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Virus Induction of Human IFNβ Gene Expression Requires the Assembly of an Enhanceosome

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Summary

We present evidence that transcriptional activation of the human interferon-β (IFNβ) gene requires the assembly of a higher order transcription enhancer complex (enhanceosome). This multicomponent complex includes at least three distinct transcription factors and the high mobility group protein HMG I(Y). Both the in vitro assembly and in vivo transcriptional activity of this complex require a precise helical relationship between individual transcription factor-binding sites. In addition, HMG I(Y), which binds specifically to three sites within the enhancer, promotes cooperative binding of transcription factors in vitro and is required for transcriptional synergy between these factors in vivo. Thus, HMG I(Y) plays an essential role in the assembly and function of the IFNβ gene enhanceosome.

Introduction

Eukaryotic cells are capable of responding specifically to large numbers of distinct extracellular signals and environmental stresses. These responses usually involve signal transduction pathways that lead to the activation of specific sets of genes. Understanding the mechanisms involved in this process is complicated by the observation that many transcription factors are activated in response to a variety of signals and, once activated, can bind specifically to regulatory sequences upstream of a large number of different genes (McKnight and Yamamoto, 1992). However, only a subset of such genes is activated in response to a given signal. Thus, a central problem in signal transduction is how this specificity is achieved.

An excellent example of this problem is provided by the transcription factor NF-κB, which can be activated in response to a large number of extracellular signals and environmental stresses (Siebenlist et al., 1994; Baeuerle and Henkel, 1994; Thanos and Maniatis, 1995b). In addition, a long and growing list of genes containing NF-κB-binding sites has been identified. Thus, how does the activation of NF-κB by a specific inducer lead to the activation of only a subset of the genes containing NF-κB-binding sites?

One answer to this question lies in the organization of transcriptional enhancers. Most inducible enhancer elements contain multiple, distinct transcription factor–binding sites that are part of a combinatorial mechanism that relies on cooperative binding of transcriptional activator proteins, transcriptional synergy, or both (for reviews see Maniatis et al., 1987; McKnight and Yamamoto, 1992; Ptashne, 1992; Tjian and Maniatis, 1994; see also Robertson et al., 1995). A number of studies have demonstrated transcriptional synergy with synthetic promoters/enhancers containing multiple copies of transcription factor–binding sites, but relatively little is known about the synergistic interactions in natural transcriptional enhancers.

An excellent model for studying this problem is provided by the virus-inducible enhancer of the human interferon-β (IFNβ) gene (reviewed by Maniatis et al., 1992; Thanos et al., 1993; Tjian and Maniatis, 1994). Virus induction requires an overlapping set of regulatory elements designated positive regulatory domains (PRDs) I through IV. PRDII, PRDIII-I, and PRDIV are recognized, respectively, by the transcription factors NF-κB, IFN-regulatory factor 1 (IRF-1), and activating transcription factor 2 (ATF-2)/c-Jun. Evidence for the involvement of these activators in virus induction of the IFNβ gene has been discussed elsewhere (Matsuyama et al., 1993; Reis et al., 1994; reviewed by Maniatis et al., 1992). The high mobility group protein HMG I(Y) also binds to two sites flanking the ATF-2/c-Jun site in PRDIV and to a site within PRDII. HMG I(Y) is required for the transcriptional activities of both NF-κB and ATF-2/c-Jun in the context of the IFNβ gene promoter, and it is required for virus induction (Thanos et al., 1993; Du et al., 1993). However, HMG I(Y) does not itself activate transcription (Thanos and Maniatis, 1992).

In vitro DNA binding studies established that HMG I(Y) binds to the PRDII and PRDIV elements through contacts in the minor groove, while the corresponding activators NF-κB and ATF-2/c-Jun bind to these sequences through contacts in the major groove. HMG I(Y) interacts specifically with both NF-κB and ATF-2 and stimulates the binding of both proteins to their binding sites in PRDII and PRDIV, respectively. Thus, both PRDII and PRDIV are composite elements containing binding sites for a transcriptional activator and HMG I(Y). Moreover, the functional synergism between NF-κB and HMG I(Y) at PRDII and between ATF-2 and HMG I(Y) at PRDIV appears to involve both protein–DNA and protein–protein interactions (Thanos and Maniatis, 1992; Du et al., 1993; Kaszubska et al., 1993). The three-dimensional structure of the enhancer may therefore be central to the mechanisms required for specific activation of the IFNβ gene, and HMG I(Y) may play an essential role in establishing this structure.

In this paper, we present the results of experiments designed to test this hypothesis. We show that the activation of the IFNβ gene in response to virus infection requires a specific set of regulatory elements that must be precisely arranged on the face of the DNA helix. In addition, we show that this arrangement of regulatory sequences is required for the cooperative in vitro assembly of an enhancer complex and for the transcriptional activity of this complex in vivo. Finally, we show that HMG I(Y) is required for the assembly of the IFNβ enhancer complex in vitro and for transcriptional synergy in vivo. Taken together,

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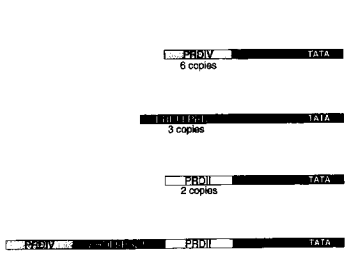

Figure 1. Comparison of the Specificity and Strengths of Synthetic and Natural Virus-Inducible Enhancers

Comparison of the transcriptional activities of the intact IFNβ promoter (bottom) with the individual homopolymeric PRDs after transient transfection into L929 cells. Each of the elements responds to virus infection as well as to other extracellular signals. By contrast, the IFNβ promoter is induced only by virus. Numbers indicate fold induction.

these observations strongly support the hypothesis that the specificity of the inducible activation of the IFNβ gene promoter requires the assembly of an enhanceosome (a term first used by Bazett-Jones et al. [1994]).

