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The role of HMG I(Y) in the assembly and function of the IFN-β enhanceosome

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Transcriptional activation of the virus inducible enhancer of the human interferon-β (IFN-β) gene in response to virus infection requires the assembly of an enhanceosome, consisting of the transcriptional activators NF-κB, ATF-2/c-Jun, IRFs and the architectural protein of the mammalian high mobility group I(Y) [HMG I(Y)]. Here, we demonstrate that the first step in enhanceosome assembly, i.e. HMG I(Y)-dependent recruitment of NF-κB and ATF-2/c-Jun to the enhancer, is facilitated by discrete regions of HMG I and is mediated by allosteric changes induced in the DNA by HMG I(Y) and not by protein–protein interactions between HMG I(Y) and these proteins. However, we show that completion of the enhanceosome assembly process requires protein–protein interactions between HMG I(Y) and the activators. Finally, we demonstrate that once assembled, the IFN-β enhanceosome is an unusually stable nucleoprotein structure that can activate transcription at high levels by promoting multiple rounds of reinitiation of transcription.

Keywords: enhanceosome assembly/HMG I(Y)/IFN-β/protein–protein interaction/transcriptional activator

Introduction

One of the key questions in understanding eukaryotic gene regulation is to explain how a relatively small number of transcriptional regulatory proteins functions to achieve the enormous diversity in gene expression required for the development of complex eukaryotic organisms. A solution to this apparent paradox lies in the ability of transcription factors to employ the principles of cooperativity and synergism; that is, the level of transcription elicited by combinations of transcription factors is significantly greater than the sum of the levels elicited by each single factor. The mixed assembly of transcription factors on regulatory elements via multiple interacting surfaces allows for the formation of unique nucleoprotein complexes in response to specific extracellular signals. Thus, cooperative interactions between a limited set of transcription factors can lead to a tremendous degree of specificity in gene activation and to a high level of transcription (reviewed in Tjian and Maniatis, 1994; Carey, 1998).

The virus inducible enhancer of the human interferon-β (IFN-β) provides one of the best-characterized examples of combinatorial interactions between distinct regulatory elements (reviewed in Thanos *et al*., 1993). This enhancer is bound by NF-κB, ATF-2/c-Jun and interferon regulatory factor (IRF) proteins, which recognize PRDII, PRDIV and PRDIII-I, respectively. Synthetic enhancers bearing multiple copies of each of these elements display unusually high basal levels of activity, are less inducible than the natural enhancer and respond to several other inducers in addition to virus infection. However, the intact IFN-β enhancer is highly inducible only following virus infection. The highly specific activation of the IFN-β gene in response to virus infection is the result of the assembly of coordinately activated transcription factors on the enhancer into a higher order nucleoprotein complex, termed the enhanceosome (Thanos and Maniatis, 1995a). Thus, virus-specific enhanceosome assembly provides the fail-safe mechanism for ensuring that the IFN-β gene is activated only by virus infection and not by the many other stimuli that can separately induce each of the activators. This finely tuned specificity in gene expression is directed by the high mobility group protein HMG I(Y), which functions as the essential architectural component required for the assembly of the IFN-β gene enhanceosome (Thanos and Maniatis, 1992, 1995a; Du *et al*., 1993; Falvo *et al*., 1995; Kim and Maniatis, 1997; Yie *et al*., 1997).

The mammalian HMG I(Y) family of proteins consists of three members: HMG I, HMG Y and HMG I-C. HMG I and HMG Y are encoded by the same gene and are generated through alternative RNA splicing, whereas a separate gene encodes HMG I-C (reviewed in Bustin and Reeves, 1996). The HMG I proteins bind specifically to the minor groove of AT-rich regions of DNA via three short basic repeats containing the core motif GRGRP or PRGRP (Geierstanger *et al*., 1994; Huth *et al*., 1997; Yie *et al*., 1997; Frank *et al*., 1998). Similar short basic repeats have been found in other proteins from bacteria, yeast, plants, insects and mammals (Bustin and Reeves, 1996). Disruption of the HMG I genes directly correlates with tumorigenesis and a null mutation of HMG I-C in mice results in the pygmy phenotype (Ashar *et al*., 1995; Schoenmakers *et al*., 1995; Zhou *et al*., 1995). Recent studies have established that the middle basic repeat of HMG I(Y) provides the basis for specific DNA binding and that the presence of the first or third repeat together with the middle repeat results in high affinity binding of a single HMG I(Y) molecule to a pair of binding sites at PRDIV and PRDII/NRDI, respectively (Figure 2A; Yie *et al*., 1997). Thus, within each pair of binding sites, high affinity DNA binding of HMG I(Y) requires intramolecular cooperative interactions. However, protein–protein interactions between these two molecules are necessary for high affinity binding of the two HMG I(Y) molecules to the intact IFN-β gene enhancer (Yie *et al*., 1997). Binding

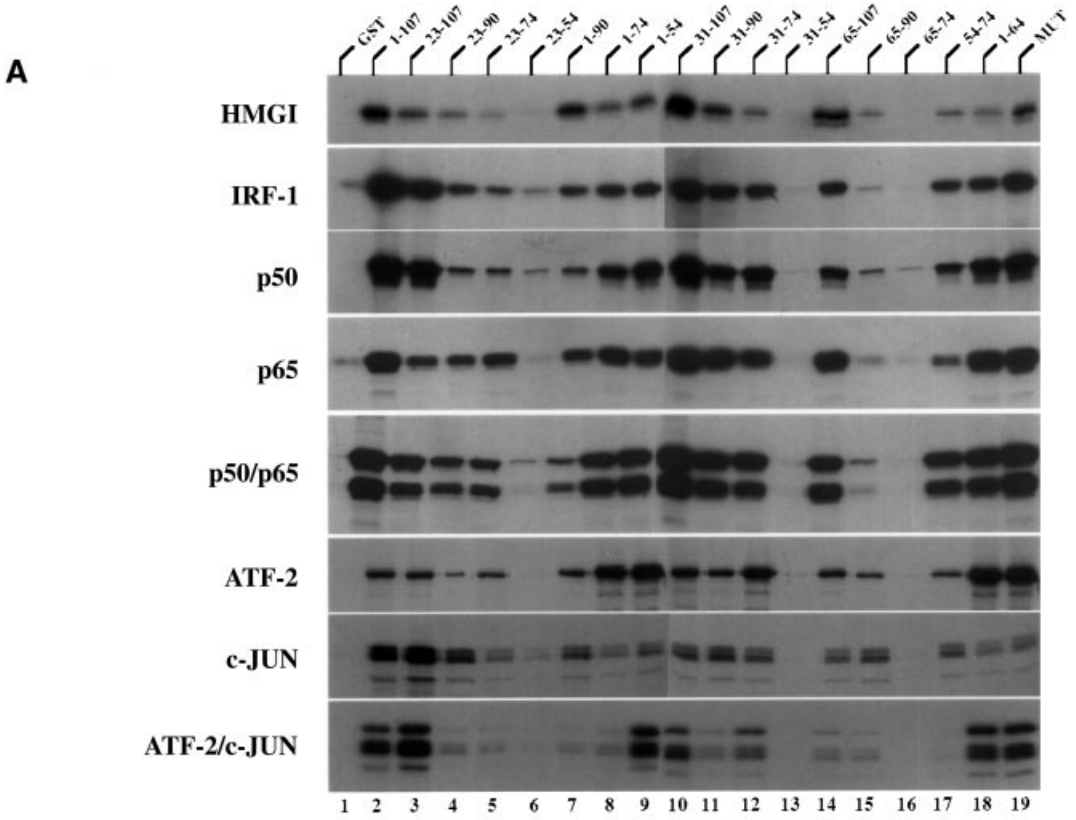
of HMG I(Y) to the enhancer alters the structure of the DNA (Falvo *et al*., 1995), allowing cooperative recruitment of the IFN-β gene activators which, together with HMG I(Y), assemble into the enhanceosome (Thanos and Maniatis, 1995a). As a result of enhanceosome assembly, the activation domains of the activators create a novel activating surface which, in turn, recruits CREB-binding protein (CBP) and CBP-associated proteins or complexes such as P/CAF (Merika *et al*., 1998; Munshi *et al*., 1998) and the *pol*II holoenzyme (Kim *et al*., 1998; our unpublished observations). Simultaneously, the activation domains also establish contacts with other components of the basal machinery. Access of the basal machinery to the promoter may be facilitated by the histone acetyltransferase activities of CBP and P/CAF via acetylation of histones in nearby nucleosomes. In fact, we have shown that the histone acetyltransferase activities of both CBP and P/CAF are required for activation of transcription from the enhanceosome *in vivo* (Munshi *et al*., 1998). Remarkably, acetylation of HMG I(Y) by CBP, but not by P/CAF, results in a decrease in its DNA binding affinity and its subsequent detachment from the enhanceosome, thus causing enhanceosome disruption and termination of IFN-β gene transcription (Munshi *et al*., 1998). Therefore, HMG I(Y) functions as the sensitive molecular switch that triggers assembly and subsequent disassembly of the enhanceosome, thus resulting in activation and postinduction repression of IFN-β gene expression. HMG I(Y) is believed to exert these effects by orchestrating a complex network of protein–DNA and protein–protein interactions that control assembly, stability and function of the IFN-β enhanceosome. However, a direct biochemical proof for HMG I(Y)'s molecular role in this model has not been obtained because the regions of HMG I(Y) required for these functions were not known.

The distinct arrangement and topology of the HMG I(Y) basic repeats on the different sites within the IFN-β enhancer suggests that different regions of the protein might be engaged in optimal protein–protein and protein–DNA interactions with each of the proximally bound IFN-β gene activators. Here we show that HMG I(Y) bears three independent protein–protein interaction surfaces that are differentially used to recruit NF-κB and ATF-2/c-Jun into the enhanceosome. Surprisingly, we demonstrated that the first step in enhanceosome assembly, i.e. recruitment of NF-κB and ATF-2/c-Jun to the enhancer, is mediated by allosteric changes induced in the DNA by HMG I(Y) and not by protein–protein interactions between HMG I(Y) and these proteins. However, we demonstrate that completion of the enhanceosome assembly process requires protein–protein interactions between HMG I(Y) and the activators. Finally, we show that once assembled the IFN-β enhanceosome is an unusually stable nucleoprotein structure that can activate transcription at high levels and can promote multiple rounds of reinitiation of transcription.

