# Isolation and Functional Characterization of Cofactors of RNA Polymerase II Transcription

by

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# Abstract

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The initiation of transcription by RNA polymerase II is the primary target for the regulation of gene expression. Significant advances have been made in recent years in the identification and characterization of the components responsible for the initiation of transcription known as the general or basal transcription factors and of the protein factors known as transcriptional activators that function through cis-acting control DNA elements to stimulate the initiation process. Additionally, an increasing number of protein factors that can not be categorized in either of these two groups but affect the level of expression of genes are being identified. These factors, collectively known as the cofactors of transcription, are implicated in regulatory mechanisms of transcription, but their mechanism of action is not well defined in general. We have established a transcription system *in vitro* with highly defined basal factors and activators in order to isolate and analyze the function of cofactors. This thesis describes characterization of two such cofactors: the 90 kDa factor and the high mobility group protein 2 (HMG-2).

The 90 kDa factor was isolated based on its stimulatory effect on the basal transcription reaction from the adenovirus major late promoter and inhibitory effect on the immunoglobulin heavy chain gene promoter. Interestingly, the 90 kDa factor is comprised of a formerly characterized negative cofactor known as Dr1/p19 and a novel 30 kDa protein homologous to a suppressor of an SRB4 mutation in yeast. The 90 kDa factor is a general negative cofactor repressing transcription from diverse promoters. It functions by forming a transcriptionally inert protein-DNA complex with TATA-binding protein (TBP) as indicated by electrophoretic mobility shift and DNase I footprinting analyses. TFIIA was shown to reverse the repression by the 90 kDa factor in a promoter specific manner, thus resulting in a change in the expression level of different promoters. This in turn suggests that the promoter specific repression can be mediated by regulation of negative cofactors such as the 90 kDa factor.

HMG-2 was identified as a required cofactor for the activated transcription mediated by Gal4-VP16. Staging analyses with immobilized transcription templates showed that it is at the step of TFIID-TFIIA-DNA (DA) complex formation where HMG-2 functions. Its mechanism of action appears to involve altering the structure of DA complexes and not simply increasing the number of DA complexes. The DNA binding and bending domain known as the HMG box was shown to be sufficient for coactivation of transcription. This fact, together with the failure to detect an increased formation of the DA complex led to the hypothesis that an HMG-2 coactivates by inducing and stabilizing the activated DA complex conformation. DNase I footprinting analyses of the DA complex in combination with the activator and HMG-box supported this hypothesis.

Thesis supervisor: Dr. Phillip A. Sharp, Salvador E. Luria Professor and Head, Department of Biology

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## *Memory*

*And you wait, awaiting the one*
*to make your small life grow*
*the mighty, the uncommon,*
*the awakening of stone,*
*the depth to be opened below.*

*Now duskily in the bookcase*
*gleam the volumes in brown and gold;*
*you remember lands you have wandered through,*
*the pictures and the garments*
*of women lost of old.*

*And you suddenly know: It was here!*
*You pull yourself together, and there*
*stands an irrevocable year*
*of anguish and vision and prayer.*

***Rainer Maria Rilke***

# Chapter I

# Introduction

Gene expression is primarily regulated at the level of transcription. Thus, the regulation of transcription is key to successful differentiation, development, and survival of organisms. For each of RNA polymerases I, II, and III (pol I, pol II, and pol III), the initiation of transcription is mediated by distinct multi-subunit enzymatic machineries whose assembly and activation are subject to regulation. The burden of regulation is particularly heavy for the RNA polymerase II (pol II) transcription system as its products, mRNAs, represent an enormously diverse group whose individual members need to be expressed differentially. Consequently, evolution has devised elaborate, multi-tiered regulatory mechanisms to control the transcription level of each of the pol II genes according to the unique demand for its expression.

The common element of the transcription for most if not all pol II promoters is the protein factors of the core enzymatic machinery. These factors are not only common among the various promoters but also are conserved in large part through the eukaryotic evolution. Much of the current knowledge about these factors has come from reconstitution of transcription *in vitro* with chromatographic fractions derived from cellular extracts. Subsequent purification of the independently necessary and collectively sufficient factors for transcription *in vitro* has led to the identification of a set of the so-called general or basal transcription factors. The various regulatory mechanisms that bring about promoter specific expression target these basal factors in the way they are recruited, assembled, and modified.

Another class of transcriptional factors operates through cis-element recognition. Known as transcriptional activators, this highly diverse group of factors amplify the level of expression of target promoters in a cognate sequence dependent manner. The proposed mechanisms of their action include derepression of promoters from a chromatin bound ground state, recruitment of basal factors to promoters, induction of activated initiation complex conformation, and promotion of elongation.

As the purification of the basal factors progressed, many other activities that affected the level of transcription *in vitro* were identified. Commonly called transcriptional cofactors these are operationally defined as factors that neither are absolutely required for the initiation of transcription nor have specific cognate DNA sequences. One group, often referred to as coactivators, is required *in vitro* in addition to transcriptional activators for efficient activated transcription. Another group, called negative cofactors, repress transcription by targeting specific components of the core enzymatic machinery. Often their action can be reversed by transcriptional activators thus elevating the relative level of expression in an activator dependent manner.

This thesis reports the identification, purification, and functional characterization of a coactivator and a negative cofactor. This introductory chapter will expand the overview presented in this section by providing a detailed summary of the current understanding of the regulation of pol II transcription.

## I. Basal Factors of Pol II Transcription

The identification of the protein factors involved in the initiation of transcription became possible with the reproduction of accurate synthesis of mRNA *in vitro* with cellular extracts (Weil et al., 1979; Manley et al., 1980; Dignam et al., 1983). This was followed by reconstitution of transcription with chromatographic fractions which established that aside from RNA polymerase II, several independent accessory protein factors are necessary (Matsui et al., 1980; Samuels et al., 1982). Of particular interest was TFIID which was shown to recognize the TATA element, often found 30 bases upstream of the initiation site. This observation led to the proposal that TFIID was the nucleator of the initiation of transcription (Fire et al., 1984; Sawadogo and Roeder, 1985).

The next breakthrough came from the observation that yeast TFIID can functionally substitute for human TFIID *in vitro* (Buratowski et al., 1988). Purification of the yeast TFIID and isolation of its cDNA clone led to identification of TATA-binding-protein

(TBP), a highly conserved protein throughout the eukaryotic kingdom (Hahn et al., 1989; Horikoshi et al., 1989; Schimidt et al., 1989; Cavallini et al., 1989; Kao et al., 1990; Hoey et al., 1990; Peterson et al., 1990). Human TFIID was shown to be a >700 kDa complex with TBP as a subunit (Samuels et al., 1982; Timmers and Sharp, 1991). Interestingly, TBP by itself was able to mediate initiation of transcription, but not able to mediate an activated level of transcription (Hoey et al., 1990; Pugh and Tjian, 1990). This in turn led to the hypothesis that attendant factors in the TFIID complex are important for mediating the activating signals from transcriptional activators. This has been demonstrated through subsequent analyses (see below).

The identification of the nucleating activity in the form of TFIID or TBP allowed defining the other basal factors in terms of the order of association with the protein-DNA complex formed at the promoter. This led to a model of the ordered assembly of basal factors into the initiation complex (Buratowski et al., 1989). TFIIA was shown to associate with TBP at the TATA site and stabilize the protein-DNA complex (Fire et al., 1984; Buratowski et al., 1989). Based on its ability to stabilize the TBP-DNA complex in native polyacrylamide gels, human and Drosophila TFIIA were purified to homogeneity and shown to consist of three peptides (35 kDa, 19 kDa, and 12 kDa species for human: Ma et al., 1993; DeJong and Roeder, 1993; Ozer et al., 1994; Sun at al., 1994 and 30 kDa, 20 kDa, and 14 kDa for Drosophila: Yokomori et al., 1993; Yokomori et al. 1994). For both the human and Drosophila TFIIA, the larger two subunits were shown to be derived from a single gene. The role of TFIIA in the initiation of transcription has been controversial for some time. The current consensus is that although it is not required for *in vitro* transcription with highly purified basal factors, TFIIA is vital in mediating the signal from transcriptional activators and blocking the repression by negative cofactors (Sun et al., 1994; Yokomori et al., 1994; Ozer et al., 1994; also see Chapter II).

The next to join the preinitiation complex was shown to be TFIIB (Buratowski et al., 1989). Originally characterized as an absolutely required protein factor for transcription *in*

*vitro*, it was shown also to associate with pol II (Reinberg and Roeder, 1987). Together, these observations led to the hypotheses that TFIIB bridges TBP and pol II. TFIIB was purified based on its absolute requirement in transcription assays *in vitro* and its association with the TBP-DNA complex (Ha et al., 1991). Cloned and expressed as a recombinant protein, TFIIB was shown to function as a monomer of 35 kDa protein (Ha et al., 1991). Domain analysis of TFIIB protein validated its proposed role as a bridging protein: roughly one half of the protein is responsible for association with TBP, and the other half, for recruiting pol II (Ha et al., 1993; Barberis et al., 1993).

Eukaryotic RNA polymerase II is a multiprotein complex, and the functional analysis of and isolation of cDNA's for its subunits are not complete (Young, 1991; Buratowski, 1994). The study of pol II has centered around the C-terminal domain (CTD) of the largest subunit which features multiple repeats of a heptapeptide sequence (Young, 1991). The CTD has been shown to be subject to regulation by phosphorylation, and the variant forms, pol IIA and pol IIO, represent nonphosphorylated and phosphorylated forms respectively (Young, 1991). It is the pol IIA form that has been shown to join the initiation complex (Laybourn and Dahmas, 1990; Lu et al. 1991). Further, pol IIA but not pol IIO was shown to interact with TBP through the CTD (Usheva et al., 1992). Together with the data that the elongating form of pol II is pol IIO, these observations led to the hypothesis that phosphorylation of the CTD is the transition event between initiation and elongation (Cadena and Dahmus, 1987; Payne et al., 1989). However, the significance of the phosphorylation as well as the role of the CTD itself is not yet clear as transcription can occur in the absence of CTD phosphorylation or even with a form of polymerase that lacks the CTD (Kim and Dahmus, 1989; Seizawa et al., 1993; Makela et al., 1995). One possible explanation is suggested by recent data which indicated that the phosphorylation may be required for only a subset of promoters (Akoulitchev et al., 1995).