Results

The Number and Types of Regulatory Elements Present in the IFNβ Gene Enhancer Determine the Specificity and Level of Induction

The combinatorial nature of the IFNβ gene enhancer is best illustrated by the observation that mutations that inactivate any one of the PRDs in the context of the IFNβ promoter result in a dramatic decrease in the level of virus induction. Moreover, a single copy of any PRD is inactive, but artificial enhancers can be created by multimerization of these elements (for review see Maniatis et al., 1992). Synthetic promoters containing multiple copies of individual PRDs are not only inducible by virus; they respond to other inducers as well (Fan and Maniatis, 1989; Leblanc et al., 1990; Du et al., 1993; Thanos and Maniatis, 1995b). For example, multiple copies of PRDII are activated by virus and by numerous other inducers of NF-κB. However, the intact enhancer responds only to virus infection. This point is illustrated in Figure 1, where synthetic reporters containing multiple copies of PRDIV, PRDIII-I, and PRDII were transfected into mouse L929 cells, which were then challenged with various inducers. The synthetic promoter containing multiple copies of PRDIV is inducible by virus and is slightly more inducible by cAMP. Similarly, a promoter containing multiple copies of the PRDIII-I element is inducible by both virus and IFNγ (M. Wathelet and T. M., unpublished data). Finally, a reporter containing multiple copies of PRDII is inducible by tumor necrosis factor α (TNFα) and virus infection. Thus, all of the synthetic enhancers respond to virus infection, but they also respond to other inducers that activate transcription factors known to bind to these sites.

In sharp contrast, the intact IFNβ promoter is efficiently induced by virus infection, but does not respond to any of the inducers that act on individual sites (Figure 1). In fact, the level of induction observed with the intact enhancer is at least an order of magnitude higher than that seen with any of the synthetic enhancers (Figure 1) (see also Leblanc et al., 1990). Thus, the specific combination and arrangement of regulatory elements in the IFNβ enhancer allow a highly specific response to virus induction and lead to a high level of induction.

This point was further illustrated by replacement of the PRDIV element with PRDII in the context of an otherwise intact IFNβ promoter (Figure 2A). The resulting construct was transfected into HeLa cells, and the activity was examined after stimulation with virus, TNFα, IFNγ, or TNFα plus IFNγ. As shown in Figure 2A, a 95-fold induction was observed with the wild-type IFNβ promoter, and this promoter did not respond to TNFα, IFNγ, or the combination of these cytokines. Remarkably, the replacement of PRDIV with PRDII not only resulted in a decrease in the level of virus induction, but the altered promoter was equally responsive to TNFα (Figure 2A). Interestingly, the combined treatment with IFNγ and TNFα revealed weak synergistic interactions between PRDII and PRDI-III, which are not evident in the wild-type promoter (Figure 2A). We conclude that the highly specific activation of the IFNβ gene in response to virus infection is a consequence of the unique arrangement and collection of transcription factor–binding sites.

Virus Induction of the IFNβ Gene Enhancer Requires Specific Helical Phasing of Individual Transcription Factor–Binding Sites

A striking characteristic of the IFNβ gene enhancer is the close spacing of individual regulatory elements. Virtually every base pair of the promoter contacts a regulatory protein, and every nucleotide is required for maximal levels of virus induction. Thus, if protein–protein interactions between the activators bound to these elements are essential for enhancer function, altering the relative positions of these elements on the DNA double helix should adversely affect enhancer function. We therefore constructed IFNβ promoters in which a half- or full-helical turn of DNA was inserted between individual PRDs. To avoid the generation of fortuitous binding sites for transcriptional activators or repressors, we randomized the inserted DNA sequence. Four different isolates with different insertion sequences were selected, linked to the bacterial chloramphenicol acetyltransferase (CAT) gene, and used in transfection experiments.

Human HeLa cells were transiently transfected with reporter genes bearing either the wild-type IFNβ promoter or promoters with insertion mutations. The level of CAT activity was determined from cell extracts derived from either mock- or Sendai virus–induced cells. Virus induction of the wild-type IFNβ promoter resulted in a 156-fold increase in CAT activity (Figure 2B, line 1). However, when slightly more than a half-helical turn (6 bp) was introduced between PRDI and PRDII, the level of virus induction was only 9-fold (Figure 2B, line 2). Remarkably, insertion of 10 bp, which reestablishes the relative positions of binding sites on the face of the DNA helix, fully restored the activity of the promoter (Figure 2B, line 3). Similarly, insertion of

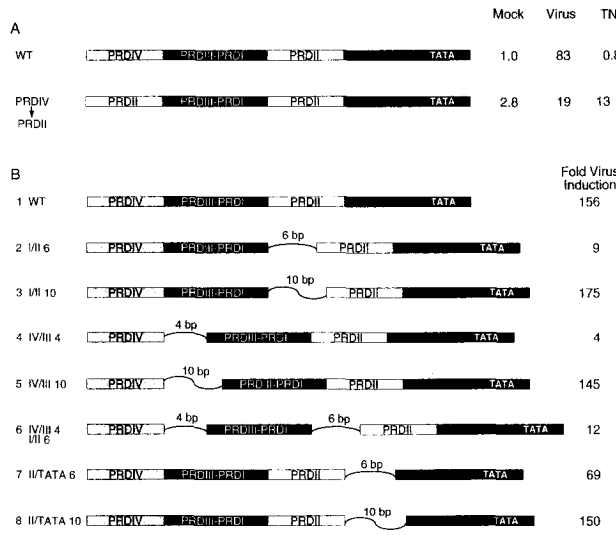

Figure 2. The Effects of Substitutions and Insertions on the Function of the IFNβ Enhancer

(A) Substitution of PRDIV by a second PRDII alters the specificity of induction. Human HeLa cells were transfected with the wild-type IFNβ–CAT reporter construct or with the indicated PRDIV to PRDII substitution. The transfected cells were induced with either Sendai virus, TNFα, IFNγ, or TNFα plus IFNγ. Fold induction is the ratio of CAT with and without the indicated inducers. Shown is the average of two independent experiments.

(B) The correct helical phasing of transcription factor–binding sites is required for virus induction of the IFNβ gene enhancer. Human HeLa cells were transfected with the wild-type IFNβ–CAT reporter construct or with the indicated insertion mutants. The transfected cells were either induced with Sendai virus or mock induced, and the CAT activity was measured from extracts of the transfected cells. Fold virus induction is the ratio of CAT with and without virus induction. Shown is the average of four independent experiments. Variability in virus induction among individual experiments and among individual constructs in the same experiment was less than 40% and 10%, respectively.

a half-helical turn between PRDIV and PRDIII virtually inactivated the enhancer (Figure 2B, line 4), whereas the insertion of a full-helical turn again restored transcriptional activity (line 5).

These experiments suggest that the activators specifically contact the basal transcription complex or that the activators specifically contact each other. With respect to the latter possibility, the proteins bound to PRDI and PRDIII could interact with those bound to PRDII and PRDIV. In fact, IRF-1 has been shown to interact specifically and synergize transcriptionally with NF-κB (Garoufalis et al., 1994; Neish et al., 1995). Alternatively, it is possible that the proteins bound to PRDII interact only with those bound to PRDIV. Consistent with this possibility is the observation that NF-κB and ATF-2 interact with each other in the absence of DNA (Du et al., 1993; Kaszubska et al., 1993).