Results

Distinct regions in HMG I(Y) are involved in interactions with the IFN-β gene activators

To identify the regions in HMG I(Y) involved in protein–protein interactions with the IFN-β gene activators, we carried out glutathione *S*-transferase (GST)-pull down and Far Western protein–protein interaction experiments using a series of HMG I deletion derivatives and *in vitro* translated ^{35}S-labeled IFN-β gene activators (Figure 1). The conclusions from these experiments are summarized as follows. In the context of the full-length protein, the N-terminus of HMG I is required for maximal interactions with itself, NF-κB (p50/p65 heterodimer) and IRF-1, but is not critical for interactions with ATF2/c-Jun (Figure 1B, compare lane 2 with lane 3). On the other hand, the acidic C-terminus is critical for association with ATF2/c-Jun, and for high affinity interactions with NF-κB and IRF-1, whereas it is not required for association of HMG I with itself (Figure 1B, compare lane 2 with lane 7). We found that the minimal region of HMG I (aa 54–74) required for interactions with NF-κB (p50/p65 heterodimer) includes the middle basic repeat and the region between the middle and the last basic repeats (Figure 1A, lane 17, and Figure 1B). In contrast, p50 and p65 homodimers separately interact with this region only weakly (Figure 1A, lane 17, and Figure 1B). In addition, this fragment of HMG I does not interact with the ATF-2/c-Jun heterodimer, although it can weakly associate with ATF2 or c-Jun homodimers separately (Figure 1A, lane 17). Thus, heterodimerization of the IFN-β gene activators results in the exposure of unique protein–protein interaction surfaces, which optimally interact with defined surfaces of HMG I. These unique patches on the surface of the heterodimeric activators appear to be different from those used by each homodimeric partner separately. The latter point is also illustrated by the fact that the minimal region of HMG I that associates with the ATF-2/c-Jun heterodimer (HMG $I_{1–54}$) interacts only weakly with c-Jun homodimers (Figure 1A, lane 9). Thus, in this case, high affinity binding of HMG I to ATF-2 suffices for interaction with the ATF-2/c-Jun heterodimer. However, this is not always the case since HMG $I_{1–74}$ interacts efficiently with ATF-2 but does not interact with the ATF-2/c-Jun heterodimer (Figure 1A, lane 8). Thus, the region of HMGI spanning aa 55–74 negatively affects interactions specifically with the ATF-2/c-Jun heterodimer. The minimal region of HMG I required for association with itself contains the last basic repeat and the carboxyl acidic tail of the protein (Figure 1A, lane 14). In contrast, high affinity interaction of HMG I with IRF-1 requires multiple overlapping regions spanning aa 31–107 (lane 10). Based on these experiments we conclude that HMG I bears multiple overlapping protein–protein interaction surfaces and that each surface is composed of a basic repeat flanked by neighboring sequences. We also found that in the context of each minimal interacting surface, removal of the flanking regions strongly decreased protein–protein interactions (compare lane 9 with 6 for all the activators, lane 12 with 17 for all the activators except p50/p65 heterodimer, and lane 14 with 15 for all the activators except ATF-2/c-Jun heterodimer and c-Jun homodimers). Thus, the basic repeats of HMG I(Y), although they are required, are not sufficient for protein–protein interactions. Importantly, similar results were obtained when the experiments were carried out in the presence of either ethidium bromide or IFN-β enhancer oligonucleotides, thus excluding the possibility that the binding site DNA may affect the strength of protein–protein interactions (data not shown). Taken together, our experiments strongly suggest that



B

	HMGI	IRF-1	p50	p65	p50/p65	ATF-2	c-JUN	ATF-2/ c-JUN
WT 1-107	+++	+++	+++	+++	+++	+++	+++	+++
23-107	+	++	+++	++	++	+++	+++	+++
23-90	+	+	+	++	++	++	++	+/-
23-74	+/-	+	+	++	++	++	+	+/-
23-54	-	-	-	-	-	-	-	-
1-90	+++	+	+	+	+	+++	+	+/-
1-74	+	++	++	++	+++	+++	+	+/-
1-54	++	++	++	++	+++	+++	+	+++
31-107	+++	+++	+++	+++	+++	+++	++	+++
31-90	++	++	++	+++	+++	+++	++	+
31-74	+	++	++	++	+++	+++	++	++
31-54	-	-	-	-	-	-	-	-
65-107	+++	++	++	+++	+++	+++	++	+/-
65-90	+	-	+/-	-	+/-	++	++	+/-
65-74	-	-	-	-	-	-	-	-
54-74	+	++	++	+	+++	++	++	-
1-64	+	++	+++	+++	+++	+++	+	+++
MUT	++	+++	+++	+++	+++	+++	+	+++

Fig. 1. Distinct regions in HMG I(Y) are required for interactions with each of the IFN-β gene activators and with itself. (**A**) Results of protein–protein interaction experiments using the GST–HMG I derivatives indicated on the top of the autoradiogram, which were incubated with *in vitro* translated ^{35}S-labeled HMG I, IRF-1, p50 homodimers, p65 homodimers, p50/p65 heterodimer, ATF-2 homodimers, c-Jun homodimers and ATF-2/c-Jun heterodimer. Specifically bound proteins were analyzed by PAGE and visualized by autoradiography. The p50/p65 and ATF-2/c-Jun heterodimers were generated by cotranslation of expression vectors for p50 plus p65 and ATF-2 plus c-Jun. The extent of heterodimerization was almost 100% as revealed by EMSA analysis (data not shown). (**B**) Summary of the protein–protein interaction experiment shown in (A). The three basic repeats in HMG I and the carboxyl acidic tail are depicted in the HMG I diagram as + and –, respectively.

different regions of the HMG I protein are engaged in optimal protein–protein interactions with each of the IFN-β gene activators.

HMG I recruits ATF2/c-Jun and NF-κB to the enhancer in the absence of protein–protein interactions

One of the roles of HMG I(Y) is to enhance the DNA binding of NF-κB and ATF-2/c-Jun to the enhancer, thus promoting enhanceosome assembly. We have shown previously that high affinity binding of HMG I to the enhancer is mediated by the middle repeat in cooperation with either the first or the last repeats depending on the nature of the binding sites (Yie *et al*., 1997). To investigate the role of intramolecular cooperative DNA binding as well as the contribution of protein–protein interactions between HMG I and the activators in recruitment of NF-κB and ATF-2/c-Jun to the enhancer, we carried out electrophoretic mobility shift assay (EMSA) experiments using several HMG I derivatives along with NF-κB or ATF-2/c-Jun. Figure 2 demonstrates that full-length HMG I protein stimulates the DNA binding activity of NF-κB (Figure 2B, compare lanes 1–5 with 6–10), ATF-2/c-Jun heterodimer (Figure 2C, compare lanes 1–4 with 9–12) and ATF-2 homodimers (Figure 2C, compare lanes 5–8 with 13–16) to the enhancer, a result consistent with previous experiments (Thanos and Maniatis, 1992; Du *et al*., 1993; Himes *et al*., 1996; Mantovani *et al*., 1998). Remarkably, HMG $I_{1–74}$, which does not interact with the ATF-2/c-jun heterodimer (Figure 1), enhances its DNA binding activity to the same degree as the wild-type protein (Figure 2C, compare lanes 1–4 with 17–20). In contrast, this derivative does not facilitate NF-κB DNA binding (Figure 2B, compare lanes 1–5 with 31–35), although it can interact with NF-κB (Figure 1). Furthermore, the two HMG I derivatives that contain the middle and the last basic repeats with or without the C-terminal acidic tail, can both stimulate the DNA binding activity of NF-κB (Figure 2B, compare lanes 51–55 with 56–60 for HMG $I_{31–107}$, and lanes 41–45 with 46–50 for HMG $I_{31–90}$), but they have no effect on ATF-2/c-Jun or ATF-2 homodimer DNA binding activity (Figure 2C, compare lanes 1–8 with 33–48). We have previously shown that HMG $I_{1–74}$ contacts PRDIV via intramolecular cooperative interactions where the first and the middle basic repeats simultaneously contact the pair of HMG I binding sites present at PRDIV (Yie *et al*., 1997). Similarly, the middle and the last basic repeats in HMG $I_{31–90}$ and HMG $I_{31–107}$ simultaneously contact the pair of HMG I binding sites at PRDII and NRDI (Figure 2A; Yie *et al*., 1997). Thus, optimal enhancement of activator DNA binding correlates with the ability of HMG I to bind via intramolecular cooperative interactions to the enhancer and not with its ability to interact with the activators. In support of this conclusion we show that HMG $I_{65–107}$ and HMG $I_{1–54}$ did not enhance NF-κB (Figure 2B, compare lanes 1–5 with 11–20) or ATF-2/c-Jun DNA binding (data not shown). Since both derivatives associate with NF-κB (Figure 1), we conclude that protein–protein interactions between HMG I and NF-κB do not suffice for stimulation of NF-κB's DNA binding activity. Interestingly, HMG $I_{31–74}$, which contains the middle basic repeat only, stimulates NF-κB and ATF-2 homodimer DNA binding, albeit with lower efficiency, but it does not enhance ATF-2/c-Jun heterodimer DNA binding (Figure 2B, lanes 31–40, and Figure 2C, compare lanes 1–8 with 25–32). Taken together, our experiments reveal that distinct regions of HMG I are required for recruitment of NF-κB and ATF-2/c-Jun to the IFN-β gene enhanceosome. In addition, activator recruitment by HMG I(Y) correlates with the ability of HMG I to bind to the enhancer via intramolecular cooperative interactions and not with protein–protein interactions between HMG I(Y) and the activators.

HMG I is required for the assembly and function of a transcriptionally competent IFN-β enhanceosome

To investigate the role of HMG I in the activation of the IFN-β gene, we carried out *in vitro* transcription experiments using HeLa nuclear extracts with recombinant activators and HMG I. Figure 3A shows that addition of a small amount of either NF-κB, IRF-1 or ATF-2/c-Jun stimulates transcription only weakly (compare lane 1 with 2–4). Furthermore, simultaneous addition of all the activators at the same amounts also did not result in synergistic activation of transcription (lane 5). In sharp contrast however, when HMG I was added along with the same amounts of the activators, a 29-fold increase in transcription was observed (lane 6). Previous studies have established that under these conditions HMG I(Y) promotes the assembly of the enhanceosome (Thanos and Maniatis, 1995a; Kim and Maniatis, 1997; Merika *et al*., 1998; Munshi *et al*., 1998). Importantly, similar results were obtained using HeLa nuclear extracts depleted of HMG I(Y) and endogenous IFN-β gene activators by oligonucleotide affinity chromatography (data not shown). Furthermore, high levels of enhanceosome-dependent transcription were also obtained when IRF-3 plus IRF-7 or IRF-7 alone (compare lane 10 with 11, and lane 13 with 14, respectively) substituted for IRF-1. Curiously, IRF-3 alone (without IRF-7) cannot participate in enhanceosome formation (data not shown). The latter proteins have been recently implicated in virus induction of the IFN-β gene (Lin *et al*., 1998; Marie *et al*., 1998; Sato *et al*., 1998; Wathelet *et al*., 1998; Yoneyama *et al*., 1998). Thus, a functional IFN-β enhanceosome can be assembled with either IRF-1, IRF-3 plus IRF-7 or IRF-7 alone. Interestingly, the observed redundancy between IRF family members in enhanceosome assembly and function contrasts with the strict requirement of specific combinations of Rel and bZip proteins (p50/p65 and ATF-2/c-Jun heterodimers) for efficient incorporation into the enhanceosome (Du *et al*., 1993; Thanos and Maniatis, 1995a,b).

