF2_{195}$, $ATF2_{192}$, c-JUN, HMG I(Y), IRF1, p50, and p65 proteins with a hexahistidine. Proteins were induced in bacteria with IPTG and were purified from cell lysates by nickel affinity chromatography as described (Thanos and Maniatis, 1992; Kim et al., 1994).

Purification of General Transcription Factors

General transcription factors TFIIA, TFIIE/F/H, and USA were fractionated from HeLa nuclear extracts by phosphocellulose (P11) chromatography (Dignam et al., 1983) and further purified as described (Meisterernst et al., 1991). RNA polymerase II was purified from HeLa nuclear pellet extracts by chromatography through Heparin-Sepharose, DEAE-cellulose, and Mono Q columns (Meisterernst et al., 1991). Flag-tagged TFIID was purified by affinity chromatography from stably transfected HeLa cells (Chiang et al., 1993). Recombinant TFIIB was expressed as a hexahistidine-tagged protein from the pET plasmid in E. coli strain BL21(DE3)pLysS and was purified by nickel affinity chromatography (Kim et al., 1995).

In Vitro Transcription Assays

The in vitro transcription reactions were performed with −110 IFNβ CAT DNA template (or mutant enhancer templates) and depleted HeLa nuclear extracts (or purified general transcription factors) in the presence or absence of PRD-binding factors (Kim and Roeder, 1997). Endogenous IFNβ enhancer- (PRD-) binding factors were depleted from HeLa nuclear extracts by DNA affinity chromatography, and HMG I(Y) was further depleted by anti-HMG I(Y) antibody chromatography (Kim and Roeder, 1994). The amounts of each recombinant PRD-binding factor were as follows (otherwise, they are indicated in the figures): ATF2, c-JUN, IRF1, p50, and p65, 100 fmol and HMG I(Y), 400 fmol. The experiments with sarkosyl or oligonucleotide competitors were performed as described (Hawley and Roeder, 1985; Hai et al., 1988). The transcription signals were analyzed by primer extension and quantitated with a phosphorimager.

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