To test the possibility that the inability of the insertion mutants to respond to virus infection is entirely due to the misalignment of the PRDII and PRDIV elements, we generated the construct shown in line 6 of Figure 2B. In this promoter, the mutations shown in lines 2 and 4 were combined by insertion of 4 bp between PRDIV and PRDIII and 6 bp between PRDI and PRDII. This double insertion restores the relative positions of the ATF-2- and NF-κB-binding sites on the face of the DNA helix. However, the function of the enhancer is not restored (compare lines 1 and 6 in Figure 2B). Thus, the inability of the insertion mutants to respond to virus infection is not simply due to the misalignment of PRDII and PRDIV. Taken together, these experiments strongly suggest that the proteins bound to PRDI-III functionally interact with the activators bound to PRDII and PRDIV. Moreover, these interactions take place only when the activators are correctly positioned on the DNA helix.

To determine whether the relative positions of the enhancer and the TATA box are required for maximal levels of virus induction, we inserted 6 or 10 bp fragments between the two elements. As shown in Figure 2B (line 7), insertion of a half-helical turn decreased the level of virus induction by only 2-fold, and the wild-type level was fully restored when a full-helical turn was inserted (line 8). The results of these helical phasing experiments indicate that enhancer function requires interactions between all the activators bound to distinct elements within the enhancer and that these interactions depend critically on the relative positions of the binding sites. By contrast, the relative positions of the enhancer and TATA box are less important. The latter conclusion is consistent with other studies showing that the IFNβ enhancer can function regardless of its distance from the TATA box or its orientation with respect to the promoter (Goodbourn et al., 1985; Falvo et al., 1995 [this issue of *Cell*]).

These observations suggest that the activators bound to the regulatory elements comprising the IFNβ enhancer are assembled as a functional unit that interacts optimally with the components of the basal transcription complex bound to the TATA box. To examine this possibility further, we compared the ability of one or two copies of the IFNβ enhancer to stimulate transcription. We found that reporters containing one or two copies of the enhancer are activated to similar levels upon virus infection (data not shown). Thus, transcriptional synergism is not observed between duplicated enhancers.

HMG I(Y) Promotes Cooperative Binding of Transcription Factors to the IFNβ Enhancer In Vitro

Previous studies have shown that the transcription factors NF-κB and ATF-2 act together with HMG I(Y) to regulate IFNβ gene expression from the PRDII and the PRDIV elements (reviewed by Maniatis et al., 1992; Thanos et al., 1993). In addition, NF-κB and ATF-2/c-Jun specifically interact in vitro (Du et al., 1993; Kaszubska et al., 1993),

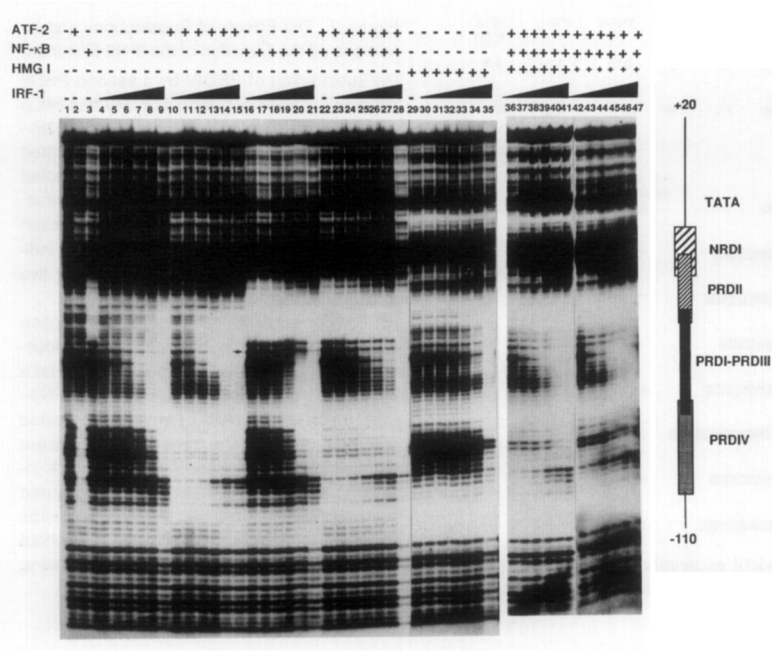

Figure 3. The Effect of HMG I(Y) on Specific Protein–DNA Interactions In Vitro

Quantitative DNase I footprinting of the wild-type IFNβ promoter using increasing amounts of recombinant IRF-1 alone (lanes 4–9) or in the presence of a constant amount of ATF-2 (lanes 10–15), NF-κB (lanes 16–21), NF-κB and ATF-2 (lanes 23–28), or HMG I(Y) (lanes 30–35). HMG I(Y) efficiently recruits IRF-1 in the presence of both NF-κB and ATF-2 (compare lanes 23–28 with 36–41 and 42–47). The amounts of recombinant proteins used were 1, 2, 5, 12, 30, and 100 ng for IRF-1; 30 ng for NF-κB; 100 ng for ATF-2; and 25 ng (lanes 36–41) and 12 ng (lanes 42–47) for HMG I(Y). All the proteins were added at the same time. The arrow indicates the region in PRDI that is not protected by IRF-1 in the presence of NF-κB.

and HMG I(Y) is required for each of their activities in the context of the IFNβ gene enhancer (Thanos and Maniatis, 1992, 1995b; Du et al., 1993).

The only known candidate for activation from the PRDIII-I element is IRF-1 (Miyamoto et al., 1988). However, the results of recent *IRF-1* gene knockout studies have shown that IRF-1 is not essential for virus induction in vivo (Matsuyama et al., 1993; Reis et al., 1994). Thus, either IRF-1 is not involved in IFNβ gene regulation or another as yet unidentified factor can substitute functionally. In the absence of other factors capable of activating transcription from PRDIII-I, and given the demonstration that IRF-1 can interact and synergize with the p50 subunit of NF-κB (Neish et al., 1995), we have used IRF-1 to study the in vitro assembly of the IFNβ gene enhanceosome.

The transcriptional synergy observed among individual regulatory elements of the IFNβ promoter may be due, at least in part, to cooperative binding of the corresponding transcription factors. To address this possibility, we carried out in vitro DNA binding experiments with purified recombinant proteins. The binding activity of each activator was determined separately in titration experiments (data not shown), and the appropriate amounts of protein were used in each experiment.