The molecular role of HMG I in the synergistic activation of transcription from the IFN-β enhanceosome was revealed in activator titration experiments where we compared the levels of transcriptional activation by NF-κB, IRF-1 and ATF-2/c-Jun in the absence or presence of HMG I. Figure 3B shows that each of the IFN-β gene activators cannot stimulate transcription significantly, even when present at high amounts (compare lane 1 with lanes 2–10). This observation is in agreement with previous transfection experiments demonstrating a requirement of all the activators for virus induction of the IFN-β gene (Thanos and Maniatis, 1995a). Furthermore, addition of all the activators maximally stimulated transcription 16-fold only when the proteins were added at the highest

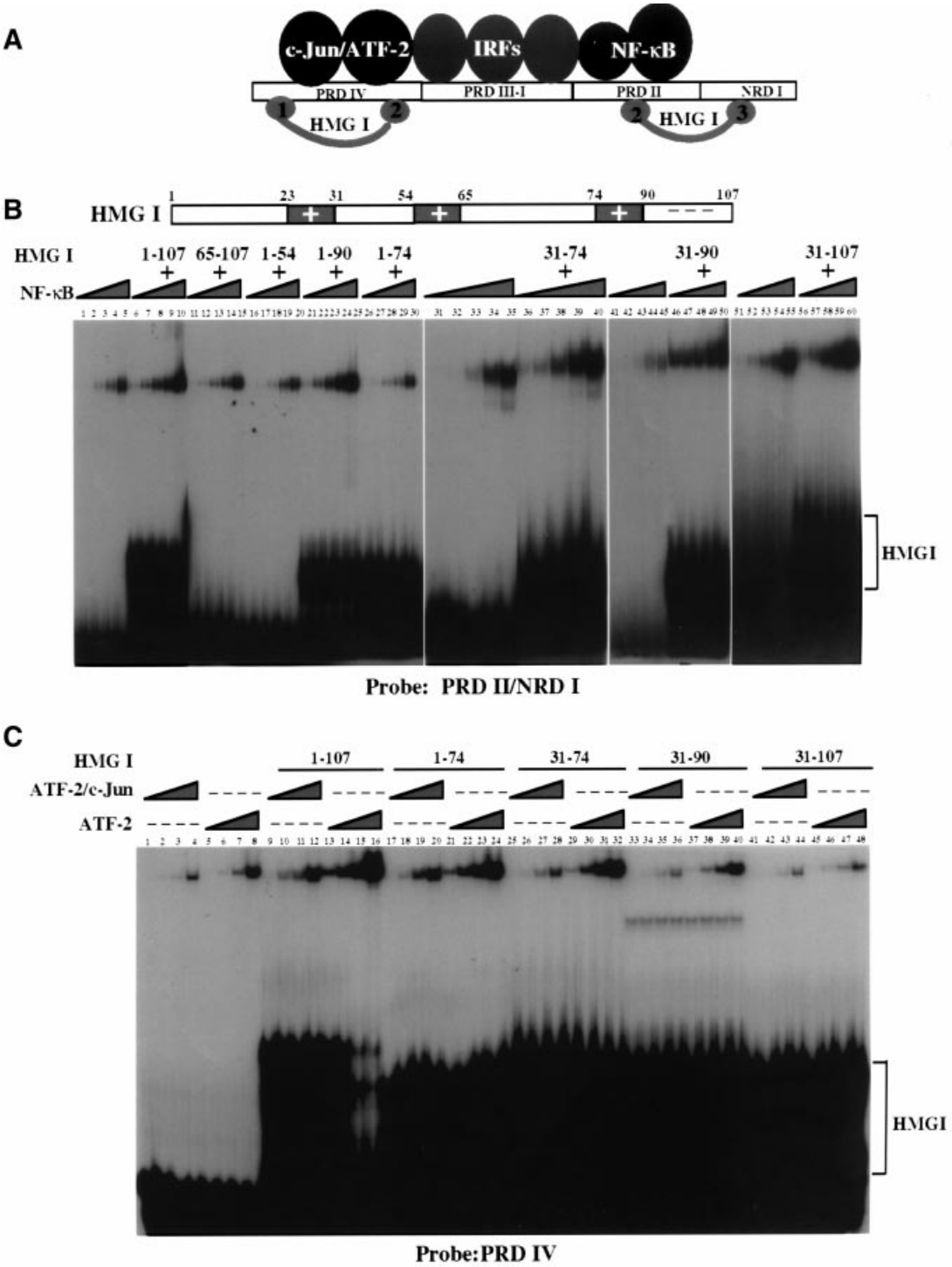


Fig. 2. Distinct regions of HMG I are used to recruit ATF-2/c-Jun and NF-κB to the enhancer. (**A**) Diagram of the IFN-β gene promoter. The enhancer region contains four positive regulatory domains (PRDI through PRDIV) bound by the transcription factors indicated. One molecule of HMG I(Y) binds to the pair of sites at PRDIV by using the first and middle basic repeats whereas a second HMG I molecule binds to the PRDII/NRDI region by using the middle and third basic repeats. One of the two possible orientations of HMG I(Y) is shown. (**B**) An EMSA experiment, using the PRDII/NRDI oligonucleotide as a probe along with increasing amounts of NF-κB either in the absence (lanes 1–5, 31–35, 41–45 and 51–55) or in the presence of the HMG I derivatives indicated, is shown. The amounts of proteins used were as follows; NF-κB: 0.05, 0.1, 0.2, 0.5 and 1 ng. Each of the HMG I derivatives was added at 20 ng. (**C**) An EMSA experiment using the PRDIV oligonucleotide as a probe along with increasing amounts of ATF-2/c-Jun (lanes 1–4, 9–12, 17–20, 25–28, 33–36, 41–44) or ATF-2 homodimers (lanes 5–8, 13–16, 21–24, 29–32, 37–40, 45–48) either in the absence (lanes 1–8) or in the presence (lanes 9–48) of the HMG I derivatives indicated. The amounts of proteins used were as follows; ATF-2/c-Jun and ATF-2 homodimers: 0.2, 0.5, 1 and 2 ng. Each of the HMG I derivatives was added at 200 ng.

amounts (lane 13). However, addition of HMG I under the same conditions resulted in a significant enhancement of transcription at lower activator concentrations (compare lane 12 with 16). HMG I on its own did not affect the basal levels of transcription (compare lane 1 with 14). The fact that HMG I exerts the greatest stimulatory effects

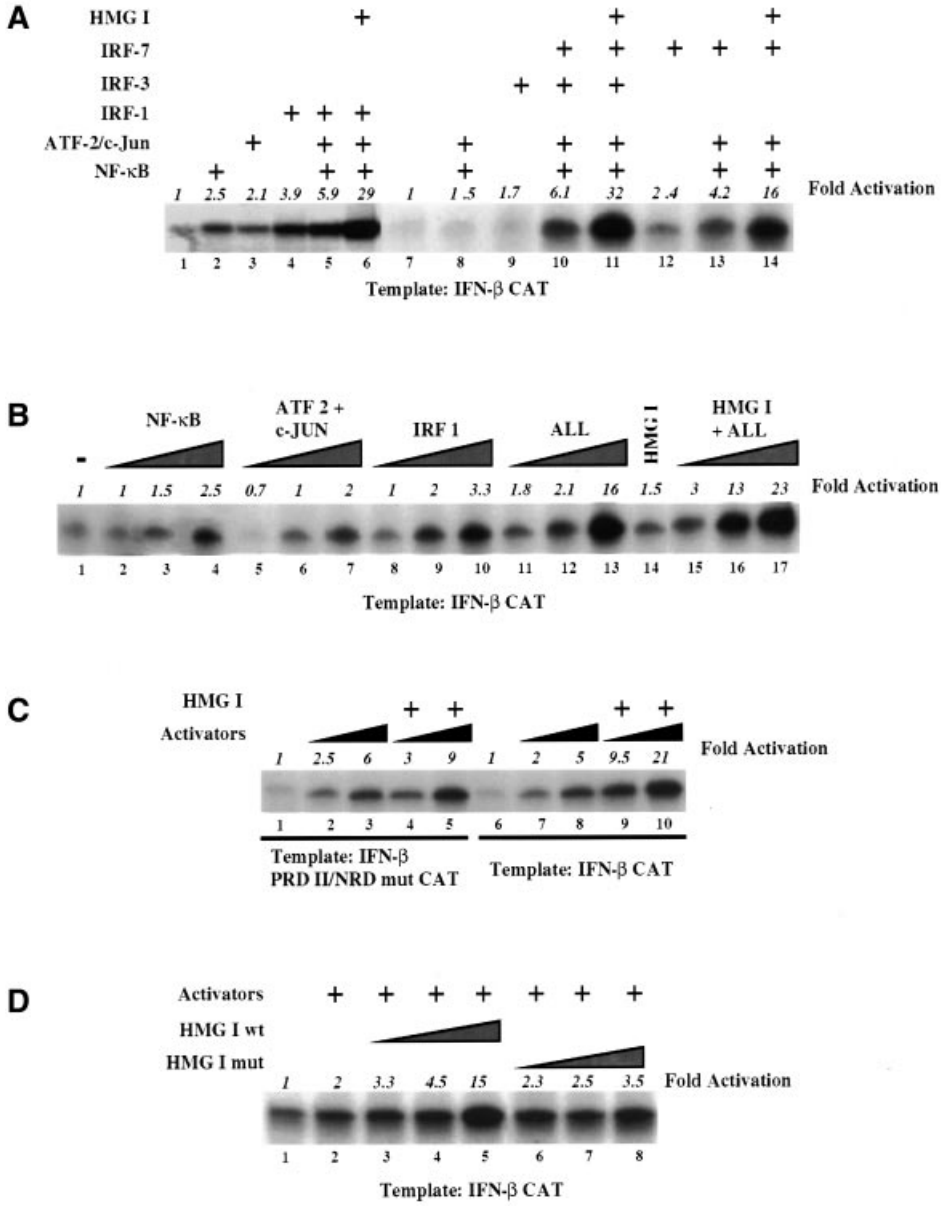


Fig. 3. HMG I(Y) is required for the assembly of a transcriptionally competent enhanceosome. (**A**) Results of an *in vitro* transcription experiment using HeLa nuclear extracts and the –110 IFN-β CAT as template DNA in the absence or the presence of the indicated recombinant proteins. The amounts of proteins used were as follows: NF-κB, 100 ng; ATF-2/c-Jun, 200 ng; IRF-1, 200 ng; HMG I, 100 ng; IRF-3, 100 ng; IRF-7, 50 ng. Correctly initiated transcripts were identified by primer extension using a CAT primer. Lanes 1–6: the basal level of transcription obtained in the absence of activators (lane 1) was set as fold activation 1. Similarly, for lanes 7–14, fold activation 1 is the amount of transcription obtained in the absence of activators (lane 7). (**B**) Results of an *in vitro* transcription experiment performed as in (A) but using increasing amounts of each activator either alone or in combination in the absence or in the presence of HMG I. The following amounts of proteins were used: NF-κB, 30, 100 and 300 ng; ATF-2/c-Jun, 50, 150 and 500 ng; IRF-1, 50, 150 and 500 ng. HMG I was added at 200 ng. (**C**) Results of an *in vitro* transcription experiment using either the wild-type IFN-β CAT template (lanes 6–10) or the template which contains the HMG I binding sites at PRDII and NRDI mutated (lanes 1–5). Activators were added at 100 ng each (lanes 2, 4, 7 and 9) and 250 ng each (lanes 3, 5, 8 and 10). HMG I was added at 200 ng. (**D**) *In vitro* transcription reactions were carried out as in (A) but using increasing amounts of either wild-type HMG I (lanes 3–5) or HMG Imut (lanes 6–8). Both proteins were added at 30, 100 and 300 ng.

at low activator concentrations is consistent with previous experiments suggesting that HMG I promotes the cooperative assembly of an IFN-β gene enhanceosome (Thanos and Maniatis, 1995a; Kim and Maniatis, 1997; Munshi *et al*., 1998).