Genetic studies with S. cerevisiae have established that the CTD is essential for viability (Young, 1991). Partial truncation provided conditional lethal mutants whose

suppressors are collectively known as the suppressors of RNA polymerase B (SRB's; Nonet and Young, 1989; also for review, see Koleske and Young, 1995). Interestingly, these SRB proteins were found as parts of a large protein complex that includes not only pol II but also TFIIB, TFIIF, and TFIIH (Koleske and Young, 1994). Further, this complex could mediate not only basal level transcription *in vitro* when combined with TFIID and TFIIE but also activated transcription if activator was added (Koleske and Young, 1994). This so-called holoenzyme provides a much simpler alternative pathway in the assembly of the initiation complex and challenges the ordered assembly model (see below).

TFIIF, TFIIE, and TFIIH were separated from one another based on their independent requirement for transcription *in vitro* (Flores et al., 1989; Flores et al., 1992). TFIIF is believed to be a dimer of heterodimers composed of 74 kDa and 30 kDa proteins (Flores et al., 1988; Sopta et al., 1989; Flores et al., 1990; Aso et al., 1992; Finkelstein et al., 1992). TFIIF was shown to interact with pol II, and the identification of its subunits as independently characterized pol II associated factors suggested that the two factors join the preinitiation complex as a preassembled form of pol II-TFIIF (Sopta et al., 1985; Flores et al., 1988). This hypothesis is supported by the fact that the small subunit of TFIIF interacts with TFIIB and recruits pol II into the preinitiation complex (Flores et al., 1991; Ha et al., 1993). TFIIF has also been shown to increase the elongation efficiency of pol II (Price et al., 1989; Bengal at al., 1991; Tan et al., 1994).

TFIIE is composed of two 56 kDa and two 34 kDa proteins (Ohkuma et al., 1990; Inostroza et al., 1991; Peterson et al., 1991; Ohkuma et al., 1991; Sumimoto et al., 1991). It also is shown to associate with pol II (Flores et al., 1989; Inostroza et al., 1991). As it joins the initiation complex after the pol II-TFIIF complex, its role has been suggested to include recruitment of TFIIH (Inostroza et al., 1991; Peterson et al., 1991; Maxon et al., 1994). Interestingly, TFIIE and TFIIH can be substituted by negative supercoiling of the template in transcription assays *in vitro* (Parvin and Sharp, 1993; Tyree et al., 1993;

Goodrich and Tjian, 1994). This in turn suggested that their role may be mediating the energy consuming steps such as open complex formation or promoter clearance. TFIIH is a multiprotein complex associated with several biological activities (Flores et al., 1992). Among these are CTD kinase activity and DNA helicase activity both of which could be related to the energy consuming step that TFIIH is believed to mediate (Lu et al., 1992; Schaeffer et al., 1993; Roy et al., 1994a ; Roy et al. 1994b; Serizawa et al., 1995). Interestingly, several subunits of the TFIIH complex have been found to be components of the nucleotide excision repair system (Schaeffer et al., 1993; Schaeffer et al., 1994; Humbert et al., 1994; for reviews see Drapkin et al., 1994 and Seroz et al., 1995). A current model envisions pol II bound TFIIH recruiting the rest of the repair machinery at the damage site where pol II would be stalled, thus accounting for the preferential repair of the transcribed strand of DNA.

The ordered assembly model is not as yet a validated model through *in vivo* analysis. As mentioned above CTD truncation mutations in S. cerevisiae led to identification of a new class of proteins known as the SRB's which in turn led to isolation of the so-called holoenzyme comprised not only of the pol II complex and SRB proteins but also of several basal transcription factors (Koleske and Young, 1994). Genetic analyses further indicate that in S. cerevisiae, the holoenzyme may be the only form of polymerase that actively participate in transcription *in vivo* (Thompson and Young, 1995). Recently, a similarly preassembled human holoenzyme has been reported (Ossipow et al., 1995; Chao and Young: personal communication). Conflicts among these reports as to components of the human holoenzyme preclude a firm conclusion regarding its composition. The generality or exclusiveness of holoenzyme as the active form of pol II with respect to promoters, cell types, and organisms as well as its exact composition remain to be determined. It should be added that even if the holoenzyme turns out to be used exclusively *in vivo*, the specific interactions between factors and their proposed role discovered through *in vitro* reconstitution are likely to remain true.

## II. Transcriptional Activators

Transcriptional activators represent a large group of proteins. Accordingly, an enormous number of studies have been carried out in investigating various aspects of their biological function. This section will focus on the regulatory role of the transcriptional activators in the transcription initiation process. Another important role of activators, derepression, will be discussed in the following section. Both the ordered assembly model and the holoenzyme model present a framework where the regulatory roles of transcriptional activators can be analyzed. Clearly, in both models, several potential steps exist whose stimulation could result in an increased production of mRNA. The role of transcriptional activators has been proposed to be precisely this: accelerating one or more of the slow steps of the initiation process.

### i) Interaction with the basal factors

The TATA biding protein (TBP) was the first basal factor shown to bind to transcriptional activators. This was demonstrated by retention of both human and yeast TBP on a viral activator VP16 affinity column (Stringer et al., 1990). The significance of this observation has been questioned because TBP was found in association with a large number of proteins which might render TBP inaccessible to activation domains. Another reason for the skepticism was that TBP was not capable of mediating activated transcription. However, several other activation domains have been shown to bind to TBP subsequently, suggesting the general importance of the interaction between TBP and activators (Emili et al., 1994; Liu et al., 1993). Furthermore, site-specific mutational analysis showed that activation domain mutants that fail to activate transcription *in vivo* also often fail to bind TBP *in vitro* (Ingles et al., 1991; Geisberg et al., 1994). Also, in one study, a yeast TBP mutant that was normal for basal transcription but defective for stimulated transcription was shown to have impaired biding to VP16 (Kim et al., 1994).

As discussed above, that TBP could not mediate activated transcription and that an apparent size difference exists between native TFIID and recombinant TBP led to the

hypothesis that TBP is in complex with several other proteins in TFIID and that these are important for the activation process (Hoey et al., 1990; Pugh and Tjian, 1990). Immunoprecipitation analysis of the Drosophila TFIID activity indeed showed that along with TBP, several proteins, now called TBP-associated factors (TAF's), were present (Dynlacht et al., 1991; for reviews see Goodrich and Tjian, 1994 and Maniatis and Tjian and Maniatis, 1994). Additionally, it was demonstrated that dissociation of TBP from the activation competent complex by subjecting it to a denaturing condition rendered TBP inert to the activator NTF-1 unless renatured TAF's were added back, thus confirming the suggested importance of TAF's in the activation (Dynlacht et al., 1991). Subsequently, human TFIID also was shown to be a complex of TBP and TAF's (Tanese et al., 1991; Zhou et al., 1992).

Further analyses focused on the interaction between TAF's and activators. Several direct interactions of activators with TAF's have been demonstrated: SP1 with TAF110, NTF-1 with TAF150, VP16 with TAF40, p53 with TAF40 and TAF160, Bicoid with TAF110 and TAF60, and Hunchback with TAF60 (Hoey et al., 1993; Goodrich et al., 1993; Chen et al., 1994; Thut et al., 1995; Sauer et al., 1995a; Sauer et al., 1995b). The importance of these interactions have been tested using TFIID subcomplexes assembled with recombinantly expressed TBP and subsets of TAF's. It was shown that the presence of target TAF or TAF's in the subcomplex was strictly required for activation by a given transcriptional activator (Chen et al., 1994; Thut et al., 1995; Sauer et al. 1995a; Sauer et al., 1995b). In addition, two activators were shown to synergistically activate transcription if and only if the target TAF's for both of the activators were present (Sauer et al., 1995a). Thus it appears that the large TFIID complex is a principal target of activation domains.

Another basal factor shown to interact with activation domains is TFIIB. Acidic and proline-rich domains can bind to and stabilize the interaction of TFIIB with the preinitiation complex (Lin et al., 1991; Lin and Green, 1991; Kim and Roeder, 1994). Furthermore, a TFIIB point mutant with a lowered affinity for VP16 was shown to mediate only basal but

not activated transcription, suggesting that a direct interaction between TFIIB and the activation domain is indeed important (Roberts et al., 1993). However, it should be noted that even though such stabilization of TFIIB occurred with both TFIID and TBP, it was only with TFIID that activation was seen (Choy and Green, 1994). Further interactions involving TAF's were proposed and demonstrated subsequently (Goodrich et al., 1993).

Acidic activation domains of VP16 and p53 were also shown to directly interact with TFIIH through one of its subunits, p62 (Xiao et al., 1994). Point mutants of VP16 with lowered transactivating activity show lowered binding to TFIIH consistent with the proposed importance of this interaction (Xiao et al., 1994). As discussed above, kinase and helicase activities of TFIIH have been implicated in the transition between initiation and elongation. These enzymatic steps represent potential rate limiting steps during the initiation process, but such stimulation by activators has yet to be reported.

The most recently discovered basal factor target of the activation domain is TFIIA. Both VP16 and another viral activator Zta were shown to directly bind TFIIA (Ozer et al., 1994; Kobayashi et al., 1995). TFIIA has been demonstrated to be required for activated transcription *in vitro*. A correlating requirement for TFIIA in stimulated formations of TFIID-promoter complex by activator has also been reported (Ozer et al., 1994; Sun et al., 1994; Yokomori, K., 1994; Lieberman and Berk, 1994). In addition, deletion derivatives of VP16 with reduced binding to TFIIA also showed reduced ability to promote the formation of the TFIID-TFIIA-DNA ternary complex (Kobayashi et al., 1994).

**ii) Mechanism of activation: recruitment**

Modeling the initiation of transcription as a multistep chemical process and demonstrating that transcriptional activators directly interact with basal factors inevitably led to the hypothesis that the role of activators is to recruit basal factors to promoters. Such recruiting would be analogous to increasing the concentration of basal factors available to join the preinitiation complex. If the recruited factor was in limiting quantities, the result would be increased production of RNA. Examples of such recruiting phenomena have

been observed. Crude preparations of TFIID were shown to bind more stably to the TATA element in the presence of activators, ATF or USF, suggesting that these activators can recruit TFIID to promoter (Sawadogo et al., 1985; Horikoshi et al., 1988). These studies were repeated with homogeneous preparations of TFIID. In the presence of either the acidic activator Gal4-VP16 or the non-acidic activator Zta, the TFIID-TFIIA-promoter complex formed more rapidly and to a greater extent suggesting that a complex with a different equilibrium constant was established by the activators (Lieberman and Berk, 1994). Similarly, the Drosophila activators, Bicoid and Hunchback were shown to promote binding of the TFIID subcomplex that contained their respective target TAF's to the promoter (Sauer et al., 1995a). Furthermore, they synergistically increased promoter occupation by a TFIID subcomplex that contained both of their target TAF's (Sauer et al., 1995a).