Figure 3 shows that increasing amounts of recombinant IRF-1 protein protect the PRDI-III element from DNase I cleavage in a concentration-dependent manner (compare lane 1 with lanes 4–9). At the highest concentrations of IRF-1, protection is extended over PRDII and a portion of PRDIV (see also Fujita et al., 1988). When the same amounts of IRF-1 are titrated in the presence of a fixed amount of ATF-2, both proteins can occupy their binding sites simultaneously, as demonstrated by the complete protection of both PRDIV and PRDI-III elements (Figure 3, lanes 14 and 15). No significant binding cooperativity is observed between the two proteins.

When the IRF-1 titration is carried out in the presence of a fixed amount of NF-κB (Figure 3, lane 3), a region within PRDI (marked by the arrow in lane 16) is no longer protected, indicating steric interference between NF-κB and IRF-1 within the PRDI site (compare lanes 4–9 with 16–21). Similarly, IRF-1 inhibited NF-κB binding to PRDII in the reciprocal experiment (data not shown). The interference between NF-κB and IRF-1 was not affected by the addition of ATF-2 (Figure 3, lanes 23–28). These results were surprising, considering that PRDII and PRDI elements were shown to function synergistically in vivo (Fan and Maniatis, 1989). To determine whether binding of HMG I(Y) to the IFNβ promoter affects the NF-κB/IRF-1 interference, we titrated IRF-1 in the presence of NF-κB, ATF-2, and HMG I(Y). As shown in Figure 3 (lanes 36–41 and 42–47), the addition of HMG I(Y) along with NF-κB and ATF-2 results in the protection of the region within PRDI that was blocked by NF-κB. We note that HMG I(Y) alone slightly inhibits binding of IRF-1 (Figure 3, lanes 30–35). Thus, HMG I(Y) induces a conformational change in a multiprotein complex that allows co-occupancy of PRDI and PRDII by IRF-1 and NF-κB, respectively.

The amount of IRF-1 required to fill PRDI was approximately the same on naked DNA and in the presence of ATF-2, NF-κB, and HMG I(Y) when all of the components were added simultaneously (Figure 3). However, a strikingly different result was obtained when the DNA was preincubated with HMG I(Y) and then mixed with increasing amounts of IRF-1 in the presence of ATF-2 and HMG I(Y). As shown in Figure 4A (lanes 14–18), much less IRF-1 was required to fill PRDI compared with the amount required to protect PRDI fully in the absence of HMG I(Y) (lanes 4–8). This HMG I(Y)–dependent cooperative binding of PRDI was not observed with a DNA fragment containing a 6 bp insertion between PRDII and PRDI (Figure 4B). HMG I(Y)–dependent cooperative binding to the IFNβ gene enhancer was also observed with ATF-2 and NF-κB alone, and this cooperativity could be abrogated by the

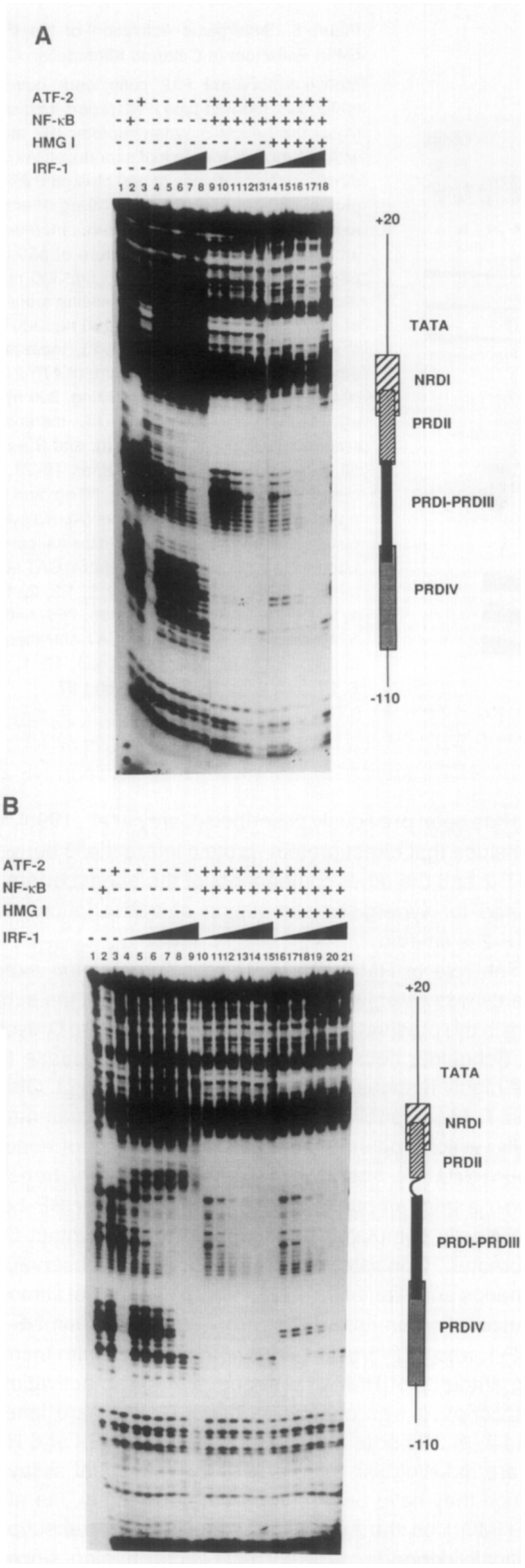

Figure 4. Preincubation of HMG I(Y) with the IFNβ Enhancer Promotes the Cooperative Binding of IRF-1

(A) Binding to the wild-type enhancer. Quantitative DNase I footprinting of the wild-type IFNβ promoter is shown, using the same amounts of recombinant proteins detailed in Figure 3, except for IRF-1, which was 1, 2, 5, 12, and 30 ng. In this experiment, HMG I(Y) (10 ng) was added to the DNA 10 min before the addition of the other proteins.

(B) Binding to the enhancer containing a half-helical turn of DNA inserted between PRDI and PRDII. Quantitative DNase I footprinting was performed as in (A), except that the probe contained 6 bp inserted between PRDI and PRDII. The amounts of recombinant proteins used were as in Figure 3.

insertion of a half-helical turn between PRDII and PRDI (data not shown).

In conjunction with other studies showing that HMG I(Y) binds specifically to the IFNβ enhancer, that it promotes the binding of both NF-κB and ATF-2 to their respective sites (Thanos and Maniatis, 1992; Du et al., 1993), and that it alters the structure of the PRDII–NF-κB complex (Falvo et al., 1995), our observations indicate that HMG I(Y) plays an essential role in the cooperative assembly of a multicomponent IFNβ enhancer complex. Moreover, this role requires the correct helical phasing of transcription factor–binding sites.