To investigate whether high affinity cooperative DNA binding of HMG I to the IFN-β enhancer is critical for the assembly of a transcriptionally active enhanceosome, we used enhancer templates that bear mutations in the pair of HMG I binding sites present at PRDII and NRDI. We have shown previously that these mutations significantly decrease HMG I binding to the enhancer, and virus inducibility *in vivo* (Yie *et al*., 1997). Figure 3C shows that in the absence of HMG I, the IFN-β activators stimulate transcription at similar levels from both templates (compare lanes 2 and 3 with 7 and 8), a result consistent with previous experiments indicating that these mutations do not affect activator DNA binding (Thanos and Maniatis, 1992; Yie *et al*., 1997). Addition of HMG I strongly activated transcription from the wild-type IFN-β enhancer,

but it did not significantly affect activation from the mutant enhancer (Figure 3C). Thus, high affinity cooperative binding of HMG I to the DNA is critical for enhanceosome assembly and function. The latter point was also verified by using an HMG I derivative (HMG Imut) that contains amino acid substitutions in the region between the second and the third basic repeats of the protein. Although HMG Imut binds DNA with a 10- to 20-fold lower affinity compared with wild-type HMG I (Munshi *et al*., 1998; data not shown), it can fully associate with all the IFN-β gene activators (Figure 1B, lane 19). As seen in Figure 3D, HMG Imut fails to support enhanceosome-dependent transcription in the same conditions under which the wild-type HMG I promotes enhanceosome assembly and function (compare lanes 3–5 with 6–8). Taken together, our experiments strongly suggest that binding of HMG I to the enhancer is critical for the assembly of a functional IFN-β enhanceosome, and that protein–protein interactions between HMG I and the activators do not suffice for enhanceosome assembly.

Protein–protein interactions between HMG I(Y) and the IFN-β activators are critical for completion of the enhanceosome assembly process

To examine the role of distinct regions of HMG I in the assembly of a functional enhanceosome, we tested several HMG I derivatives for their ability to facilitate enhanceosome assembly *in vitro* and *in vivo*. First, enhanceosome assembly was investigated by DNase I footprinting experiments where we measured recruitment of IRF-1 to the IFN-β enhancer in the presence or the absence of NF-κB, ATF-2/c-Jun and HMG I(Y), as described previously (Thanos and Maniatis, 1995). Figure 4A shows that increasing amounts of IRF-1 bind to the PRDIII-I element in a dose-dependent manner (lanes 4–9). When the same amounts of IRF-1 were titrated in the presence of a fixed amount of ATF-2/c-Jun and NF-κB (lanes 2, 3 and 10) a small decrease in IRF-1's DNA binding activity was observed (compare lanes 4–9 with 11–16). We have shown previously that this decrease is due to steric interference between NF-κB bound to PRDII and IRF-1 bound to PRDI (Thanos and Maniatis, 1995a; Escalante *et al*., 1998). However, addition of wild-type HMG I(Y) to the binding reactions greatly facilitated recruitment of IRF-1, thus leading to enhanceosome assembly (lanes 17–22). Remarkably, HMG $I_{1–74}$, which does not interact with ATF-2/c-Jun (Figure 1), did not facilitate enhanceosome assembly (lanes 23–28). Furthermore, HMG I derivatives which lack the ability to bind to the enhancer via intramolecular cooperative interactions (HMG $I_{31–107}$ and HMG $I_{31–74}$) also did not promote enhanceosome assembly (lanes 29–34 and 35–39). These results are consistent with the idea that HMG I(Y) induces a conformational change in both DNA and the activators in a way that allows formation of the enhanceosome.

Additional evidence for the role of protein–protein interactions between HMG I(Y) and the activators in enhanceosome assembly was provided by *in vitro* transcription experiments. Figure 4B shows that in the absence of HMG I, the IFN-β activators do not form the enhanceosome (compare lanes 1–4 with 5). Addition of increasing amounts of wild-type HMG I promotes enhanceosome assembly and transcriptional activation (compare lane 5 with 6–8). Remarkably, HMG $I_{31–74}$ and HMG $I_{31–107}$, which can recruit NF-κB but not ATF-2/c-Jun to the enhancer (Figure 2), respectively, fail to promote enhanceosome assembly (lanes 15–17 and 9–11). Similarly, HMG $I_{1–74}$, which recruits ATF-2/c-Jun but not NF-κB, also does not facilitate enhanceosome-dependent transcription (lanes 12–14). Thus, it appears that the two HMG I(Y) molecules bound to the IFN-β enhancer must simultaneously and independently recruit NF-κB and ATF-2/c-Jun to the enhancer. Therefore, recruitment of NF-κB alone by HMG I(Y) does not suffice for recruitment of ATF-2/c-Jun and vice versa, despite the ability of these activators to interact with each other (Du *et al*., 1993). Strikingly, simultaneous addition of HMG $I_{1–74}$ and HMG $I_{31–107}$ also did not result in assembly of a functional enhanceosome (lanes 18–20). Furthermore, HMG $I_{1–90}$, which enhances NF-κB's DNA binding activity in the absence of strong protein–protein interactions with the factor (Figures 1 and 2), strongly inhibited enhanceosome assembly in a dose-dependent manner (lanes 21–23). This inhibition is probably due to the property of HMG $I_{1–90}$ to bind non-specifically to the DNA when present at high concentrations (Yie *et al*., 1997). Similar results were obtained in transient transfection experiments with *Drosophila* Schneider cells that are devoid of HMG I-like proteins (Thanos and Maniatis, 1995). As seen in Figure 4C, expression of wild-type HMG I facilitated enhanceosome assembly, whereas HMG $I_{1–74}$, HMG $I_{31–107}$ and HMG $I_{31–74}$ did not. The inability of HMG $I_{1–90}$ to inhibit the IFN-β enhanceosome *in vivo* could be due to its sequestration at multiple sites in the genome (Yie *et al*., 1997). Taken together, our *in vivo* and *in vitro* experiments strongly suggest that the two HMG I molecules bound to the IFN-β enhancer work independently to recruit NF-κB and ATF-2/c-Jun to the enhancer at the onset of enhanceosome assembly. Recruitment of both activators by HMG I is critical for enhanceosome assembly. Finally, the inability of the combination of HMG $I_{1–74}$ and HMG $I_{31–107}$ to nucleate enhanceosome assembly, taken together with their distinct patterns of protein–protein interactions, suggests that enhanceosome assembly is completed by protein–protein interactions between the HMG I molecules and between HMG I and the activators (see Discussion).

HMG I(Y) is present in enhanceosome complexes both in vivo and in vitro

The experiments described above taken together with previous reports strongly suggest, but do not prove, that HMG I is present in the transcriptionally active enhanceosome complex. This assumption was based on the fact that enhancer complexes formed in the presence of HMG I display a slight retardation of their electrophoretic mobility when compared with complexes formed in the absence of HMG I (Figure 2; Thanos and Maniatis, 1992; Du *et al*., 1993; Munshi *et al*., 1998), suggesting the presence of HMG I in these complexes. Alternatively, HMG I could function by a 'hit and run' mechanism involving transient binding to DNA, thus inducing a conformational change that allows tighter association of the activators with the DNA. To investigate the presence of HMG I in complexes containing NF-κB or ATF-2, we carried out Western blot analysis using proteins that were purified from preparative EMSAs (Figure 5A). Figure 5A