The significance of TFIID recruitment was tested *in vivo*. A fusion protein of the LexA DNA binding domain and TBP was shown to produce an activated level of transcription in a LexA biding site dependent manner (Chatterjee and Struhl, 1995). In an analogous experiment using the DNA binding domain of RFX1 and TBP fused to the leucine zipper motifs of max and myc respectively, an activated level of transcription was obtained in an RFX1 binding site dependent manner (Klages, N. and Strubin, M., 1995). As activation domains were absent in these fusion proteins, it was argued that the role of activation domains was no more than a tether between DNA binding domain and TBP used to recruit TBP to promoters.

Other proposed targets of recruitment by activators include TFIIB and the holoenzyme. An acidic activator was shown to have no effect on binding of TBP but increased the proportion of promoters occupied by TFIIB (Lin and Green, 1991). Likewise, proline-rich activator CTF1 also resulted in recruitment (or stabilization ) of TFIIB to the initiation complex (Kim and Roeder, 1994). Transcription mediated by the holoenzyme can be stimulated by Gal4-VP16 unlike reactions containing pol II and basal factors purified as

separate components (Koleske and Young, 1994). Single step recruitment of the holoenzyme was proposed as a mechanism of activation, and supporting evidence came from the observation that the holoenzyme was specifically retained by a VP16 affinity column (Hengartner et al., 1995). Another line of support for the model of activation by holoenzyme recruitment comes from analysis of a mutation of Gal11, a subunit of the holoenzyme (Barberis et al., 1995). A mutation in Gal11 resulted in the ability to interact with the dimerization domain of Gal4. An activation of transcription occurs in the yeast strains with this mutation in a Gal4 binding site dependent manner without requiring the activation domain. Since the activation domain could be replaced by an artificial tether between DNA binding domain and the holoenzyme, it is thus argued that activation domains function as recruiting modules for the holoenzyme.

**iii) Mechanism of activation: conformational change**

A direct consequence of modeling the role of activators as simple recruiting of limiting basal factors is that increasing the concentration of the proposed target factor should obviate the need for the activator. This hypothesis was tested in the case of TFIIB by titrating TFIIB over several logs of concentration into activated transcription reactions. The result clearly indicated that increasing TFIIB cannot circumvent the need for activators (Choy and Green, 1993; Shykind et al., 1995). Another prediction of the recruitment theory is that under conditions where recruitment is seen, activation of transcription also should be seen if the rest of the basal factors are provided. Although TFIIB recruitment was seen in conjunction with TBP and an activator, no activation of transcription was obtained unless TBP was replaced with TFIID (Choy and Green, 1993). Likewise, under conditions where recruitment of TFIID by activator was seen, addition of the rest of the basal factors resulted in no activation unless additional factors were included (Lieberman and Berk, 1994; see below about cofactors). These observations indicate that simple recruitment of basal factors to promoters by activators cannot be equated to the entire activation process.

The failure to circumvent the need for activators by increasing the concentration of a basal factor suggests that there are other slow steps that may be stimulated by activators. One such step is proposed to be a conformational change, or isomerization to an activated state of protein -DNA complex. That a conformational change occurs in pol II transcription is suggested by a kinetic study of TBP binding to DNA (Hoopes et al., 1992). The binding of TBP was shown to display a kinetic behavior characteristic of a two step process. It is proposed that initial binding is followed by an isomerization into a more stable complex. Thus, the step commonly depicted as a single step binding could in fact represent two or more steps subject to potential regulation by activators. Such alteration is not always detectable by assays designed to assess a quantitative increase in the number of protein-DNA complex. If the interaction of an activator and basal factor stimulates the association of the basal factor with the promoter and also induces conformational change, simply increasing the concentration of the basal factor will not fully compensate for the absence of the activator. This is particularly so if the conformational change step is much slower than the binding step.

Several studies have shown that activators can affect the conformation of the preinitiation complex. In the studies with crude TFIID, Gal4 derived synthetic transcriptional activators altered the interaction between TFIID and adenovirus E4 promoter qualitatively (Horikoshi et al., 1988). Specifically, the activator induced downstream protection in footprinting analysis that was the past initiation site and was not seen when TFIID and Gal4 were tested individually. Thus, an induction of a new conformation could be inferred. Similar induction of extended alteration of footprint is also seen with the activator Zta and a homogeneous preparation of TFIID (Lieberman and Berk, 1994). A conformational change by activator is also seen with TFIIB. In a partial proteolysis analysis, the V8 protease cleavage pattern of TFIIB was shown to be altered in the presence of the activator Gal4-VP16 (Roberts and Green, 1994). This phenomenon was proposed

to reflect a conformational change which promotes subsequent recruitment of pol II-TFIIF into the initiation complex.

**iv) Regulation at multiple steps**

In previous sections, several specific interactions between activators and basal factors were described. Also, conformational changes were proposed as potential regulatory effects of activators. Collectively, these observations suggest that activators can affect several steps in the initiation process rather than a single step. The analysis of the interaction between activators and TFIIB is instructive. It was shown that activators promote TFIIB binding to promoters preoccupied with either TBP or TFIID (Lin and Green, 1991; Choy and Green, 1994). Activators were also shown to recruit down stream basal factors such as pol II, TFIIF, and TFIIE (Choy and Green, 1994). Interestingly, the recruitment of the latter three factors required the presence of TAF's as TBP was unable to mediate the recruitive effect (Choy and Green, 1994). It was thus demonstrated in a given *in vitro* system that activators can affect more than a single step of the initiation.

Further support for the multistep regulation model comes from synergistic behavior of activators. Synergism refers to a greater than additive effect of individual activators (For review see Herschlag and Johnson, 1993). This phenomenon has been seen both with promoters that often have binding sites for several distinct activators and with promoters with multiple binding sites for a single activator (Lin et al., 1990; Carey et al., 1990). The basis of synergism is unlike that seen with prokaryotic transcription factors where binding of one factor can promote binding of another. Rather, Gal4 derived activators synergized under saturating conditions consistent with the hypothesis that the synergism was due to simultaneous interaction between activators and multiple targets (Carey et al., 1990). Kinetic modeling argues that in a multistep pathway, accelerating any step would contribute to an increase in the overall rate of the reaction. Furthermore, the slower a given step is, the bigger the effect of stimulation will be on the overall rate. Most importantly, the effect of an individual stimulatory event will be compounded rather than added (Herschlag and

Johnson, 1993; Kingston and Green, 1994). Thus, the synergistic effect of multiple activators most likely reflects stimulation of several slower steps rather than a single slowest step.

### III. Cofactors of transcription

Transcriptional cofactor is an operational term used to describe a group of protein factors that are neither basal transcription factors nor have specific DNA binding sites but nevertheless affect the level of transcription. As genetic and biochemical analyses reveal additional factors that fit such a loose description, it is recognized that a highly diverse group of proteins make up this group. Most likely, equally diverse mechanisms are awaiting to be unraveled. This section will summarize the current understanding of several groups of cofactors. Particular attention will be given to their regulatory interaction with basal factors and activators.

#### i) Structural and regulatory proteins of chromatin

Most of the *in vitro* analysis thus far described has used naked plasmid DNA as the transcription template. *In vivo*, DNA is found complexed with chromosomal proteins, most notably with the histone proteins which in association with DNA form nucleosomes, the structural unit of chromatin. Genetic and biochemical data indicate that one level of gene regulation occurs through regulation of the nucleosome structure (for reviews, see Workman and Buchman, 1993; Paranjape et al., 1994; Wolffe, 1994). In yeast, depleting histone H4 *in vivo* was shown to result in the activation of several promoters (Han and Grunstein, 1988). In addition, mutations of the N-terminal domains of histones H3 and H4 raised the expression level of distinct subsets of genes (Mann and Grunstein, 1992; Roth et al., 1992). These *in vivo* observations were in good agreement with repression of basal transcription by reconstituted nucleosomes *in vitro* (Knezetic and Luse, 1986; Workman and Roeder, 1987). Similar repression was also achieved with histone H1 alone (Croston et al., 1991; Croston et al., 1992; Lorch et al., 1992).

These observations led to the hypothesis that one step involved in transcription initiation *in vivo* is overcoming the structural impediment presented by nucleosomes. Indeed, preincubation of the template with TFIID or a set of basal factors prevented transcriptional repression by nucleosome assembly (Workman and Roeder, 1987). Likewise, sequence specific factors were shown to counteract the repression mediated by nucleosomes or histone H1 (Laybourn and Kadonaga, 1991; Workman et al., 1991; Croston et al, 1991; Croston et al., 1992; Lorch et al., 1992; Dusserre and Mermod, 1992). Furthermore, it was shown that activation domains were necessary for derepression of promoter activity indicating that derepression is not a simple competition for DNA binding (Workman et al., 1991; Croston et al., 1992). An interesting consequence of the derepression activity of activators is that the relative ratio of activation becomes higher as the consequence of concomitant repression of the basal reaction (Workman et al., 1991; Laybourn and Kadonaga, 1991). This suggests that *in vivo* such mechanism could be used to render a gene hyper-responsive to activators from its nucleosome-repressed ground state. Another interesting observation was that if transcription on nucleosome-bound template was reconstituted with highly purified basal factors, the anti-repression activity of the activator was not seen (Croston et al., 1992; Lorch et al., 1992; Dussserre and Mermod, 1992). This indicated that the competitive interaction between transcription factors and nucleosomes likely involved other cofactors.