Synergistic Activation of the IFNβ Promoter in Cotransfection Experiments Requires the Correct Helical Phasing and HMG I(Y)

To determine whether the cooperative interactions between transcriptional activator proteins observed in vitro are reflected by synergistic interactions between these factors in vivo, we performed cotransfection experiments in mouse embryonal P19 cells. These cells were chosen because they lack endogenous IRF-1 and NF-κB (Harada et al., 1990; Thanos and Maniatis, 1995a). The wild-type IFNβ reporter plasmid was cotransfected into these cells along with increasing amounts of expression vectors encoding each activator alone or a combination of all three activators. Previous studies have shown that NF-κB and IRF-1 synergistically activate the IFNβ gene promoter in cotransfection experiments (Garoufalis et al., 1994; Neish et al., 1995). As shown in Figure 5A, increasing amounts of transfected NF-κB (lanes 2–5), IRF-1 (6–9), and ATF-2/c-Jun (10–13) weakly activated transcription. In sharp contrast, cotransfection of the combination of the expression vectors encoding NF-κB, IRF-1, and ATF-2/c-Jun led to a synergistic activation of the IFNβ enhancer (Figure 5A, lanes 14–17). For example, transfection of 100 ng of NF-κB (Figure 5A, lane 2), IRF-1 (lane 6), or ATF-2/c-Jun (lane 10) did not stimulate transcription from the intact IFNβ promoter. However, when the same amounts of the activator expression constructs were cotransfected into the cells, a 47-fold stimulation of transcription was observed (Figure 5A, lane 14). More important, when even smaller amounts of expression vectors were cotransfected (amounts at which virtually no activation was observed with each activator alone), a high level of synergy was still observed (Figure 5A, lanes 18–21). These results are consistent with our in vitro binding experiments showing cooperative binding of the IFNβ activators.

To determine whether the helical constraints that interfere with virus induction and in vitro binding also interfere with transcriptional synergy, we examined the effect of inserting DNA between PRDI and PRDII on the expression of the reporter gene in P19 cells. As shown in Figure 5B, cotransfection of the IFNβ gene activators led to an additive activation, as opposed to the synergistic activation

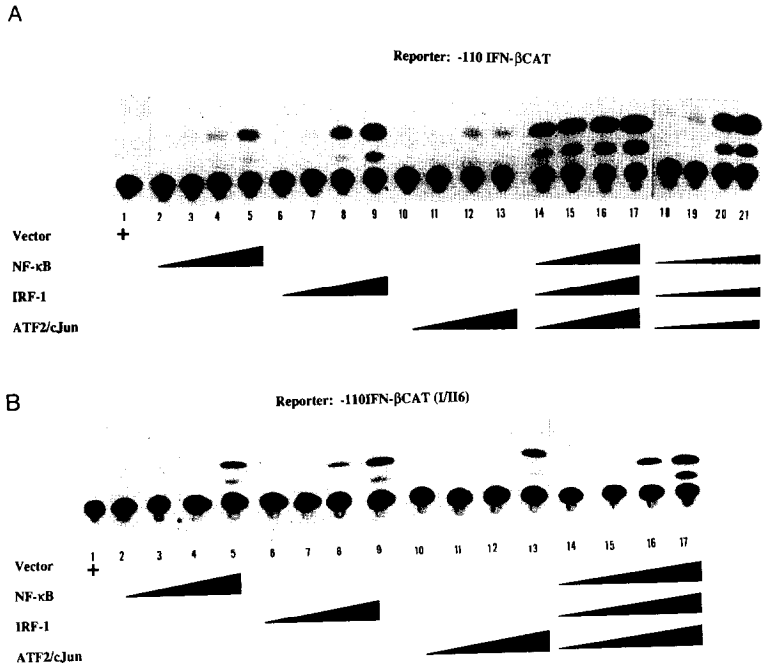

Figure 5. Synergistic Activation of the IFNβ Gene Enhancer in Cultured Mammalian Cells

Mouse embryonal P19 cells were cotransfected with the wild-type IFNβ reporter plasmid (A) or the helical mutated reporter (B), along with increasing amounts of expression vectors directing the synthesis of p50 plus p65, ATF-2 plus c-Jun, and IRF-1. Lane 1, 9 μg of empty expression vector. Lanes 2–5, increasing amounts of an equimolar mixture of p50 and p65 expression plasmids (100 ng, 300 ng, 1 μg, and 3 μg). Lanes 6–9, increasing amounts of *IRF-1* expression plasmid (100 ng, 300 ng, 1 μg, and 3 μg). Lanes 10–13, increasing amounts of an equimolar mixture of *ATF-2* and *c-jun* expression plasmids (100 ng, 300 ng, 1 μg, and 3 μg). Lanes 14–17, increasing amounts (100 ng, 300 ng, 1 μg, and 3 μg) of all the expression vectors. Lanes 18–21, increasing amounts (3 ng, 10 ng, 30 ng, and 100 ng) of all the expression vectors (A). No synergistic activation is observed with the helical mutated IFNβ promoter (B). The relative CAT activities for lanes 1–21 in (A) are: 1, 1.3, 2, 4, 9, 1, 2, 12, 28, 1, 1.5, 3, 5, 47, 135, 256, 540, 2, 5, 40, and 157. The relative CAT activities for lanes 1–17 in (B) are: 1, 1, 2, 2.4, 12, 1, 1.5, 6, 21, 1, 1.8, 2, 12, 2, 3, 14, and 67.

obtained with the wild-type promoter. We conclude that synergistic transcriptional activation in vivo of the natural IFNβ enhancer requires cooperative binding of multiple transcriptional activators, a result consistent with our in vitro reconstitution experiments.