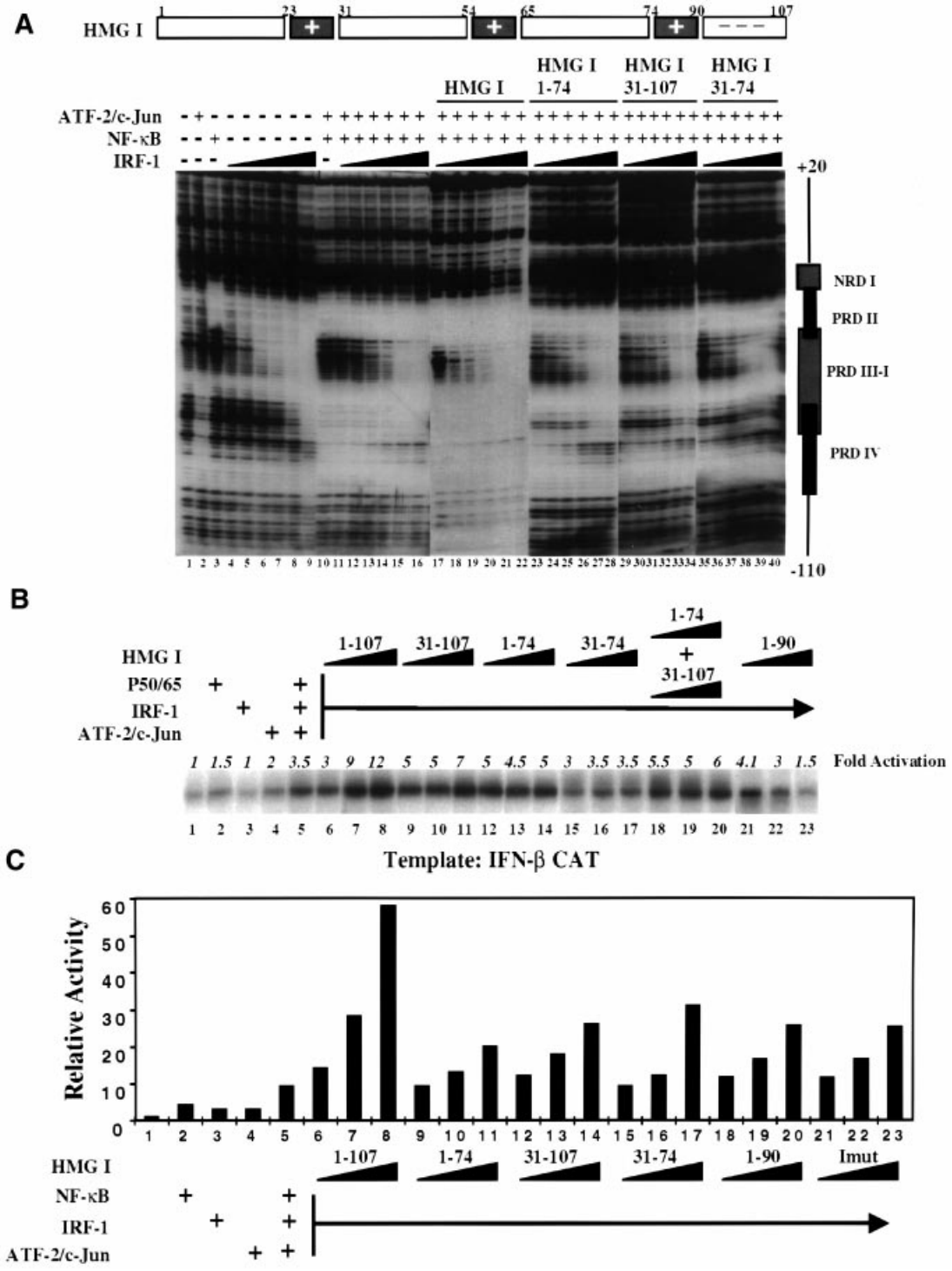


Fig. 4. The role of protein–protein and protein–DNA interactions between HMG I(Y) and the activators in the assembly of the IFN-β enhanceosome. (**A**) Quantitative DNase I footprinting using increasing amounts of recombinant IRF-1 alone (lanes 4–9) or in the presence of a constant amount of NF-kB and ATF-2/c-Jun (lanes 11–16) along with either wild-type HMG I(Y) (lanes 17–22), HMG $I_{1–74}$ (lanes 23–28), HMG $I_{31–107}$ (lanes 29–34), or HMG $I_{31–74}$ (lanes 35–40). The amounts of proteins used were 2, 6, 20, 50 and 150 ng of IRF-1, 20 ng NF-κB, 200 ng ATF-2/c-Jun, 30 ng each of the HMG I(Y) derivatives. (**B**) Results of an *in vitro* transcription experiment carried out as described in Figure 3A using increasing amounts of the indicated HMG I derivatives. All the HMG I derivatives were added at 30, 100 and 300 ng. (**C**) *Drosophila* Schneider cells were transfected with the IFN-β CAT reporter plasmid (100 ng) along with expression vectors encoding the IFN-β gene activators and several HMG I derivatives. The amounts of expression vectors were as follows: 100 ng of an equimolar mixture of pPAC p50 and pPAC p65, 800 ng of pPAC IRF-1, 600 ng of an equimolar mixture of pPAC ATF-2 and pPAC c-Jun and 200 ng, 600 ng and 2 μg of the indicated HMG I derivatives. Vector DNA (pPAC) was added as necessary to achieve a constant amount of transfected DNA. The cells were harvested 48 h after transfection and the CAT activity was determined and normalized based on β-galactosidase activity obtained from the cotransfected hspLacZ (200 ng) plasmid. The average of three independent experiments is shown and the variability from experiment to experiment was <20%.

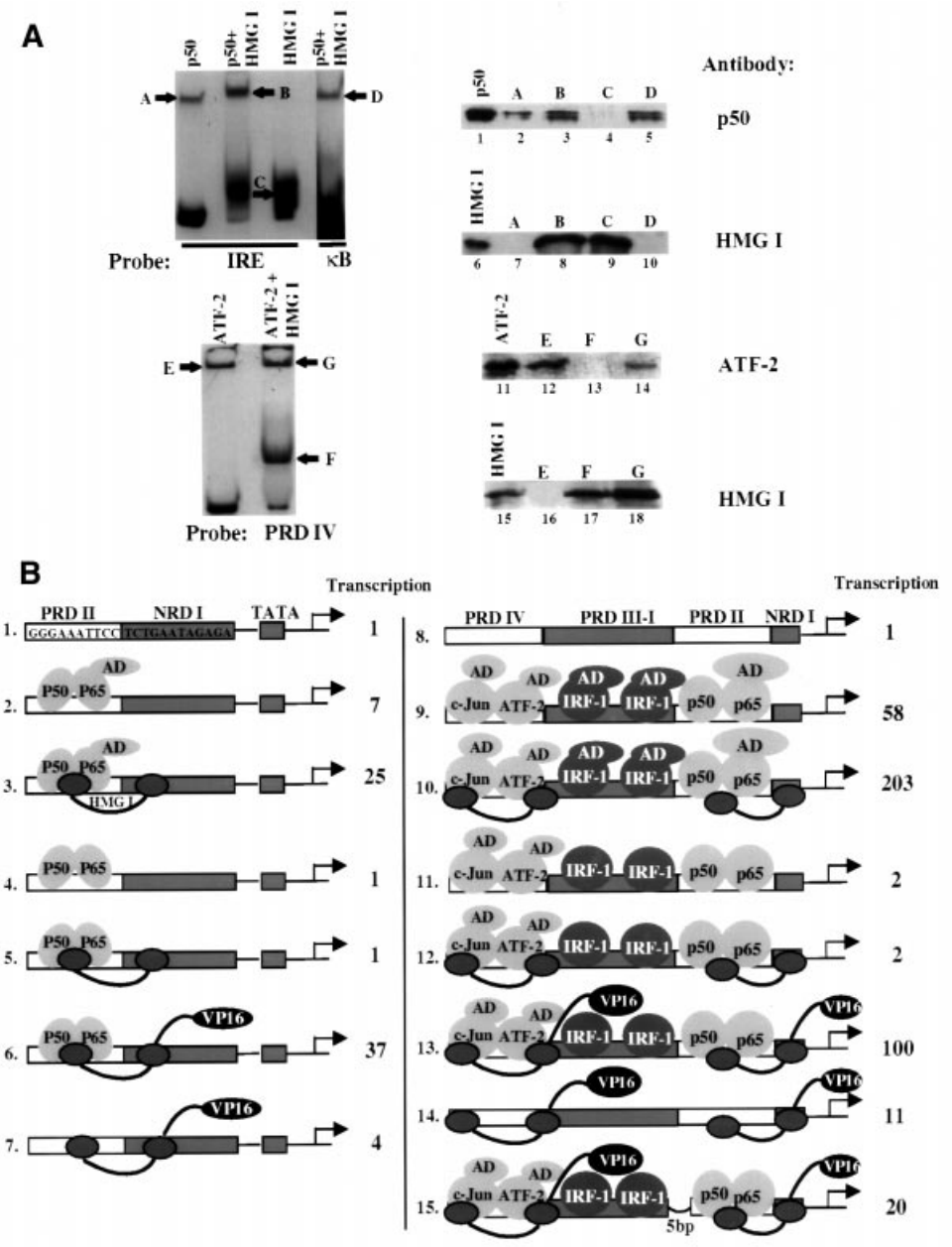


Fig. 5. HMG I(Y) is present in enhanceosomes complexes both *in vivo* and *in vitro*. (**A**) A preparative EMSA (left) using the IRE or IgκB (top) and PRDIV (bottom) oligonucleotides as probes and the indicated combination of recombinant proteins. Radioactive nucleoprotein complexes (depicted as A to F) were excised, the proteins were precipitated by adding TCA and were detected by Western blot analysis (right) using the indicated antibodies. Lanes 1, 6, 11 and 15 contain recombinant p50, HMG I, ATF-2 and HMG I, respectively, used as positive controls. (**B**) Mouse P19 cells were cotransfected using the PRDII/NRDI CAT (lanes 1–7) or the –110IFN-βCAT (lanes 8–14) or –110IFN-βCAT I/II6 (lane 15) as reporters (300 ng) along with the indicated expression vectors. The amounts of expression vectors used were as follows: NF-κB (50 ng), NF-κBΔAD (100 ng), HMG I (2 μg), VP16-HMG I (2 μg), IRF-1 (250 ng), IRF-1ΔAD (250 ng), ATF-2/c-Jun (400 ng). Vector DNA (pCDNA3) was added as necessary to achieve a constant amount (4 μg) of transfected DNA.

shows that HMG I is present in the same complexes that contain NF-κB and ATF-2 (lanes 8 and 17). Importantly, HMG I is not present in the NF-κB complex purified from the immunoglobulin κB site probe (IgκB, lane 10), thus excluding the possibility that the presence of HMG I with NF-κB at PRDII is due to protein–protein interactions between HMG I and NF-κB. We have shown previously that HMG I does not bind to the IgκB site (Thanos and Maniatis, 1992). Thus, HMG I(Y) not only recruits NF-κB and ATF-2/c-Jun to the enhancer, but also forms stable nucleoprotein complexes with these activators, at least *in vitro*.

To investigate whether HMG I(Y) and the IFN-β gene activators can also form complexes *in vivo* on the promoter, we carried out transfection experiments using the NF-κB and HMG I derivatives shown in Figure 5B. We deleted the activation domain of NF-κB (NF-κBΔAD) and fused the VP16 activation domain to HMG I. Thus, a ternary complex formed *in vivo* on the promoter between NF-κBΔAD and VP16-HMG I should activate transcription.

Figure 5B (line 2) shows that transfection of a small amount of wild-type NF-κB activates transcription from the PRDII reporter 7-fold, and these levels are increased further to 25-fold by coexpression of HMG I (line 3). Since HMG I does not activate transcription on its own (Thanos and Maniatis, 1992), the stimulation observed is likely to be due to the enhanced DNA binding of NF-κB by HMG I. Deletion of NF-κB's activation domain abolished its ability to activate transcription either in the absence or the presence of coexpressed HMG I (Figure 5B, lines 4 and 5). Interestingly, cotransfection of NF-κBΔAD and VP16-HMG I stimulated transcription 37-fold (line 6), thus indicating the assembly of a NF-κBΔAD/VP16-HMG I complex on the promoter *in vivo*. Furthermore, VP16-HMG I alone activated transcription only weakly (line 7), presumably due to sequestration of the protein to multiple DNA binding sites in the genome (Yie *et al*., 1997). However, in the presence of coexpressed NF-κBΔAD, both proteins bind cooperatively and specifically to PRDII. The latter conclusion is also supported by the inability of VP16-HMG I to form cooperative complexes with NF-κB on the IgκB binding site (data not shown).