One such cofactor may be the SWI/SNF complex. SWI and SNF genes were originally identified from independent genetic screens (for review, see Winston and Carlson, 1992). Subsequent analysis showed clear functional interdependence among the members of the two groups in activating transcription from diverse promoters (Hirschhorn et al., 1992; Peterson and Herskowitz, 1992). Subsequent purification of several SWI/SNF proteins as a large complex confirmed that these genes share a common function (Cairns et al., 1994; Peterson et al., 1994; Cote et al., 1994). Their role appeared to involve reconfiguration of chromatin structure for the purpose of transcriptional activation

as mutations in several SWI/SNF genes resulting in reduced transcription could be suppressed by mutations in histone genes (Hirschhorn et al., 1992; Kruger et al., 1995). *In vitro* analysis provided further support. Purified yeast and human SWI/SNF complexes stimulated binding of Gal4 transcription factors in the presence of nucleosomes (Cote et al., 1994; Kwon et al., 1994). In addition, the human SWI/SNF complex also stimulated binding of TBP to the promoter (Imbalzano et al., 1994). Recruitment of the SWI/SNF complex to the promoter may in fact be carried out by activators. Immunoprecipitation of the glucocorticoid receptor from wild type but not SWI mutant strains showed that SWI3 was coprecipitated (Yoshinaga et al., 1992). Interestingly, a recent report indicated that the yeast holoenzyme contains SWI2/SNF2, SWI3, SNF5, and SNF11 and is active in reconfiguration of nucleosomes (Wilson et al., 1996). This observation suggests that recruiting of the holoenzyme by activators would bring SWI/SNF activity to promoter, and the SWI/SNF complex would in turn stabilize the binding of activators and TBP.

Another protein implicated in the regulation of chromatin structure for transcriptional activation is HMG-17, a member of a non-histone chromosomal high mobility group proteins (HMG; Paranjape et al., 1994; Paranjape et al., 1995). HMG-17 was shown to preferentially associate with nucleosomes over naked DNA and to activate transcription in the presence of Gal4-VP16 from nucleosome-bound promoters. This potentiation of activation required the presence of HMG-17 during the assembly of nucleosomes and was dependent on the activation domain of Gal4-Vp16. Thus, a mechanism by which HMG-17 controls the accessibility of nucleosome bound promoters to transcription factors has been proposed (Paranjape et al., 1995).

**ii) Negative cofactors of pol II transcription**

Genetic experiments in yeast have implicated several activities as negative cofactors of pol II transcription. The SPT4 to STP6, TSF1 to TSF6, and SUD1 genes were discovered as suppressors to mutations of upstream controlling elements of specific promoters (Swanson and Winston, 1992; Chen et al., 1993; Yamashita, 1993). Mutations in these

genes often elevated the level of transcription from multiple promoters and consequently induced pleiotropic effects in growth and mating behaviors. Thus, they are proposed to be repressors of pol II transcription with broad specificity. Their mechanism of action is unknown, but it may involve a dynamic competition for DNA binding with activators. This hypothesis is consistent with the design of the genetic screens used in discovering them.

The MOT1 gene was identified in yeast by mutations that led to increased basal expression of many genes (Davis et al., 1992). Subsequent identification of this gene with the biochemically defined activity, ADI (for ATP-dependent-inhibitor), allowed preliminary analysis of its mechanism of action (Auble et al., 1994). MOT1 removes TBP from DNA in an ATP dependent fashion. Its dissociation activity is not promoter specific and can be blocked by TFIIA. In addition, the lethality of the dominant mutant can be suppressed by an overexpression of TBP and TFIIA. Another negative cofactor complex consists of NOT1-4 gene products (Collart and Struhl, 1994). Originally identified as mutants that affect initiation site usage in the HIS3 promoter, these genes were further characterized as general negative cofactors regulating diverse promoters. The fact that NOT cofactor complex preferentially represses the uninducible initiation site over the activator-inducible initiation site is consistent with a mechanism involving a coordinated interaction with transcriptional activators.

Fractionation of mammalian cellular extracts also led to the identification of several negative cofactors. NC1 and NC2 (for negative cofactor)were purified from the USA (for upstream factor stimulatory activity; see below) activity which along with basal factors and activators were required for a high level of activation *in vitro* (Meisterernst et al., 1991; Meisterernst and Roeder, 1991). The presence of NC1 or NC2 in the activated transcription reactions raised the relative level of activation, thus suggesting that their *in vivo* role may be rendering promoters more sensitive in response to activators. Both of them were shown to complex with TBP on the promoter. Interestingly, NC1-TBP and

NC2-TBP complexes were shown to form competitively with the TBP-TFIIA complex. This is consistent with the proposed role of TFIIA in mediating the derepression from negative cofactors. It is thus possible that the derepressing activity of TFIIA is organized by transcriptional activators as one of the regulatory steps that activators target.

Another well-characterized negative cofactor is Dr1 (Inostroza et al., 1992). Purified on the basis of its ability to associate with TBP on promoters, this a negative cofactor of transcription was proposed to be composed of four identical subunits of a 19 kDa phosphoprotein. Subsequent *in vitro* and *in vivo* analyses indicated that Dr1 may be subject to competitive regulation by transcriptional activators (Kraus et at., 1994; Yeung et al., 1994). A recent report indicated that NC2 contains Dr1/p19 as its component (Kim et al., 1995). Chapter II of this thesis describes the purification and functional analysis of a novel heteromeric negative cofactor complex that contains Dr1/p19 as a subunit (also see below).

**iii) Positive cofactors of pol II transcription**

Positive cofactors are often aptly referred to as coactivators. They are not necessary for the basal reaction but potentiate activated transcription. In this regard, TAF's, SRB's and even TFIIA can be considered coactivators. Their roles in activated transcription have been described above. The aforementioned USA was discovered by assaying for complementation of activated transcription *in vitro*. Several positive cofactors, PC1 to PC4, that initially copurified with TFIID and NC1-2 were separated and purified from USA fraction (Meisterernst et al, 1991; Meisterernst and Roeder, 1991). These activities can independently potentiate activated transcription reactions which otherwise show little response to transcriptional activators.

Best characterized among the PC's is PC4. A 15 kDa protein, PC4 has domains that are homologous to viral transcriptional regulators, ICP4 and IE180, implicated in regulation of the immediate-early genes of herpes simplex virus (Kretzschmar et al., 1994; Ge and Roeder, 1994). It has DNA binding activity that appears to be important for its

coactivator function as both activities can be regulated by phosphorylation (Kaiser et al., 1995; Ge et al., 1994)). In addition, PC4 was shown to interact independently with free or DNA bound Gal4 -VP16 and DNA bound TBP-TFIIA complexes but not with free TBP (Kretchmar et al., 1994; Ge and Roeder, 1994). Order of addition experiments indicated that PC4 acts at TFIIA-TFIID-promoter (DA) stage of the initiation process in part by increasing the number of the DA complexes (Kaiser et al., 1995). Along with these observations, its ability to complement several different activators suggests that it is a general coactivator that bridges the activator and the initiation complex (Kretzschmar et al., 1994; Ge and Roeder, 1994).

PC3 also was purified and cloned. Surprisingly, PC3 was determined to be DNA topoisomerase I (Kretzschmar et al., 1993; Merino et al., 1993). PC3 repressed basal transcription and stimulated activated transcription simultaneously thereby leading to a large fold of activation (Kretzschmar et al., 1993). Topoisomerase I from vaccinia virus, E. coli, and yeast could not substitute for the human form, and mutant human topoisomerase I without enzymatic activity could function as coactivator (Merino et al., 1993). Its physical interaction with TBP and requirement for TFIIA for coactiviation suggests that its stage of action is at DA complex step (Merino et al., 1993). Recent data suggest that topoisomerase I stimulates formation of DA complex that includes multiple topoisomerases at specific sites in the promoter in the presence of Gal4-VP16 (B. Shykind: personal communication).

Using a similar complementation of activation assay, HMG-2 was identified as a coactivator (Shykind et al., 1994). HMG-2 and its homologue HMG-1 define a class of high mobility group (HMG) proteins unrelated to the aforementioned HMG17. The key feature of HMG-1/2 proteins is a small domain called the HMG box (Landsman and Bustin, 1993). This domain, found in several transcriptional activators, is responsible for binding to DNA. The HMG-box binds to DNA in the minor groove and induces bending, an observation which led to the proposal that LEF-1, a transcription factor with a HMG box, functions as an architectural factor (Giese et al., 1995; Love et al., 1995). Chapter III

will describe preliminary characterization of HMG-1/2 as a coactivator proposed to function at the DA complex formation step by stabilizing the activated conformation. Appendices A and B will present analyses of the structure of the activated complex involving TFIID, TFIIA, Gal4-VP16, and HMG-1/2 proteins.

The architectural role of coactivator has a well-characterized precedent. A screen of a cDNA expression library for proteins that interact with the PRDII regulatory element of the human interferon-β (IFN-β) gene led to isolation of HMG-I(Y) as a potential factor in IFN-β regulation (Thanos and Maniatis, 1992). HMG-I(Y) represents another group of the high mobility group proteins distinct from HMG-17 and HMG-1/2. HMG-I(Y) binds to the PRDII element specifically through the minor groove but does not stimulate transcription from the IFN-β gene by itself. It is shown to interact specifically with NF-κB and ATF, two transcriptional activators required for the stimulation of IFN-β gene and to promote the binding of the two factors to their binding sites in the regulatory region of the IFN-β promoter (Thanos and Maniatis, 1992; Du et al., 1993; Thanos and Maniatis, 1995). Interestingly, both *in vitro* assembly of the complex involving NF-κB, ATF, and HMG-I(Y) and *in vivo* transcriptional activation presumably mediated by this complex are critically dependent on the precise positional relationship between individual binding sites (Thanos and Maniatis, 1995). Thus, it is proposed that HMG-I(Y) is a coactivator that functions by facilitating the formation of the structure of protein-DNA complex required for activation of the IFN-β gene.