A further test for synergistic interactions between transcriptional activators on the IFNβ gene enhancer was provided by a comparison of the transcriptional activities of two naturally occurring ATF-2 isoforms generated by alternative splicing within the basic region of the bZIP domain. ATF-2_{195} binds specifically to PRDIV and interacts with HMG I(Y) in the absence of DNA, and its affinity for PRDIV is enhanced by HMG I(Y) (Du and Maniatis, 1994). By contrast, ATF-2_{192} binds to PRDIV, but does not interact with either NF-κB or HMG I(Y). HMG I(Y) actually inhibits the binding of ATF-2_{192} to PRDIV (Du and Maniatis, 1994). As expected in cotransfection experiments, a high level of expression was observed with the ATF-2_{195} isoform (Figure 6, 0.25 and 0.5 μg). By contrast, ATF-2_{192} fails to synergize with the other IFNβ gene activators at the low levels of transfected plasmids. The difference between these two isoforms does not appear to be a result of differences in the level of expression, since the ATF-2_{192}/c-Jun heterodimer stimulates transcription from a reporter containing multiple PRDIV sites at levels (4-fold) comparable with that of the ATF-2_{195}/c-Jun (data not shown). However, at much higher concentrations of ATF-2_{192}, the level of expression approaches that observed with ATF-2_{195}. These results indicate that protein–protein interactions are essential for transcriptional synergism at lower levels of activator. At very high levels of ATF-2_{192}, these interactions do not appear to be essential for synergism. A precedent for transcriptional synergism in the absence of cooperative binding has been previously described (Carey et al., 1990). We conclude that direct protein–protein interactions between ATF-2 and the other components of the enhancer are required for synergistic stimulation of transcription when ATF-2 is limiting.

The role of HMG I(Y) in these cotransfection experiments was established after transfection of all the activators in the presence or absence of HMG I(Y) into Drosophila Schneider cells. We chose these cells because they are highly responsive to exogenous HMG I(Y) (J. Olesen and T. M., unpublished data), in contrast to mammalian cells, which apparently contain higher levels of endogenous HMG I(Y). As shown in Figure 7 (lanes 2–4), transfection of a small amount of NF-κB, ATF-2/c-Jun, or IRF-1 only marginally stimulated transcription from the intact IFNβ promoter. Consistent with our previous observations (Thanos and Maniatis, 1992), HMG I(Y) does not stimulate transcription on its own (Figure 7, lane 5). When NF-κB, IRF-1, and ATF-2/c-Jun were cotransfected with increasing amounts of HMG I, a strong synergistic activation of transcription was observed (Figure 7, compare lanes 6 and 7–9). We note that the activities of HMG I and HMG Y are indistinguishable in all of the functional assays in which they have been compared. Importantly, the effect of HMG I on the synergistic stimulation of transcription critically depends on the correct helical phasing, since the addition of HMG I does not promote synergistic activation of transcription on a promoter containing 6 bp between PRDI and PRDII (Figure 7, compare lanes 15 and 16–18). On the contrary, transfected HMG I represses this promoter, perhaps by introducing unfavorable DNA conformational changes. Thus, the HMG I(Y) protein is required in vivo for the synergistic stimulation of transcription

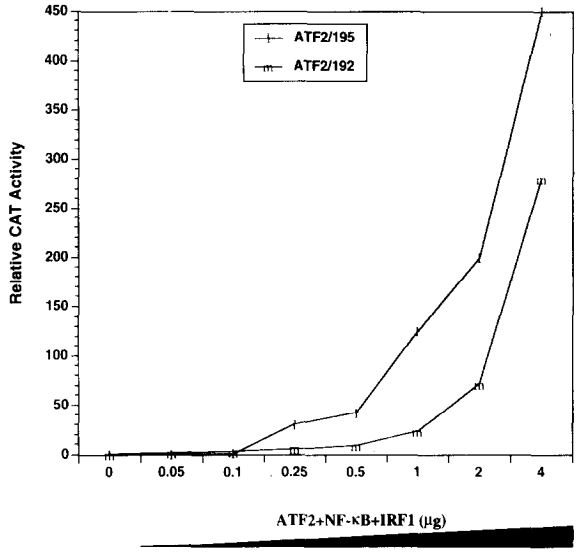

Figure 6. Direct Protein–Protein Interactions between ATF-2 and NF-κB Are Required for the Synergistic Activation of the IFNβ Gene Promoter

Mouse embryonal P19 cells were cotransfected with the wild-type IFNβ reporter along with increasing amounts (50 ng, 100 ng, 250 ng, and 500 ng) of an equimolar mix of p50 plus p65, *IRF-1*, and *c-jun* plus either ATF-2_{195} (ATF-2/195) or ATF-2_{192} (ATF-2/192). Synergistic activation is obtained only with the form of ATF-2 that interacts with NF-κB (compare ATF-2_{195} with ATF-2_{192}).

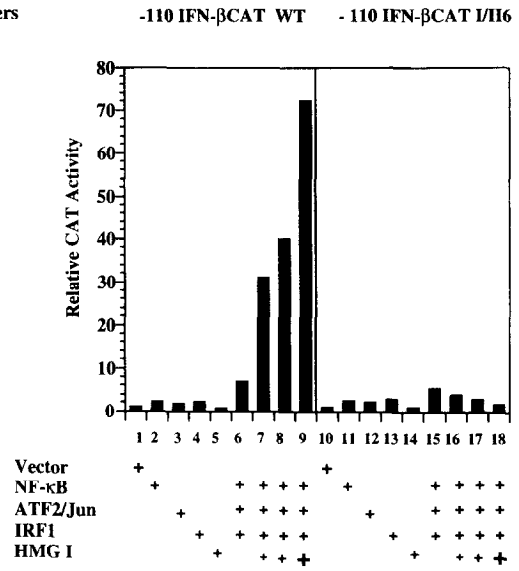

Figure 7. HMG I(Y) Is Required for the Synergistic Activation of the IFNβ Gene Promoter in Drosophila SL2 Cells

Drosophila SL2 cells were transfected with the reporter construct containing the IFNβ wild-type (lanes 1–9) or the helical mutant promoters (lanes 10–18) along with the indicated Drosophila expression vectors. The cells were harvested 48 hr after transfection, and the CAT activity was determined. Lanes 1 and 10, 8 μg of empty expression vector; lanes 2 and 11, 1 μg of an equimolar mixture of p50- and p65-expressing plasmids; lanes 3 and 12, 1 μg of *IRF-1* expression vector; lanes 4 and 13, 1 μg of an equimolar mixture of *ATF-2* and *c-jun* expression vectors; lanes 5 and 14, 2 μg of HMG I expression vector. Lanes 6 and 15 received the indicated expression plasmids in the same amounts as lanes 2–5 and 11–14. Lanes 7–9 and 16–18 received the same amount of activators as in lanes 6 and 15, plus increasing amounts of HMG I–expressing plasmid (1, 2, and 4 μg).

by the IFNβ gene activators, a result consistent with our previous observations (Thanos and Maniatis, 1992; Du et al., 1993).