The presence of HMG I was also investigated in the context of the natural IFN-β enhanceosome *in vivo*. P19 cells were transfected with NF-κBΔAD and IRF-1ΔAD in the presence or the absence of coexpressed VP16-HMG I. Figure 5B (lines 9 and 11, respectively) shows that cotransfection of wild-type but not mutant activators leads to high levels of activated transcription. We have previously shown that these high levels of transcription are due to the assembly of the enhanceosome (Thanos and Maniatis, 1995; Merika *et al*., 1998). Deletion of the NF-κB and IRF-1 activation domains results in abolishment of transcription. However, coexpression of VP16-HMG I, but not native HMG I, along with the NF-κBΔAD and IRF-1ΔAD partially restored activation of transcription (Figure 5B, compare line 12 with 13). This effect is due to enhanceosome assembly since insertion of a half helical turn of DNA between PRDI and PRDII strongly decreases transcriptional activation (line 15). Finally VP16-HMG I on its own activated transcription only weakly (line 14). Thus, HMG I is a component of the transcriptionally active IFN-β enhanceosome *in vivo*.

The enhanceosome is extraordinarily stable during transcription

The main prediction of the enhanceosome model (Thanos and Maniatis, 1995a) is that HMG I(Y) functions by promoting the cooperative assembly of a remarkably stable higher order nucleoprotein structure that can activate transcription synergistically. To investigate whether the high levels of enhanceosome-dependent transcription also correlate with an increase in its stability, we carried out *in vitro* transcription experiments in the absence or the presence of cold oligonucleotide competitors. The IFN-β enhanceosome was allowed to assemble for 30 min on the wild-type IFN-β enhancer or on a mutant enhancer containing a half helical turn of DNA inserted between PRDI and PRDII (Thanos and Maniatis, 1995a). The wild-type enhanceosomes and the mutant 'enhanceosomes' were then challenged with 100-fold excess of an IFN-β enhancer oligonucleotide for 10 min before the start of the transcription reaction. As seen in Figure 6A, the wild-type enhancer directs high levels of transcription upon enhanceosome assembly (compare lane 1 with 3). However, when the mutant enhancer was used as template, the levels of transcription were significantly reduced (Figure 6A, compare lane 3 between top and bottom panels). Remarkably, the IFN-β enhanceosome assembled on the wild-type enhancer but not on the mutant enhancer is resistant to the competitor DNA (Figure 6A, compare lanes 3 and 4 between top and bottom panels). Furthermore, activation of transcription by NF-κB or IRF-1 alone was almost completely eliminated upon addition of the competitor DNA (Figure 6B and C, lanes 1–4), thus resembling activation by the helically permutated enhancer (Figure 6A, bottom panel). Therefore, the correct helical phasing of the activator and HMG I binding sites is required for both high levels of transcription and the stability of protein–DNA and protein–protein interactions. These experiments demonstrate that the wild-type IFN-β enhanceosome is an exceptionally stable higher order nucleoprotein structure. Addition of the oligonucleotide competitor after the assembly of the preinitiation complexes did not affect the levels of transcription from the enhanceosome but significantly decreased activation by the helically permutated enhancer (Figure 6A, compare lanes 5–8 between top and bottom panels). In contrast, we demonstrate that NF-κB and IRF-1 alone readily dissociate from the promoter during sequential rounds of transcription (Figure 6B and C, lanes 5–8). This observation suggests that the enhanceosome remains intact during multiple rounds of transcription. Finally, single round transcription experiments in the presence of sarkosyl revealed that following preinitiation complex (PIC) assembly, the competitor oligonucleotides can remove IRF-1 but not NF-κB or the enhanceosome (Figure 6B and C, lanes 9–12). Thus, IRF-1 but not NF-κB can be removed from a committed PIC. Taken together, our experiments suggest that the stability and the transcriptional properties of the enhanceosome differ from those of its components when studied either separately or outside of the natural enhancer context.

A dramatic illustration of the enhanceosome's extraordinary stability was provided by DNase I footprinting competition experiments. Figure 6D compares the off rates of NF-κB and enhanceosome on the IFN-β enhancer. Thus, nucleoprotein complexes containing either NF-κB or the enhanceosome were assembled on a radiolabeled IFN-β enhancer and then challenged with 150-fold cold competitor for different amounts of time, followed by the addition of DNase I and gel analysis. As seen in Figure 6D, NF-κB dissociates from the enhancer after 30 min of incubation with the competitor DNA (lane 4). Amazingly, the IFN-β enhanceosome remains stable even after 10 h of incubation with the competitor enhancer DNA (lanes 11–19).

The extraordinary stability of the enhanceosome permits multiple rounds of transcription

The experiments described above suggested that the tremendous stability of the enhanceosome might allow more rounds of transcription compared with those obtained by NF-κB or IRF-1 alone. To test this idea, we carried out *in vitro* transcription experiments using low amounts of the detergent sarkosyl, which inhibits PIC assembly but

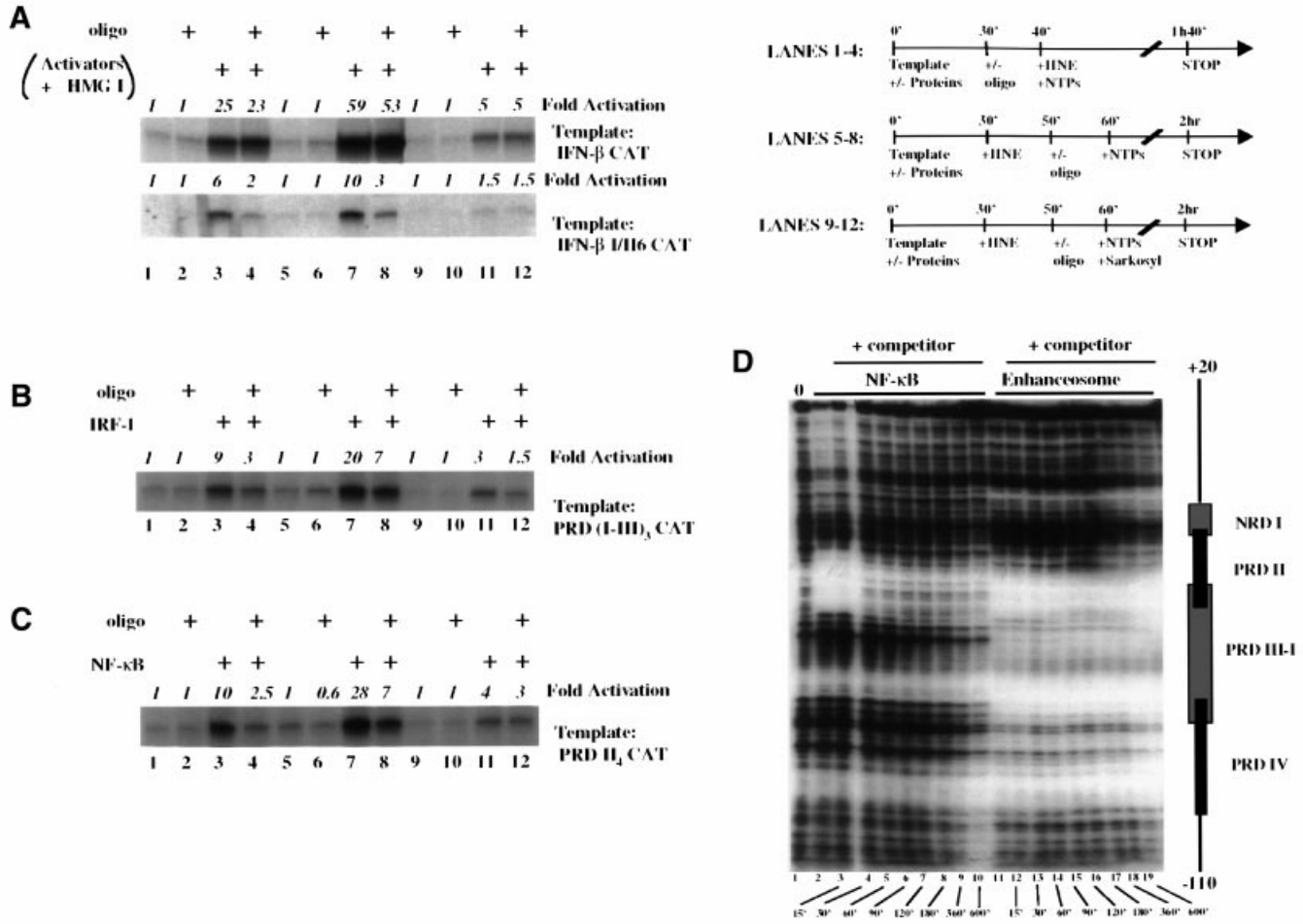


Fig. 6. The enhanceosome is extraordinarily stable during transcription. (**A**) Results of *in vitro* transcription experiments using either the wild-type (top) or the helically permutated (bottom) –110IFN-β CAT templates. The amounts of proteins used were as in Figure 3. The kinetic scheme shown at the top right of the figure indicates the order of addition of activators, HeLa nuclear extract (HNE) and the competitor oligonucleotides (100-fold excess). Sarkosyl was added at 0.05%. (**B**) Same as in (A), but the reporter contains three copies of the PRDI-III element. IRF-1 was added at 1 μg to achieve high levels of activation. (**C**) Same as in (A), but the reporter contains four copies of the PRDII element. NF-κB was added at 750 ng to achieve high levels of activation. In each set (lanes 1–4, 5–8 and 9–12), the basal level of transcription was set as fold activation 1. (**D**) Quantitative DNase I footprinting experiment comparing the stability of the NF-κB/DNA (lanes 2–10), or the enhanceosome (lanes 11–19) complexes. Competitor enhancer DNA was added at 150-fold excess for the indicated at the bottom of the gel amounts of time. Lanes 2 and 11 received no competitor.

does not affect elongation of transcription (Hawley and Roeder, 1985). DNA templates bearing either the enhanceosome, NF-κB or IRF-1 alone were incubated with nuclear extract for 30 min to promote PIC formation followed by the addition of nucleotide triphosphates (NTPs) to initiate elongation of transcription. Sarkosyl was then added at different time points and elongation was allowed to proceed for an additional 60 min. Given that essentially only one round of transcription will occur when sarkosyl is added immediately after NTPs, the total number of rounds of reinitiation of transcription corresponds to the ratio of total transcription obtained in the absence of sarkosyl to that obtained in its presence. Figure 7 shows that the enhanceosome supports six rounds of transcription in the 60 min incubation period. In contrast, in the absence of the enhanceosome, the basal IFN-β promoter supported 2.5 rounds of transcription only (compare lanes 1–5 with 6–10). This effect is due to completion of the enhanceosome assembly process since HMG I derivatives that fail to assemble a stable enhanceosome (Figure 4) do not allow for maximal reinitiation of transcription (compare lanes 6–10 with 11–30). This point was illustrated further using mutant IFN-β enhancer templates that fail to direct assembly of an enhanceosome. Thus, either a helically permutated enhancer (lanes 31–40) or an enhancer bearing mutations in HMG I binding sites (41–50) promoted only two to three rounds of transcription, similar to the extent observed with NF-κB or IRF-1 alone (lanes 51–70). These results, taken together with the data of Figure 6, strongly support the idea that the extraordinary stability of the IFN-β gene enhanceosome directly correlates with its property to stimulate multiple rounds of transcription reinitiation. Furthermore, the property of NF-κB or IRF-1 activators to support the same number of rounds of transcriptional reinitiation as the basal promoter correlates with previous studies using the GAL4-VP16 activator (White *et al.*, 1992). Thus, due in part to its great stability, the enhanceosome displays unique transcriptional properties when compared with each of its components in isolation.