Genetic screens also led to identification of coactivators. One screen took advantage of growth inhibition by an overexpression of the activator Gal4-VP16 which was proposed to mirror the preferential inhibition of activated transcription over basal transcription *in vitro* by high concentrations of Gal4-VP16 (Berger et al., 1993). It was hypothesized that Gal4-VP16 was titrating out basal factors and that mutations in proteins that bridge activators and basal factors would lead to alleviation of activator induced toxicity. Five independent genes, ADA1, ADA2, ADA3, ADA5, and GCN5 were identified as potential adaptors

(Berger et al., 1992; Pina et al., 1993; Maracus et al., 1994). Of these, genetic and biochemical data suggest that ADA2, ADA3, and GCN5 function in a complex (Marcus et al., 1994; Horiuchi et al., 1994). An ADA2 mutant strain was defective in mediating activation by VP16 or GCN4 *in vivo*, and extracts from the strain showed poor activation *in vitro* as well, consistent with its proposed role as a coactivators (Berger et al , 1992). Interestingly, the ada2 strain was normal in response to the activators HAP4 and Gal4 indicating that a measure of specificity exists with this cofactor (Berger et al., 1992; Pina et al., 1993). In addition, parts of ADA2 and GCN5 show homology to the zinc-finger domain and the bromo domain respectively which are motifs also seen in other transcriptional coactivators, again consistent with the proposed role of ADA's and GCN5 as coactivators (Kwok et al., 1994; Marcus et al., 1994).

Another group of coactivators appear to function in a promoter specific fashion despite lacking DNA binding domains. These proteins function by associating with transcriptional activators that bind to specific cognate DNA targets. Some of the well known examples include CBP (for CREB binding protein), VP16, and Bob1. Analyses of their function and mechanism of action provide paradigms of transcriptional regulation through promoter specific coactivators. CBP associates with the cAMP regulated enhancer binding protein (CREB) subject to regulation through phosphorylation (Kwok et al., 1994). Interestingly, the activity of CBP and its homologue, p300, is regulated by the viral oncogene product E1A (Arany et al., 1995; Lundblad et al., 1995). Thus, targeting coactivators such as p300 may be used in regulating a set of genes involved in cellular functions such as cell cycle regulation. VP16 is a potent Herpes Simplex viral activator. Chimeric activators using its activation domain have been widely used. VP16 is recruited to immediate early genes of the virus by the cellular activator Oct-1 and is utilized in assembling a higher order structure involving additional cellular factors (Kristie et al., 1989; Kristie and Sharp, 1990). This assembly process is proposed to be a control point for the activity of the immediate early genes. Bob-1 is a lymphoid specific coactivator that associates with Oct-1 and Oct-2 which

recognize the DNA element, 5'ATGCAAAT3', known as the octamer element (Gstaiger et al., 1995; Strubin et al., 1995). Paradoxically, the octamer element has been shown to be key in determining lymphoid specific expression of immunoglobulin genes as well as in expression of several house-keeping genes. The discovery of Bob-1 led to the proposal that the tissue-specificity of immunoglobulin gene expression is primarily determined by the presence of Bob-1 as it is expressed in lymphoid specific manner and potentiates transcription only from the immunoglobulin genes. Thus, these promoter specific coactivators in combination with their cognate DNA binding factors can result in an added level of specificity to promoter expression.

## IV. Conclusion and Perspectives

Defining a set of basal factors sufficient for the accurate initiation of transcription established a context in which regulatory mechanisms can be studied. Many of the mechanistic studies discussed in this chapter utilize *in vitro* analyses where one regulatory mechanism can be studied in isolation from a myriad of other ongoing cellular events. This is also the principal approach taken in the investigations of two cofactors described in the rest of this thesis. In chapter II, purification and characterization of a 90 kDa negative cofactor, one subunit of which is the previously cloned cofactor Dr1, are described. The results suggest a utilization of this negative cofactor in a promoter specific manner. Chapter III presents the characterization of HMG-1/2 proteins as coactivators of transcription. The formation of an activated initiation complex mediated by HMG-1/2 is proposed, and its structure is investigated through linking number change analysis and DNase I footprinting analysis.

Most of the cofactors discovered through biochemical means and several discovered through genetic screens have noticeable common features despite their distinct identities. First, many of them work at an early stage of the assembly of the initiation complex, particularly at the DA complex stage. The 90 kDa factor and HMG-1/2 are no exceptions.

Secondly, many of them have non-specific DNA binding activities. Again, the 90 kDa factor and HMG-1/2 act likewise. These features suggest that modification of the TFIIA-TFIID-DNA complex in combination with activators may be the mechanism of action for many cofactors. This would be a particularly engaging possibility if the holoenzyme turns out to be the sole form of active polymerase *in vivo*. If so, a model in which activators, TFIIA, TFIID, and coactivators cooperatively form a primed target of the holoenzyme may be envisioned. Chromatin structures and other negative cofactors would then likely function as dynamically regulated competitive elements to the formation of primed early complexes.

Future studies can take several directions. One is establishing a more precise quantitative model of transcription initiation. The availability of basal factors in highly defined forms should allow a precise measurement of thermodynamic and kinetic parameters of each step in the assembly of the initiation complex. The effect activators and coactivators have on each of the steps could then be determined and interpreted in terms of changes in these parameters. Even complex phenomena such as synergism by multiple activators and conformational alterations subsequent to association also could be quantitatively described. Such efforts may clarify several confounding observations in the field. For example, a quantitative approach may establish the functional significance of each of the reported interactions between activators and basal factors. Also, the controversy surrounding the necessity for coactivators in stimulated transcription may be settled. One likely outcome of these investigations is that considerable variations exist among promoters. For example, depending on the exact sequence of the TATA element, TFIID association may or may not be a rate limiting step. Thus, recruiting of TFIID by activators will have variable contributions to the overall level of activation depending on the exact nature of the TATA element. Similar variations in response to cofactors could be envisioned. In fact, for both the 90 kDa factor and HMG-2, promoter specific effects are reported.

Another future direction *in vitro* analysis could take is reproducing various features of the *in vivo* situation. For example, synthetic promoters used in most of the *in vitro* studies for the purposes of simplifying the variables and amplifying the desired effects should be replaced with wild type promoters. As the aforementioned preliminary analyses suggest, promoter specificity may be a significant aspect of the function of cofactors. If so, the use of the DNA elements in the configuration of wild type promoters would provide more valid information about the true role of cofactors. Also, the ability to recreate nucleosomes should make the analysis of the effect of cofactors on chromatin bound promoters possible. Several chromosome associated proteins such as the high mobility group proteins and topoisomerase I have been implicated as transcriptional cofactors, and their precise mechanism of action may become clear only in the context of chromatin bound promoters. The knowledge obtained from quantitative analyses of the interaction between various factors and from reproducing *in vivo* conditions should then be integrated to gain a further understanding of the role of transcriptional cofactors in the regulation of gene expression.

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# Chapter II

# A Negative Cofactor Containing Dr1/p19 Modulates Transcription with TFIIA in a Promoter Specific Fashion

This chapter has been submitted to the Journal of Biological Chemistry

## Summary

An activity which modulated the relative levels of transcription from the adenovirus major late promoter (MLP) and the immunoglobulin heavy chain μ promoter (μ) was purified as a 90 kDa factor. This factor is suggested to be a heterotetramer of two subunits: a 20 kDa polypeptide identical to the previously described Dr1/p19 and a novel 30 kDa polypeptide. The Dr1/p19 protein has been characterized as a repressor of transcription, and the 30 kDa protein is related to a recently identified yeast gene proposed to encode a repressor of transcription. The 90 kDa factor forms a complex with TBP on DNA and at high concentrations of both factors protects over a 150 bp region around the promoter from DNase I cleavage. The conformation of this complex as assayed by footprinting analysis is altered by TFIIA on the MLP but not on the μ promoter. Similarly, TFIIA reverses the repression of transcription by the 90 kDa factor on the MLP but not on the μ promoter. Thus, the interactions of TBP, TFIIA, and the 90 kDa factor are promoter specific.

## Introduction

Transcription reactions *in vitro* were developed to analyze the synthesis of pre-mRNA from promoter DNA. Reconstitution of transcription with chromatographic fractions has allowed purification of general transcription factors and isolation of the genes encoding the corresponding polypeptides (for review, see Buratowski, 1994; Maldonado and Reinberg, 1995; Zawel and Reinberg, 1995). Also identified were activities dispensable for the basal levels of transcription that affected the absolute amounts of product RNA. Initially, activities that suppressed transcription *in vitro* were largely ignored, but as addition of these activities was shown often to increase the relative response to sequence-specific transcriptional activators, interest in their identity and mechanism grew (Meisterernst et al., 1991; Meisterernst and Roeder, 1991; Merino et al., 1993). These are collectively referred to as negative cofactors of transcription and are distinct, at least operationally, from silencer element dependent proteins in that they repress a wide variety of promoters.

Several lines of evidence suggest that repression of transcription is a general and important aspect of transcriptional regulation. Both genetic and biochemical experiments suggest that chromatin structure represses transcription (for review, see Croston and Kadonaga, 1993; Paranjape et al., 1994). Furthermore, studies of SWI/SNF complexes show that controlling the accessibility of chromatin bound promoters to transcription factors is one of the regulatory steps of transcription (Hirschhorn et al., 1992; Winston and Carlson, 1992; Kwon et al., 1994; Imbalzano et al., 1994). Genetic screening for yeast mutants with elevated basal transcription and for suppressors of mutations to specific upstream activating sequences has led to the identification of several so-called global repressors of polymerase II (pol II) transcription (Swanson and Winston, 1992; Chen et al., 1993; Yamashita, 1993; Collart and Struhl, 1994; Auble et al., 1994). Mutations to many of them have pleiotropic effects, often elevating the level of transcription from multiple promoters. The SRB family of proteins, found as suppressors of C-terminal

domain (CTD) truncation mutations of pol II, are implicated as important components of transcription initiation *in vivo* (for review, see Koleske and Young, 1995). Several of SRB genes have been shown to be essential for survival, and in particular the analysis of SRB 4 gene suggested a direct involvement in the transcription of most genes (Thompson et al., 1993; Thompson and Young, 1995). A conditional loss of function mutation in SRB4 gene can be suppressed by mutations in other genes, suggesting the presence of a repression system operating in opposition to the SRB proteins (R. Young: personal communication).