Discussion

The human IFNβ gene is specifically activated in response to virus infection. Here, we provide evidence showing that this process requires the assembly of an enhanceosome, a higher order nucleoprotein complex containing at least three types of transcription factors and HMG I(Y). First, we show that a specific type and number of transcription factor–binding sites and their correct positioning on the face of the DNA double helix are required for virus induction, synergistic activation by transfected transcriptional activators, and the correct assembly of an enhancer complex in vitro. It is important to note that enhancers inactivated by the insertion of a half-helical turn can be fully reactivated by the addition of a second half-helical turn, which reestablishes the normal helical phasing of transcription factor–binding sites. Second, we show that HMG I(Y) is required for the synergistic interactions between transcriptional activators in cotransfection experiments in vivo and for cooperative interactions in enhanceosome assembly in vitro. Strikingly, these functions of HMG I(Y) also depend on a specific arrangement of protein-binding sites on duplex DNA.

Multiple protein–protein and protein–DNA interactions, and the formation of a highly specific three-dimensional structure, provide a self-editing mechanism for ensuring that the IFNβ gene is activated by virus and not by the many inducers of NF-κB or of the other transcriptional activators that bind to the enhancer. In addition, the cooperativity of enhanceosome assembly could determine the threshold levels of transcription factors required for transcriptional activation, and these levels could be inducer specific.

According to the model of enhanceosome structure illustrated in Figure 8, enhanceosome assembly would require the coordinate induction of all the IFNβ gene activators and the presence of HMG I(Y). In addition, specific members of each transcription factor family would be selected for assembly. For example, the PRDII and the PRDIV elements are recognized by a number of different Rel and bZIP protein family members, respectively. However, a number of observations indicate that the p50/p65 heterodimer is the optimal Rel family member involved in the activation of the IFNβ gene (Thanos and Maniatis, 1995a). Similarly, the only proteins detected in virus-inducible gel shifts of PRDIV are ATF-2 homodimers and ATF-2/c-Jun heterodimers (Du et al., 1993). The JunD–FosB heterodimer is capable of binding to PRDIV in vitro, but this interaction is strongly inhibited by HMG I(Y) (Du et al., 1993). As discussed above and elsewhere (Matsuyama et al., 1993; Reis et al., 1994) the protein(s) required

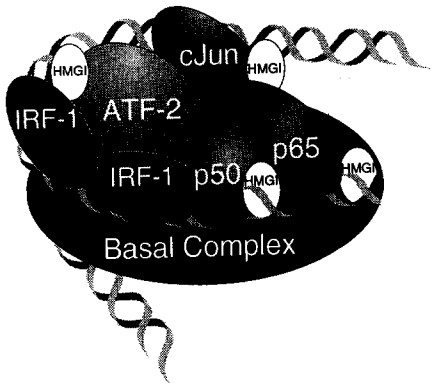

Figure 8. A Model for the IFNβ Gene Enhanceosome

The enhanceosome is a 60 bp segment of DNA bound to the transcriptional activators NF-κB (p50/p65), IRF-1, and ATF-2/c-Jun and HMG I(Y). HMG I(Y) is bound to the minor groove of DNA, while the transcriptional activators are bound to the major groove. The binding of HMG I(Y) to DNA and to NF-κB and ATF-2/c-Jun facilitates the conformational changes required to form the complex. Once assembled, the enhanceosome makes multiple contacts with the basal transcription complex. The relative sizes of the proteins and the length of the DNA covered are not drawn to scale.

for activation via PRDI and PRDIII has not been definitively identified. For the purpose of discussion here, however, IRF-1 is capable of promoting enhanceosome assembly in vitro and synergistic interactions in vivo. The formation of transcription factor complexes consisting of specific members of different families of transcription factors could, therefore, provide the means of integrating multiple signaling pathways to target the activation of specific genes.

An essential feature of the model outlined in Figure 8 is the role of HMG I(Y) (Thanos and Maniatis, 1992; Du et al., 1993). HMG I(Y) binds to the indicated sites in vitro and stimulates the binding of both NF-κB and ATF-2/c-Jun to their respective binding sites. An additional HMG I(Y)–binding site was recently identified between the NF-κB site and the TATA box, and mutations in this site also interfere with HMG I(Y) binding and significantly decrease the level of virus induction (D. T. and T. M., unpublished data). In an accompanying paper, we examine the structural role of HMG I(Y) in the IFNβ gene enhanceosome (Falvo et al., 1995). We show that PRDII has an intrinsic bend, which is reversed in a dimer-specific fashion by NF-κB in conjunction with HMG I(Y). The reversal of this bend correlates with the cooperative binding of NF-κB and HMG I(Y) to PRDII and with the ability of NF-κB to mediate virus induction of the IFNβ enhancer. We also show that PRDIV is intrinsically bent and that this bend is reversed by the binding of ATF-2/c-Jun. The DNA conformation in this complex is further modulated by HMG I(Y) (Falvo et al., 1995). Thus, the IFNβ enhancer appears to be prebent in an inactive configuration, and enhanceosome assembly leads to conformational changes required for the formation of an active complex.

The multiple protein–protein interactions portrayed in the model of Figure 8 are also supported by the observations that NF-κB and ATF-2/c-Jun (Du et al., 1993; Kaszubska et al., 1993; data not shown) and IRF-1 and NF-κB (Neish et al., 1995) interact specifically in solution in the absence of DNA. The fact that interactions between NF-κB and ATF-2 are stimulated by HMG I(Y) (data not shown) is not reflected in the model, since the mechanism of this stimulation is not known. The role of specific protein–protein interactions between HMG I(Y) and ATF-2 and between NF-κB and ATF-2 was directly demonstrated in vivo by the inability of the ATF-2_{192} variant (which does not interact with HMG I(Y) or NF-κB) to support synergistic activation of the IFNβ promoter at lower levels of ATF-2 expression.

Generality of Enhanceosomes

Previous studies have shown that alterations in the relative positions of upstream activator–binding sites and core promoters can adversely affect the activity of some prokaryotic (reviewed by Perez-Martin et al., 1994) and eukaryotic promoters (Windle and Sollner-Webb, 1986; Xie and Rothblum, 1992; Takahashi et al., 1986; Wu and Berk, 1988). However, in early studies, alterations in the helical relationships and relative orientations of transcription factor–binding sites within eukaryotic enhancers (Fromenthal et al., 1988; Ondek et al., 1988) and promoters (McKnight, 1982) did not significantly affect transcription. In addition, multiple copies of transcription factor–binding sites can mimic the properties of the intact enhancers and promoters from which they were derived (McKnight and Yamamoto, 1992). Thus, higher order structures were not thought to be essential for enhancer and promoter function.