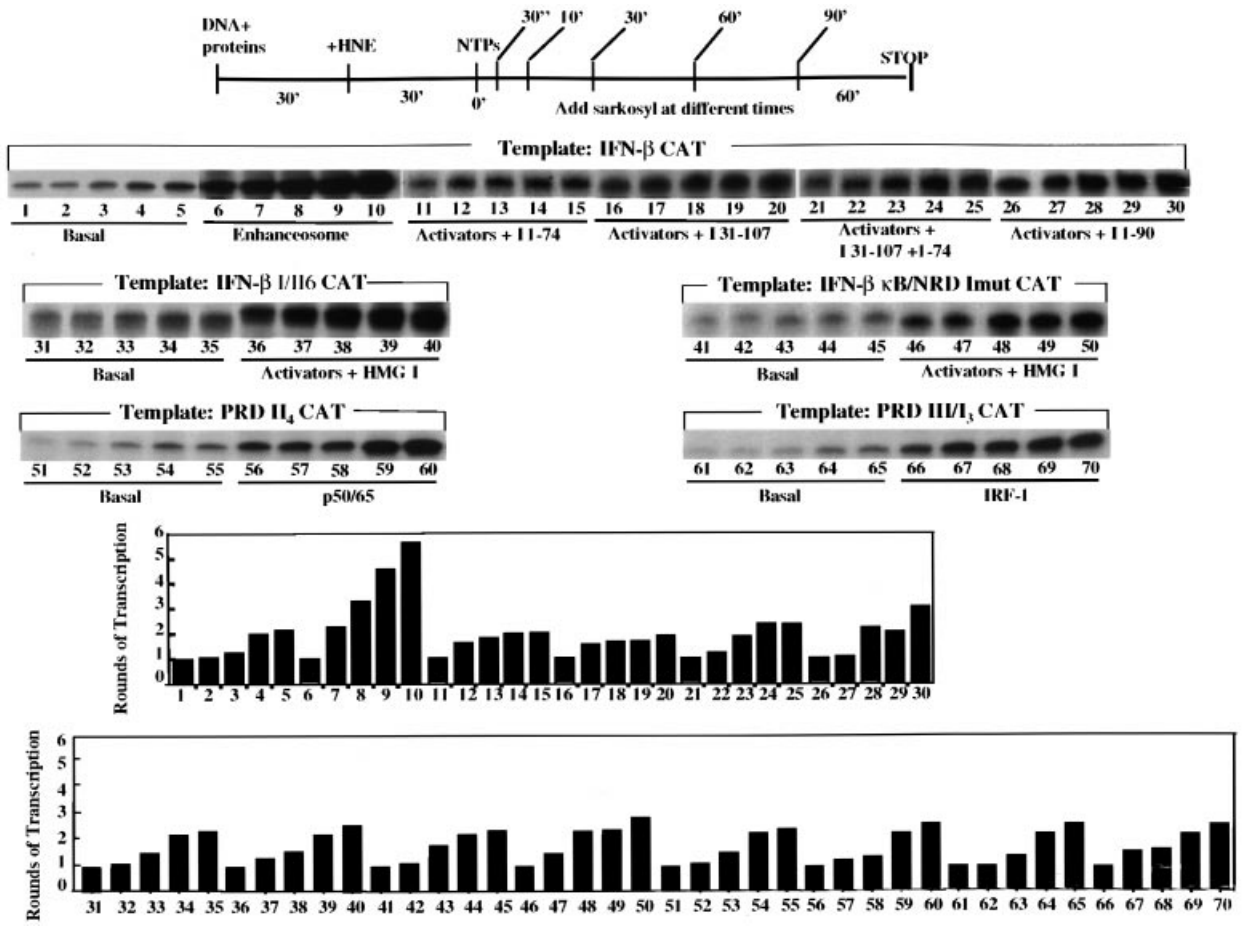


Fig. 7. The extraordinary stability of the enhanceosome permits multiple rounds of transcription. Results of *in vitro* transcription experiments using –110IFN-β CAT as template along with either wild-type HMG I(Y) (lanes 6–10), HMG $I_{1–74}$ (lanes 11–15), HMG $I_{31–107}$ (lanes 16–20), HMG $I_{31–107}$ plus HMG $I_{1–74}$ (lanes 21–25), or HMG $I_{1–90}$ (lanes 26–30). The –110IFN-β(I/II6)CAT (lanes 31–40), IFN-βPRDII/NRDImut (lanes 41–50), $PRDII_4CAT$ (lanes 51–60) and $PRDI\text{-}III_3CAT$ (lanes 61–70) were used as templates. Template DNA (100 ng) was incubated with recombinant proteins for 30 min followed by the addition of HNE for an additional 30 min. Next, NTPs were added followed by the addition of 0.05% Sarkosyl at the times indicated in the kinetic scheme shown on the top of the figure. The radioactive bands were quantitated and the total number of rounds of reinitiation of transcription, which corresponds to the ratio of transcriptional activation obtained at 90 min to that obtained at 30 s, was plotted over time.

Discussion

In this paper, we have examined the mechanisms by which the architectural protein HMG I(Y) functions to promote assembly of the IFN-β gene enhanceosome. The IFN-β enhanceosome comprises a short segment of DNA (65 bp) bound by the three distinct transcriptional activators: NF-κB, IRFs and ATF-2/c-Jun. The role of two molecules of HMG I(Y), which bind to four sites within the enhancer, is to promote the cooperative binding of the activators to the DNA, thus forming the enhanceosome. Once the enhanceosome has formed, the activating regions of the factors function by recruiting the transcriptional apparatus. We show here that the first step in enhanceosome assembly, i.e. the HMG I-dependent recruitment of NF-κB and ATF-2/c-Jun to the enhancer, is mediated by discrete regions of HMG I and is governed by protein–DNA rather than protein–protein interactions. However, completion of the enhanceosome assembly process necessitates the establishment of protein–protein interactions between the activators, between HMG I molecules and between HMG I and the activators. The multiplicity of protein–DNA and protein–protein interactions is required for a conformational change induced on DNA and on the activators, thus allowing enhanceosome assembly. The outcome of these interactions is a remarkably stable structure that is capable of activating transcription at high levels and for multiple rounds.

How HMG I works

Our experiments revealed the critical and complex role of HMG I(Y) in the assembly and function of the IFN-β gene enhanceosome. We showed that HMG I(Y) bears multiple interacting surfaces used for protein–protein and protein–DNA interactions. We have identified the interacting surfaces of HMG I(Y) with each of the IFN-β gene activators and examined the role of these protein–protein interactions in the assembly of a functional enhanceosome. Contrary to our expectations, we found that HMG I(Y) derivatives that are deficient or severely compromised in their interactions with NF-κB or ATF-2/c-Jun are still capable of enhancing their DNA binding activity as efficiently as the wild-type protein. These observations contrast with our classical view of cooperative DNA binding where a given protein is directed to a weak DNA

binding site by interacting with a second protein bound to a nearby sequence (Ptashne and Gann, 1998). Instead, we demonstrated that the ability of HMG I to stimulate activator DNA binding depends on the quality of contacts between HMG I and DNA. Thus, in the ATF-2/c-Jun heterodimer case, HMG I must contact the DNA by positioning simultaneously the first and the middle basic repeats on the pair of binding sites at PRDIV. We imagine that the intramolecular cooperative binding of HMG I to the DNA optimally reverses PRDIV's intrinsic DNA bend in a way that permits high-affinity DNA binding of ATF-2/c-Jun to the site. In the NF-κB case, however, binding of the middle repeat of HMG I at PRDII only, suffices for enhancement of NF-κB's DNA binding activity. Again, protein–protein interactions between HMG I(Y) and NF-κB are not required for recruitment of the latter to the enhancer. Thus, effective reversal of the intrinsic bend at PRDIV, but not at PRDII, requires binding of HMG I(Y) at both sites, a result consistent with previous experiments (Falvo *et al*., 1995). This difference between ATF-2/c-Jun and NF-κB is probably due to the positioning of the HMG I sites relative to the activator binding sites. Thus, HMG I(Y) binds at the central AT-rich part of PRDII, and NF-κB recognizes the flanking GC-rich portion of the site, whereas the pair of HMG I binding sites at PRDIV flank the ATF-2/c-Jun binding site (Thanos and Maniatis, 1992; Du *et al*., 1993). Therefore, based on these observations we propose that the mechanistic basis for the observed differences in HMG I-dependent recruitment of NF-κB and ATF-2/c-Jun to the enhancer depends on the spatial arrangement of HMG I binding sites and its effective reversal of the intrinsic DNA curvature. Therefore, this type of cooperative DNA binding between HMG I(Y) and transcription factors depends on allosteric changes induced on DNA by HMG I(Y), and not on protein–protein interactions between HMG I(Y) and the activators (see model in Figure 8). A similar model has been proposed to explain the cooperative DNA binding observed between separated homeo- and POU-domains of the octamer factor, in the absence of protein–protein interactions between these domains (Klemm and Pabo, 1996). Interestingly, however, protein–protein interactions between HMG I(Y) and other transcription factors such as SRF and NF-Y suffice for enhancement of their DNA binding activity (Currie, 1997; Chin *et al*., 1998). Taken together, these results suggest that HMG I(Y) can enhance the DNA binding activity of several DNA binding proteins either by inducing allosteric effects on DNA or by interacting directly with the factors. These mechanisms of HMG I(Y) action are versatile and adaptable. For example, depending on the identity and architecture of the transcription factor/HMG I(Y) composite element, transcriptional regulators could be tethered to promoters either via HMG I(Y)-induced allosteric alterations on DNA or via protein–protein contacts with HMG I(Y). In the latter case, HMG I(Y) could serve as an allosteric effector on transcription factors, thus influencing dimerization (Du and Maniatis, 1994) and/or active conformation.