Chromatographic fractionation of extracts from mammalian cells identified several activities that repress transcription *in vitro* (Meisterernst et al., 1991; Meisterernst and Roeder, 1991; Merino et al., 1993). Among these negative cofactors of transcription is Dr1 (Inostroza et al., 1992). It was described as a homotetramer of a 19 kDa phosphoprotein capable of forming protein-DNA complexes with TBP and blocking transcription. The inhibition of transcription was not reversible by addition of any basal factors including TFIIA. Transfection assays indicated that Dr1/p19 can repress transcription *in vivo* also and may be a target of regulation by upstream activators (Kraus et al., 1994; Yeung et al., 1994). Specifically, Dr1/p19 repressed pol II promoters generally when expressed in cells by transfection, and this repression was reversed by cotransfection of activators such as VP16 and $E1A_{13S}$. Additionally, NC2, a negative cofactor, with properties similar to Dr1 including its binding to TBP has been described by Kim et al to contain Dr1 (Kim et al., 1995). Immunoprecipitation of labeled extracts with specific antiserum indicated that Dr1/p19 exists inside the cell in association with several proteins (Inostroza et al., 1992). The activities of complexes containing these proteins have not been characterized.

We have purified a 90 kDa transcriptional cofactor consisting of Dr1/p19 and a novel protein with a molecular mass of 30 kDa. This report describes the initial characterization of the cofactor complex. The results suggest that it is primarily a negative cofactor with

differential effects depending on the identity of the core promoter element. The mechanism of the repression involves formation of complexes with TBP at the promoter, and variations between core promoters were manifest in the presence of TFIIA.

## Experimental Procedures

**Purification of the 90 kDa factor**- The 90 kDa factor was purified by following its stimulatory activity on MLP transcription from the HeLa nuclear extract derived, 0.3 M KCl DEAE fraction (C1) prepared as previously described (Parvin et al., 1994) . The C1 fraction was adjusted to buffer A [20 mM Hepes-NaOH (pH7.9), 20% glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] plus 500 mM KCl and applied to a superdex 200 26/60 column (FPLC, Pharmacia). Fractions corresponding to the molecular mass of 90 kDa were pooled, equilibrated to buffer A plus 100 mM KCl, and loaded onto a Mono-S HR 5/5 column (FPLC, Pharmacia). The bound material was eluted with a 15-ml linear gradient of 100 to 400 mM KCl in buffer A. The active fractions, found between 200 from 300 mM KCl, were pooled, equilibrated to buffer A plus 100 mM KCl, and then applied to a Mono Q HR 5/5 column (FPLC, Pharmacia). The loaded material was eluted with a 12-ml linear gradient from 100 to 500 mM KCl in buffer A. The activity was eluted near 400 mM KCl. The active fractions were dialyzed to buffer A plus 500 mM KCl and loaded onto a Superdex 200 16/60 column (FPLC, Pharmacia). The active fractions of the 90 kDa factor are shown in figure 1A.

**Binding to immobilized DNA-** The DNA fragment containing the MLP region was the Hind III and Xmn I fragment of the MLP transcription template plasmid (Buratowski et al., 1988; Timmers and Sharp, 1991). Coupling of the biotin-labeled DNA to magnetic beads has been described (Shykind et al., 1995). The protein preparations were incubated with the immobilized DNA in the transcription buffer plus 0.1 % NP-40 in a total volume of 30 µl. 120 ng of yeast TBP (Chasman et al., 1993) and 60 ng of the 90 kDa factor preparation were used. The protein-DNA complexes were subsequently isolated and washed three times with 30 µl of the incubation buffer. Subsequently, a semi-stringent wash with 30 µl of the transcription buffer with 150 mM KCl and 0.1% NP-40

was done. The washed beads were resuspended in SDS-PAGE buffer, boiled, and loaded onto the SDS-gel directly.

**Amino acid sequencing of p30-** An estimated amount of 2.5 μg of p30 was acetone-precipitated from the final gel filtration chromatography fractions and resolved on 10% SDS gel. After being transferred to nitrocellulose membrane, proteins were visualized by Ponceau S staining. Subsequently, the band representing p30 was excised, destained, and digested by endoproteinase Lys-C. The resulting peptides were separated on a C4 column using an Applied Bioscience (Biosystems) HPLC. Amino acid sequencing was done by Richard Cook (MIT Biopolymers Laboratory) with an Applied Biosystems model 477A protein sequencer with on-line model 120 PTH amino acid analyzer.

***In vitro* transcription-** Preparations of the recombinant transcription factors, yeast TBP, human TBP, TFIIB, TFIIE, and TFIIF have been described (Chasman et al., 1993; Parvin et al., 1994). RNA polymerase II was the highly purified preparation from CHO cells (Carthew et al., 1988). Preparation of TFIIA and TFIIH has also been described (Shykind et al., 1995). The reaction conditions and typical amounts of general transcription factors were as previously described (Parvin et al., 1994). The amounts of TBP, TFIIA, and the 90 kDa factor were varied and accordingly indicated (see figure legends). The transcription templates used also have been described (Timmers and Sharp, 1991; Parvin et al., 1994).

**DNase I footprinting, EMSA, and Western assay-** A pair of primers (5'-dG CACAAGCTTCCCAGTCACGACGTTGTAAAACGACGG-3'; 5'-dCGCTACTCGAGA GGAATAATGAGGAAAGGAGAGTAG-3') was used to generate a 250-bp PCR product from the Gal4(3)-μ transcription template (Shykind et al., 1995). The DNA fragment representing the Gal4(3)-μ promoter region from -190 to +55 was produced by Hind III

and Xho I digestion of the PCR product and was inserted into pBSII-SK+ plasmid. The probe used for footprinting assay and EMSA was generated from the plasmid by cleavage with Xho I and Klenow-end filling with [$^{32}$P] dCTP followed by Hind III digestion and gel purification. The similarly produced MLP probe was the previously described Hind III-Xho I fragment of Gal4(3)-MLP labeled at the Xho I site with [$^{32}$P]-dCTP (Shykind et al., 1995). DNase I footprinting assays were done under essentially transcription conditions supplemented with 0.5 mg/ml of bovine serum albumin (BSA) and 10 mM β-mercaptoethanol in the absence of non-specific carrier such as poly(dGdC). The DNase I digestion and urea gel analysis were done as described (Shykind et al., 1995). Incubation for EMSA was also done under essentially transcription conditions supplemented with 0.15 mg/ml of BSA and 10 mM DTT in the absence of poly(dGdC). The protein-DNA complexes were resolved by 4% acrylamide TGE (tris-glycine-EDTA) gel electrophoresis. The EMSA gel in figure 8 was exposed at room temperature without drying. Gel slices were excised and soaked in 20 ul of 5X SDS loading buffer at 60$^{o}$C for 15 minutes. Subsequently, the slices were fitted into the wells of an SDS gel directly. The electrophoresis and transfer to nitrocellulose membrane were done following standard protocols. The blot was probed with anti-TFIIA antiserum (kind gift of H. Handa) and visualized with ECL (Amersham).

## Results

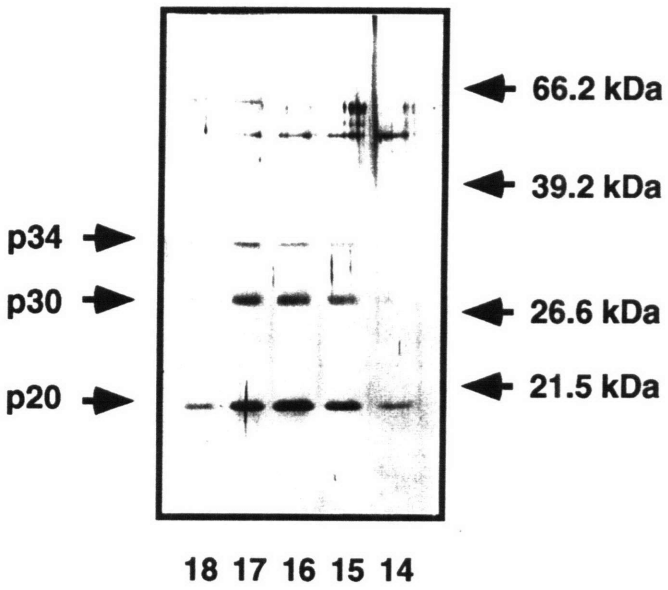
**Purification of the 90 kDa factor**- An activity with a molecular weight of 90 kDa which modulated basal transcription was previously identified (Parvin et al., 1992). Specifically, the activity enhanced transcription from the Major Late Promoter (MLP) of adenovirus and inhibited transcription from the μ promoter of the immunoglobulin heavy chain gene (μ). In addition, the 90 kDa activity formed protein-DNA complexes in association with TBP on the MLP. This activity was purified to near-homogeneity by assaying for its stimulatory effect on the MLP. The final preparation contained three polypeptides, 20 kDa (p20), 30 kDa (p30), and 34 kDa (figure 1A). In the Mono Q chromatographic step prior to the gel filtration chromatographic step, the 34 kDa protein did not copurify with the other two proteins or with the MLP-stimulating activity (data not shown) and thus is most likely not a component of the activity. Coomassie brilliant blue and Ponceau S stainings of the proteins in the preparation indicated one-to-one stoichiometry of p20 and p30 (data not shown). The purified activity chromatographed as a protein factor of 90 kDa as reported for the initial preparation (Parvin et al., 1992).

Several lines of circumstantial evidences suggested that the p20 was the previously cloned transcriptional cofactor, Dr1/p19 (Inostroza et al., 1992). These included the similarity of chromatographic behavior, the ability to form protein-DNA complexes with TBP, and the modulatory effect on transcription. Their identity was confirmed by a Western assay using an anti-Dr1 antiserum (gift of D. Reinberg; figure 1B) and partial peptide sequencing (data not shown).

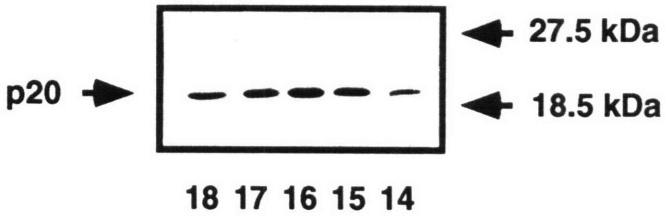
Dr1 was originally described as a homotetramer of the p19 phosphoprotein (Inostroza et al., 1992). Thus, it was of interest to determine whether p30 was associated with Dr1/p19 (p20) or was an incidentally copurifying species. The association of the two polypeptides was tested by a DNA binding assay, taking advantage of the proposed

**Figure 1. The Purified 90 kDa factor.** (A) Silver stain SDS-polyacrylamide gel of the 90 kDa factor preparation. The fractions from the last chromatographic step (gel-filtration) are shown. Fraction #16 corresponds to a molecular weight of 90 kDa. (B) Western assay on identical fractions as shown in the silver stain gel. Anti-Dr1/p19 antiserum (kind gift of D. Reinberg; 21) was used.