More recent studies, however, have shown that changes in the relative positions of transcription factor–binding sites within enhancers and promoters can interfere with transcription (Natesan and Gilman, 1993; Giese et al., 1995; Meacock et al., 1994; Reith et al., 1994). Moreover, proteins that alter DNA structure play an essential role in enhancer function (Natesan and Gilman, 1993; Giese et al., 1992, 1995; Giese and Grosschedl, 1993; for recent review see Grosschedl, 1995) and lead to either activation or repression. These individual cases show the general importance of higher order structure in enhancer function, but they differ significantly from the IFNβ gene. First, the IFNβ enhancer contains multiple HMG I(Y)–binding sites, while the other enhancers contain a single binding site for an architectural protein. Second, HMG I(Y) interacts specifically with all three of the transcriptional activators that bind to the IFNβ enhancer. Third, HMG I(Y) does not induce a sharp DNA bend when it binds to the IFNβ enhancer. Rather, HMG I(Y) facilitates the reversal of intrinsic DNA bends in PRDII and PRDIV (Falvo et al., 1995).

A helical phasing requirement has also been demonstrated for TNFα induction of the E-selectin promoter (Meacock et al., 1994), a promoter which has been shown to contain HMG I(Y)–binding sites (Lewis et al., 1994; Whitley et al., 1994). Mutations in at least one of these sites, which abolish HMG I(Y) binding, also adversely affect TNFα induction (Lewis et al., 1994; Whitley et al., 1994).

Thus, HMG I(Y)–dependent enhanceosome assembly may be a characteristic of many inducible enhancers.

It is important to note, however, that strict spatial requirements for transcription factor–binding sites are not a property shared by all enhancers. For example, enhancer elements can be distributed over many thousands of base pairs of DNA (Goto et al., 1989; Corbin and Maniatis, 1990), and enhancers containing distinct organizations of the same binding sites can mediate expression in the Drosophila neuroectoderm (Gonzalez-Crespo and Levine, 1994). In fact, the requirements for spatial organization of a specific set of enhancer elements can be germ layer–specific during embryogenesis (Szymanski and Levine, 1995). Thus, enhancers can display considerable flexibility in their organization, perhaps a reflection of the mechanisms involved in the creation of novel patterns of gene expression during evolution. On the other hand, enhancers required for inducible on/off switches that respond to distinct extracellular signals may have evolved special mechanisms for achieving a high level of specificity.

Experimental Procedures

Cell Culture and Transfections

HeLa or P19 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, antibiotics, and L-glutamine (2 mM) in a humidified incubator containing 5% CO_2. Transient transfections, virus inductions, and CAT assays were performed essentially as described previously (Thanos and Maniatis, 1992). HeLa cells were transfected with 12 μg of reporter plasmid, 3 μg of CMV–β-gal expression vector, and 5 μg of pSP73 carrier plasmid. CAT activity was normalized for transfection efficiency by β-galactosidase activity (Miller, 1972). P19 cells were plated in 6-well plates 1 day prior to transfection and were approximately 30% confluent at the time of transfection. The cells were refed with fresh media 3 hr before transfection. The transfection cocktail contained 1 μg of reporter plasmid, 100 ng of CMV–β-gal, and the indicated amounts of expression vectors. The total amount of DNA was kept constant by including the appropriate amounts of the empty expression vector. Inductions with Sendai virus, TNFα, IFNγ, and cAMP were performed as previously described (Fan and Maniatis, 1989; Du et al., 1993; Thanos and Maniatis, 1995a).

Drosophila melanogaster SL2 cells were maintained in Schneider media (GIBCO BRL) and supplemented with 12% fetal calf serum and antibiotics at ambient temperature. SL2 cells were plated in 6-well dishes 24 hr prior to transfection and transfected by the method of Chen and Okayama (1988). Cells were harvested 48 hr posttransfection, and CAT activity was determined from cell extracts. Normalization for transfection efficiency was performed in all experiments based on β-galactosidase activity. The transfection cocktail contained 1 μg of the −110IFNβ–CAT reporter construct, 100 ng of hsp82lacZ plasmid (which was used as an internal control for transfection efficiency) (Abel et al., 1992), and the indicated activators. Vector DNA (pPAC) was added as necessary to achieve a constant amount of transfected DNA.

Plasmid Constructions

Insertion and substitution mutants of the IFNβ promoter were constructed by using the PCR methodology described previously (Thanos and Maniatis, 1992), and all mutations were verified by DNA sequencing. The PRDIV to PRDII substitution was constructed by replacing the ATF-2/c-Jun binding site (−100 to −91) with PRDII (−64 to −55). The I/II6 and I/II10 constructs contain insertions between nucleotides −65 and −64 from the start site of transcription. The IV/III4 and IV/III10 constructs have insertions between −91 and −90. Finally, the II/TATA6 and II/TATA10 contain insertions between −38 and −37. The mammalian expression vectors for p50, p65, ATF-2, c-Jun, and IRF-1 were as previously described (Thanos and Maniatis, 1992, 1995a; Du et al., 1993). To construct the Drosophila expression vectors, we cloned the entire open reading frames for p50, p65, *ATF-2*, *c-jun*, *IRF-1*, and HMG I in the BamHI site of the pPAC plasmid (Krasnow et al., 1989).

Assembly of the Transcription Enhancer Complex and DNase I Footprinting Assays

Bacterially expressed and purified p50, p65, ATF-2, and HMG I(Y) proteins were as described previously (Thanos and Maniatis, 1992; Du et al., 1993). To construct the *IRF-1* expression plasmid, the entire open reading frame of *IRF-1* was cloned in the bacterial expression vector pRSETA (Invitrogen) in-frame with the His-6 moiety. After refolding, the proteins were concentrated (1 mg/ml, except p50/p65, which was 300 ng/ml) and extensively dialyzed in DB buffer without BSA but supplemented with 0.3% NP-40 and 0.5 mM PMSF. For the DNase I footprinting experiments, IFNβ promoter fragments (20,000 cpm) from −110 to +20 were end labeled at the noncoding strand and incubated with the indicated proteins in 20 μl of DB buffer (10 mM Tris [pH 8.0], 15 mM HEPES [pH 7.9], 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) and 1 mg/ml BSA, 5% glycerol on ice for 30 min. DNase I (Worthington) was diluted in 50 mM $CaCl_2$, 20 mM HEPES (pH 7.9) to 2 ng/ml, and 2 μl was added directly to the binding reaction. Digestion was allowed for 5 min on ice, followed by the addition of 200 μl of stop buffer (2.5 M ammonium acetate, 25 μg/ml sonicated salmon sperm DNA). The DNA was ethanol-precipitated, dried, resuspended in 3 μl of formamide dye, and loaded on a 6% sequencing gel.

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