Although protein–protein interactions between HMG I(Y) and the IFN-β activators are not critical for their efficient recruitment to the enhancer, we found that such interactions are required for completion of the enhanceosome assembly process. For example, we showed that

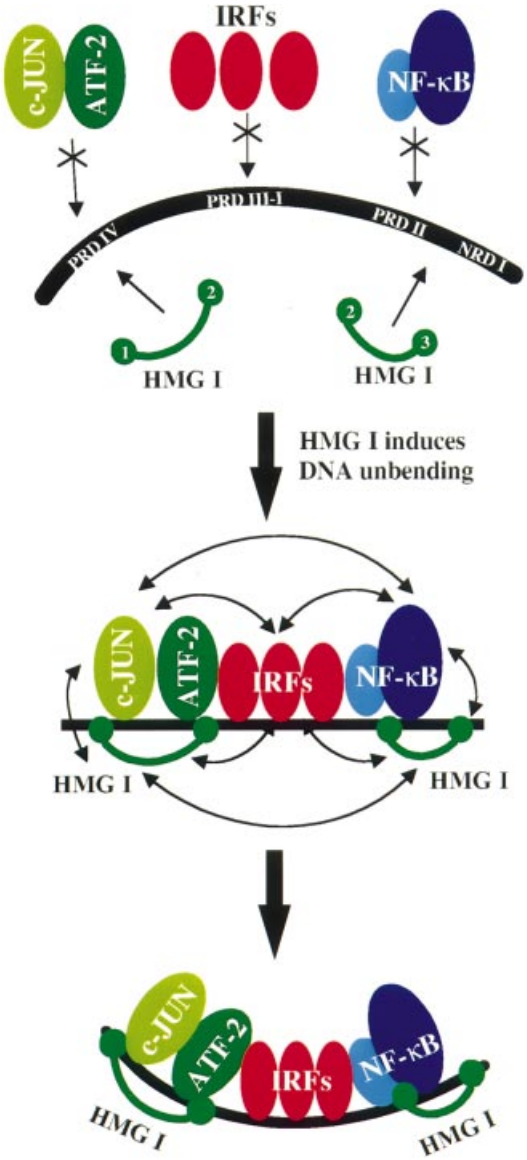


Fig. 8. A two-step model for enhanceosome assembly. Due to the unfavorable intrinsic DNA curvature of the promoter, the IFN-β activators recognize their binding sites with low affinity. However, binding of HMG I(Y) to the promoter unbends the DNA, thus lowering the free energy required for activator DNA binding. This allosteric effect on DNA results in a significant enhancement of activators' promoter binding affinity in the absence of protein–protein interactions with HMG I(Y). Enhanceosome assembly is completed by protein–protein interactions (arrows) between all the components, thus leading to a remarkably stable nucleoprotein structure, the enhanceosome.

HMG $I_{1–74}$ and HMG $I_{31–107}$, which can separately recruit ATF-2/c-Jun and NF-κB to the enhancer, respectively, do not suffice for enhanceosome assembly when they are added together in assembly reactions. The most likely explanation for these observations stems from the fact that these derivatives, especially HMG $I_{1–74}$, display a reduced affinity for interactions with several components of the enhanceosome, such as ATF-2/c-Jun, IRF-1 and HMG I itself, thus implying that these interactions are critical for enhanceosome assembly. Taken together, these experiments revealed that in the process of enhanceosome assembly, HMG I(Y) does not act by simply raising the

local activator concentration by enhancing their DNA binding activity, but it also orchestrates the complex network of protein–protein interactions required for enhanceosome assembly. Furthermore, although HMG I bears multiple interaction surfaces with each activator, it appears that due to the precise spatial arrangement of the enhanceosome components, only one of these patches is actually used with each partner in the context of a fully assembled enhanceosome. Therefore, deletion of any one of these surfaces is expected to affect a specific set of protein–protein interactions with detrimental effects on enhanceosome assembly, a prediction verified from our experiments. In summary, our experiments are consistent with a two step model for enhanceosome assembly. The first step is the HMG I(Y)-dependent recruitment of the activators to the enhancer via allosteric changes induced on DNA by HMG I(Y). We demonstrated that this step is independent of protein–protein interactions between HMG I(Y) and the activators. The second step involves the establishment of a complex network of mutual protein–protein interactions between the activators and HMG I(Y), thus leading to completion of enhanceosome assembly (Figure 8).

Enhanceosome, cooperativity and specificity in gene activation

A remarkable feature of the IFN-β enhanceosome is that it is composed of proteins that can also be activated individually by other extracellular signals in addition to virus infection. However, the IFN-β gene is not activated by these other signals. The highly specific activation of IFN-β gene transcription is due to the fact that virus infection provides the only signal that can coordinately activate all the IFN-β gene activators, which together with HMG I(Y) assemble into the enhanceosome. Assembly, stability and optimal functioning of the IFN-β gene enhanceosome require a precise helical relationship between individual transcription factors and HMG I(Y) binding sites. In fact, we showed that HMG I(Y) promotes cooperative enhanceosome assembly in a manner that depends on the precise arrangement of activators and HMG I(Y) binding sites on the DNA helix. Furthermore, the stability of preformed enhanceosomes also depends on the same parameters. These unusual properties of the enhanceosome are directly reflected by the ability of the wild-type enhanceosome to activate transcription at very high levels and promote multiple rounds of reinitiation of transcription. On the other hand, we showed that the IFN-β gene activators, when tested either separately or together but in the context of helically permutated enhancers or with mutant HMG I(Y) proteins, are capable only of low levels of transcription and fewer rounds of reinitiation of transcription. This is due, at least in part, to the decreased stability of these enhancer complexes. Therefore, we propose that the HMG I(Y)-dependent cooperative assembly and increased stability of the enhanceosome is responsible for the high levels of synergistic transcription elicited during virus infection.

Transcripts of IFN-β mRNA are not detected in uninfected cells, but after virus infection the gene is activated to very high levels and then undergoes a rapid postinduction turnoff. Considering these observations, we are confronted with the question of why activation of IFN-β gene transcription requires an extraordinarily stable structure, such as the enhanceosome. On the surface, our conclusion that the enhanceosome is an extraordinarily stable structure is inconsistent with the dynamic regulation of IFN-β gene transcription. One solution to this apparent paradox lies, at least in part, in our previous observation that recruitment of CBP into the enhanceosome leads to acetylation of HMG I(Y) followed by its detachment from the enhanceosome, thus causing enhanceosome destabilization and termination of transcription (Munshi *et al*., 1998). Furthermore, virus infection not only stimulates enhanceosome assembly and therefore IFN-β gene transcription, but in parallel, activates the expression of several other genes encoding known repressors of IFN-β gene transcription, such as p50 homodimers, IκBα, IRF-2, PRDIIBF1 and PRDIBF1 (Maniatis *et al*., 1992; Tran *et al*., 1997). However, none of these factors is strong enough to disassemble the enhanceosome. Therefore, we propose that the extraordinary stability of the IFN-β enhanceosome not only permits high levels of transcription by allowing multiple rounds of reinitiation in the presence of newly synthesized repressor proteins, but allows for instantaneous enhanceosome disruption and subsequent shut-off upon acetylation of HMG I(Y) by CBP.

An important feature of the cooperative enhanceosome assembly is that it is governed by a series of relatively weak protein–protein and protein–DNA interactions. Thus, multiple protein–protein and protein–DNA interactions can produce a highly stable and specific complex composed of proteins that individually display low affinities and specificities for interactions with each other and with DNA. Evidently, an effective use of this process requires that the concentration of the activators be tightly controlled. Consistent with this, we have shown that HMG I(Y) promotes the cooperative assembly and stabilizes the enhanceosome when the activators are present in limiting amounts. Thus, the cooperativity of enhanceosome assembly could determine the threshold levels of transcription factors required for transcriptional activation, and these levels are inducer specific. In fact, uninfected cells contain very low, sometimes undetected, nuclear amounts of IFN-β gene activators, but following virus infection these amounts are increased but do not exceed the threshold levels required for activation of transcription on their own. We propose that HMG I(Y) functions by coordinating the cooperative assembly of these factors into a transcriptionally active enhancer complex at subthreshold activator concentrations.

Materials and methods

In vitro transcription

Recombinant proteins were expressed and purified as described previously (Thanos and Maniatis, 1995; Yie *et al*., 1997). *In vitro* transcription reactions were carried out as follows. Template DNA (100 ng) was incubated with recombinant proteins for 30 min at room temperature followed by the addition of 40 μl of transcription mix containing 0.5 mg/ml bovine serum albumin (BSA), 5 mM dithiothreitol (DTT), 0.5 mM NTPs, 4 mM $MgCl_2$, 35 mM HEPES pH 8.2, 80 mM KCl and 10 μl HeLa nuclear extract (8 mg/ml). The reactions were incubated at 30°C for 1 h and stopped by the addition of 100 μl mix containing 0.4 M sodium acetate, 10 mM EDTA, 0.2% SDS, 5 μg tRNA and 20 μg proteinase K at 37°C for 15 min. The RNA was extracted by phenol/chloroform and ethanol precipitated. The RNA pellet was resuspended in 20 μl hybridization mix containing 40 mM PIPES pH 6.4, 1 mM

EDTA, 400 mM NaCl, 0.2% SDS, 0.1 pmol γ-^{32}P-labeled CAT primer followed by incubation at 37°C overnight. The hybridization reaction was precipitated by adding 180 µl of 0.5 M ammonium acetate and isopropanol. The RNA–primer hybrid was resuspended in 10 µl reverse transcription mix containing 50 mM Tris pH 8.3, 74 mM KCl, 6 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, 1 U AMV reverse transcriptase and was incubated at 42°C for 1 h. The products were analyzed on 8% acrylamide:bisacrylamide(19:1) gel containing 5 M urea in 0.5× TBE.

Protein–protein interactions

Protein–protein interaction experiments were carried out essentially as described (Merika *et al*., 1998). Briefly, the indicated proteins were *in vitro* translated using a coupled transcription–translation kit (Promega) in a 50 µl reaction containing [^{35}S]methionine and other reagents recommended by the manufacturer. Beads containing normalized amounts of HMG I derivative GST-fusion proteins, expressed and purified as described previously (Yie *et al*., 1997), were equilibrated in buffer A (50 mM NaCl, 20 mM Tris–Cl pH 8.0, 0.05% NP-40, 0.25% BSA, 1 mM PMSF and 1 mM DTT) for 30 min at 4°C. Subsequently, 3 µl *in vitro* translated protein was added, and the reactions were incubated at 4°C for 2 h. The beads were washed 3 times with buffer A, once with buffer A without BSA, and bound ^{35}S-labeled proteins were visualized on a 10–15% SDS–PAGE gel, stained and destained, submerged in fluorographic solution (Amplify; Amersham), dried and exposed overnight at –80°C.

Electrophoretic mobility shift assays, DNase I footprinting and Western blot analysis

EMSAs and DNase I footprinting were performed as described previously (Thanos and Maniatis, 1992, 1995). For the preparative gel shown in the Figure 5, the binding reactions were scaled up 20-fold. The wet gel was exposed on X-ray film and the indicated bands were excised. The gel slices were eluted by using Model 422 Electro-Eluter (Bio-Rad) and the proteins were precipitated by adding 25% trichloroacetic acid. The precipitated proteins were loaded on 10% (p50 or ATF-2 Western) or 15% (for HMG I detection) SDS–PAGE. The proteins were transferred onto membrane by using standard protocols and detected by p50 and ATF-2 specific antibodies (Du *et al*., 1993; Thanos and Maniatis, 1995b).

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