A


66.2 kDa
39.2 kDa
p34
p30
26.6 kDa
p20
21.5 kDa
18 17 16 15 14

B


27.5 kDa
p20
18.5 kDa
18 17 16 15 14

interaction between TBP and the 90 kDa activity on the MLP (figure 2). A biotin-labeled DNA fragment containing the MLP immobilized on streptavidin-coupled magnetic beads was incubated with either no protein (lanes 3, 7, and 11), recombinant yeast TBP alone (lanes 4, 8, and 12), the 90 kDa activity preparation alone (lanes 5, 9, and 13), or TBP and the 90 kDa activity preparation (lanes 6, 10, and 14). Following incubation under transcription conditions, protein-DNA complexes were isolated and washed with transcription buffer containing 150 mM KCl. The supernatants, the washes, and the isolated protein-DNA complexes (eluates) were analyzed on a silver-stained SDS gel. TBP bound to the promoter DNA as it was cleared from the supernatant upon incubation (lane 4). TBP binding to DNA was partially labile to the 150 mM KCl wash (lane 8), but a substantial fraction was recovered from the eluate (lane 12). None of the proteins in the 90 kDa activity preparation were capable of binding to DNA with significant affinity as all three were recovered in the supernatant (lane 5). However, when TBP was present, both p20 and p30 polypeptides tightly associated with DNA (lane 6) and were quantitatively recovered in the eluate (lane 14). In addition, in the presence of p20 and p30, TBP bound to the promoter more strongly as none of it eluted in the wash (lane 10) and was recovered quantitatively in the eluate (lane 14). Similar results were obtained using recombinant human TBP instead of yeast TBP. Consistent with distinct chromatographic characteristics, the 34 kDa protein did not associate with the promoter DNA even in the presence of TBP (lane 6). Together with the copurification, the DNA binding assay confirmed that p20 and p30 are in a single complex. Furthermore, copurification with the transcriptional activity and association with TBP strongly suggest that the p20/p30 complex constitutes the 90 kDa factor.

P30 was blotted to nitrocellulose membrane and digested with endoproteinase Lys-C, and the resulting peptides were separated by HPLC. Primary amino acid sequences were obtained from several of these peptides (figure 3A). Database searching resulted in identification of a partial rat cDNA clone (Express Sequence Tag ID: EST107790;

**Figure 2. Binding assay to immobilized DNA.** Promoter containing DNA fragment was immobilized to magnetic beads. Various combinations of yeast TBP and the 90 kDa factor preparation were incubated under transcription conditions. The supernatants after binding and isolation of the beads (lanes 3-7), semi-stringent washes with 150 mM KCl (lanes 7-10), and the final eluates (washed beads in SDS loading buffer; lanes 11-14) were analyzed by silver stain SDS gel. Lanes 1 and 2 show the preparations of yeast TBP (120 ng) and 90 kDa factor (60 ng) respectively. The 90 kDa preparation contained 200 μg/ml of carrier insulin.

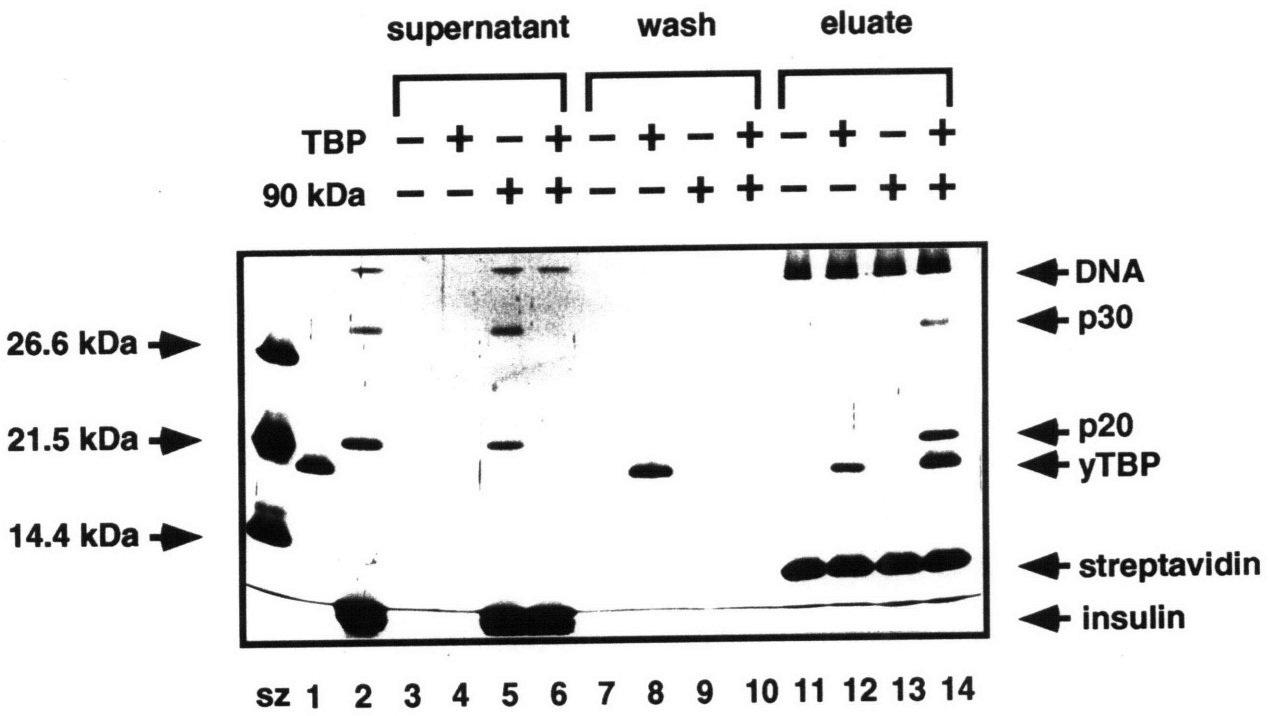

supernatant
wash
eluate
TBP – + – + – + – + – + – +
90 kDa – – + + – – + + – – + +
26.6 kDa
21.5 kDa
14.4 kDa
DNA
p30
p20
yTBP
streptavidin
insulin
sz 1 2 3 4 5 6 7 8 9 10 11 12 13 14

**Figure 3. Sequence Analysis of p30.** (A) Amino acid sequences of p30 were obtained from tryptic peptides separated by HPLC. "X" designates unidentified residues. (B) Predicted amino acid sequence of the rat cDNA clone EST107790. Carboxyl terminal 133 amino acids are shown. Regions of identity to the peptide sequences are underlined. Comparative analyses of nucleotide and amino acid sequences between EST107790 and DRAP1 (for Dr -associated polypeptide 1; Mermelstein et al., 1996; D. Reinberg: personal communication) indicate that EST107790 has undergone a deletion of 161 nucleotides. As a consequence, the sequence for the carboxyl terminal 25 amino acids likely represents an incorrect sequence. (C) Protein sequence alignments between the rat cDNA clone and homologous proteins. HAP5 is a subunit of yeast CAATT binding protein complex (McNabb et al., 1995). Yer159c is a hypothetical protein found within a yeast cosmid clone (GenBank ID: U18917).

A

Peptide-4: KTMTTSHL

Peptide-12: KXLVASVPDMQG

Peptide-26: KXXIELEQQFDFL

Peptide-28: KLXGTDSEQED

Peptide-36: KXXYATXDFTLLELN

B

N A R F P P A R I K K I M Q T D E E I G

K V A A A V P V I I S R A L E L F L E S

L L K K A C Q V T Q S R N A K T M T T S

H L K Q C I E L E Q Q F D F L K D L V A

S V P D M Q G D G E D N H T D G D K G P

R R G R K P L S E P S N S L H A L H L S

S A F T P S T S R P L S T *

C

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EST107790   4 F P P A R I K K I M Q T D E E I G K V A A A V P V
             F P P A + + K K I M Q T D E + I G K V +   A   P V
Yer159c    53 F P P A K V K K I M Q T D E D I G K V S Q A T P V

             I I S R A L E L F L E S L L K K A C Q V T Q S R N
             I     R + L E   F +     L + K K +   + +   +   +
             I A G R S L E F F I A L L V K K S G E M A R G Q G

             A K T M T T S H L K Q C I E L E Q Q F D F L K D 77
               K   + T       L K +   I     + + + F D F L + +
             T K R I T A E I L K K T I L N D E K F D F L R E 126
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EST107790   5 P P A R I K K I M Q T D E E I G K V A A A V P V I
             P   A R I + K + M + T D E + +     + + A     P + I
HAP 5     135 P F A R I R K V M K T D E D V K M I S A E A P I I

             I S R A L E L F L E S L L K K A C Q V T Q S R N A
               + + A   E + F +     L     + A     V   +
             F A K A C E I F I T E L T M R A W C V A E R N K R

             K T M T T S H L K Q C I E L E Q Q F D F L K D L V
             + T +     +   +   +   + +         F D F L   D + V
             R T L Q K A D I A E A L Q K S D M F D F L I D V V

             A S V P 83
                   P
             P R R P 213
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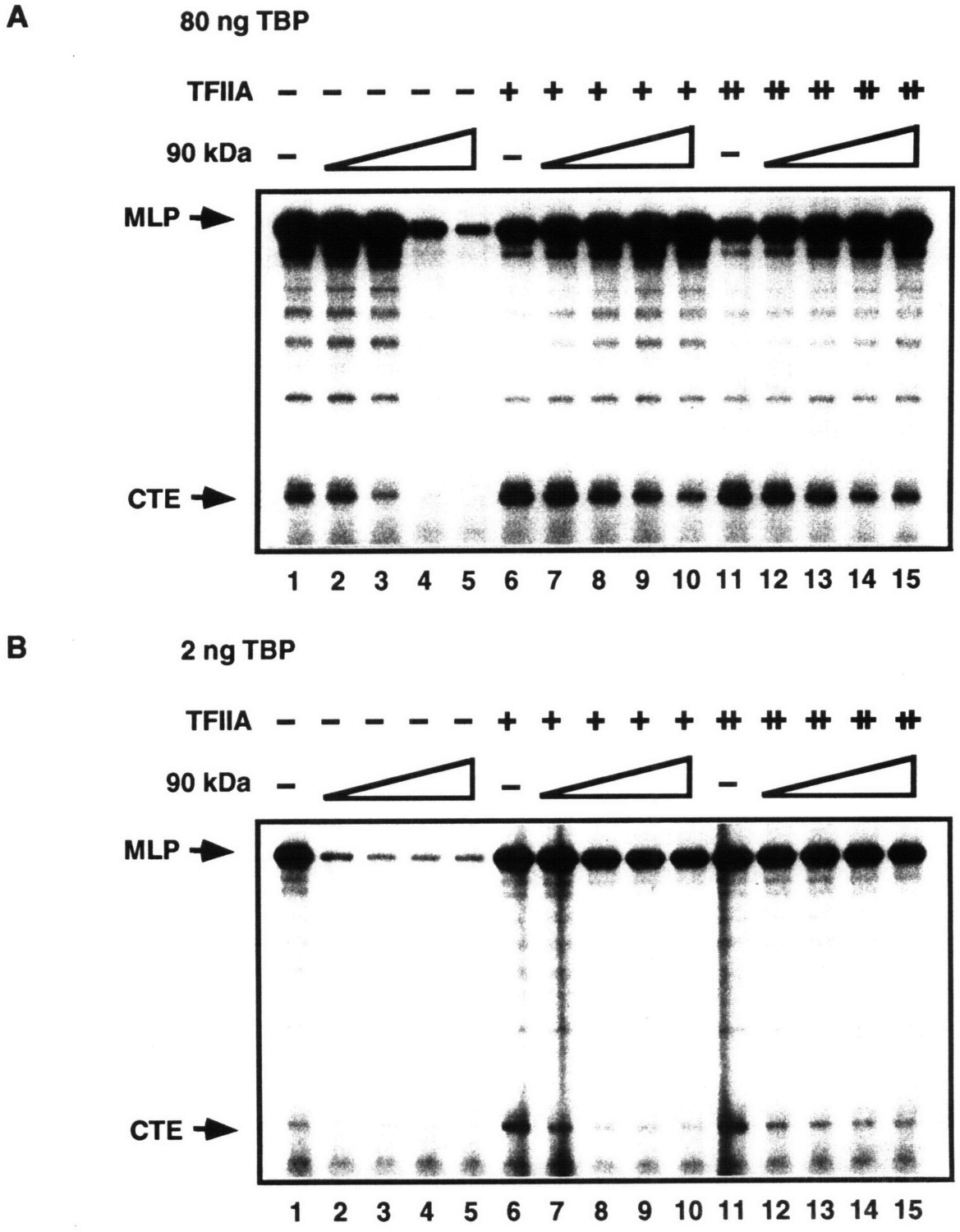
GenBank ID: H32566; figure 3B) from the Express Sequence Tag cDNA library (Lee et al., 1995). Several peptides derived from p30 show identity to the predicted amino acid sequence from the cDNA. Interestingly, the cDNA clone itself shows a strong homology to HAP5 (32% identity; score of 142 using BLOSUM62 matrix; p value versus Non-Redundant Data Base as of Feb.29, 1996 of 2.6e-12), a subunit of yeast CCAAT binding heteromeric protein complex also containing HAP2, HAP3, and HAP4 (McNabb et al., 1995). Considering that Dr1/p19 (p20) has homology to HAP3, another subunit of the CCAAT binding complex, the homology between the cDNA and HAP5 further suggests that Dr1/p19 (p20) and p30 form a complex. The rat cDNA clone also shows a strong homology to a *S. cerevisiae* protein (gene Yer159c; GenBank ID: U18917) of unknown function. In fact, the sequence relationship of the rat cDNA clone to the yeast gene is greater than that to HAP 5 (48% identity; score of 191; p value of 7.2e-22). Interestingly, this protein has been identified as a suppressor of mutations in the SRB4 gene which encodes a component of the pol II holoenzyme in yeast (see discussion; R. Young: personal communication).

**The 90 kDa factor in transcription-** Dr1 has been described as a negative cofactor of basal transcription (Inostroza et al., 1992), yet the 90 kDa factor has been purified based on its stimulatory effect on the MLP (Parvin et al., 1992). Systematic transcription assays with a defined set of transcription factors revealed that the 90 kDa factor stimulated transcription only in the presence of TFIIA and saturating levels of TBP (data not shown). Further analysis indicated that the stimulatory activity of the 90 kDa factor was due to a derepression from an inhibitory effect of TFIIA. Specifically, in the presence of saturating levels of TBP, TFIIA repressed transcription from the MLP (figure 4A: compare lanes 1, 6, and 11), and the addition of increasing levels of the 90 kDa factor relieved this inhibition (figure 4A: lanes 6 to 10 and lanes 11 to 15). This was despite the fact that identical levels of the 90 kDa factor by itself inhibited transcription (figure 4A: lanes 1 to 5).

**Figure 4. Modulation of basal transcription from MLP by the 90 kDa factor.** (A) Transcripts were generated with 25 ng of linearized MLP plasmid in the presence of 80 ng of human recombinant TBP. The 90 kDa factor was titrated (0, 1, 2, 3, 4 ng) in the absence of TFIIA (lanes 1-5) or in the presence of 2 ng (lanes 6-10) or 4 ng (lanes 11 to 15) of TFIIA. Transcripts from MLP and the internally initiated transcripts from CTE were separated by urea-PAGE. (B) Transcripts were generated with 25 ng of linearized MLP plasmid in the presence of 2 ng of human recombinant TBP.


A
80 ng TBP
TFIIA
90 kDa
MLP
CTE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
2 ng TBP
TFIIA
90 kDa
MLP
CTE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

TFIIA has been associated with a stimulation of transcription, and both the repression by TFIIA and the derepression by a second repressor were paradoxical. An insight was obtained by observing another transcript of 90 nucleotides. This internally initiated transcript originating within the G-less cassette is probably driven by a TATA-like sequence, 5'-TATATTT-3' (designated as CTE for cryptic TATA element), located 30 bases upstream of its initiation site (data not shown). TFIIA stimulated transcription from the CTE under conditions in which it repressed transcription from the MLP ( figure 4A: compare lanes 1, 6, and 11) as if it was modulating a competition between the MLP and the CTE. TBP-saturating conditions would induce a competitive environment for the promoters in incorporating the other limiting basal factors. Under such conditions, a promoter-specific alteration of the affinity of the other basal factors to TBP-promoter complexes by TFIIA would alter the ratio of the levels of expression of the promoters. Consistent with this model, TFIIA still stimulated CTE but did not repress transcription from the MLP when TBP was added at subsaturating levels and the other basal transcription factors were abundant relative to TBP (figure 4B: compare lanes 1, 6, and 11).

The 90 kDa factor likely prevents the progression of the initiation complex assembly beyond TBP binding for both the MLP and the CTE in the absence of TFIIA. The presence of TFIIA blocked the repression by the 90 kDa factor on the MLP but not on the CTE. Under TBP saturating conditions, this block likely resulted in more basal factors available for the MLP thus accounting for the stimulation of the MLP (figure 4A: lanes 6 to 10 and lanes 11 to 15), and under TBP limiting conditions, it resulted in the maintenance of the level of transcription from the MLP (figure 4B: lanes 6 to 10 and lanes 11 to 15). The inability of TFIIA to function equivalently at the CTE resulted in the repression of transcription from the CTE at all levels of TBP and TFIIA. Thus, it appears that TFIIA has a variable effect on different core promoters. On the CTE, it has a net stimulatory effect but cannot block the repression from the 90 kDa factor. On the MLP, TFIIA does not have net

stimulatory activity but restored (figure 4A) or maintained (figure 4B) the capability of TBP to associate with the other basal factors in the presence of the 90 kDa factor. Again, consistent with this interpretation, under limiting condition of TBP relative to the other basal factors, addition of the 90 kDa factor did not stimulate the MLP at any level of TFIIA (figure 4B).

The above results indicated that both TFIIA and the 90 kDa factor differentially affect core promoters. To study this possibility, the effect of the 90 kDa factor in combination with TFIIA was tested on a series of core promoters. In the absence of TFIIA, the 90 kDa factor repressed transcription from all of the tested promoters (figure 5: lanes 1 to 5 for each promoter). It should be noted that the repression showed highly variable efficiencies. The adenovirus E4 promoter (E4) was completely shut off in the presence of 2 ng of the 90 kDa factor while at the same level, the interleukin-2 (IL2) promoter showed substantial residual activity. In the presence of TFIIA (lanes 6 to 10 for each promoters), the E4 and the IL2 promoters were not repressed by the 90 kDa factor. However, repression by the 90 kDa factor was not blocked by TFIIA for either the immunoglobulin heavy chain μ (μ) or the immunoglobulin light chain κ (κ) promoter. Thus, TFIIA and the 90 kDa factor show promoter specific effects individually and together result in a large change in the ratio of expression levels of various promoters.

**Protein-DNA complexes on promoters formed by the 90 kDa factor, TBP, and TFIIA**- Interactions of TBP, TFIIA, and the 90 kDa factor with the core promoters were examined by DNase I footprinting assay. TBP protected the TATA region (-18 to -35) of the MLP as expected (figure 6A, compare lanes 1 and 2). As the 90 kDa factor was titrated, a series of DNase I hypersensitive sites and protected regions emerged (figure 6A: lanes 2 to 5). Specifically, at the highest level of the 90 kDa factor (figure 6A: lane 5), several hypersensitive sites (-140, -115, -100, -85, -75, -60, -15, and +10) either became more sensitive to or maintained the level of cleavage by DNase I, but in between these sites the bases were protected from DNase I digestion. Within the TATA region the

**Figure 5. Promoter specific modulation of transcription by the 90 kDa factor.** The repressive effect of 90 kDa factor was assayed in the absence (lanes 1-5) and in the presence (lanes 6-10; 4 ng) of TFIIA on Immunoglobulin heavy chain promoter (μ), Adenovirus E4 promoter (E4), interleukin-2 promoter (IL2), and immunoglobulin light chain promoter (κ). Each reaction contained 80 ng of human recombinant TBP and 25 ng of linearized template plasmid. The 90 kDa factor was titrated (0, 1, 2, 3, 4 ng) as in figure 4.

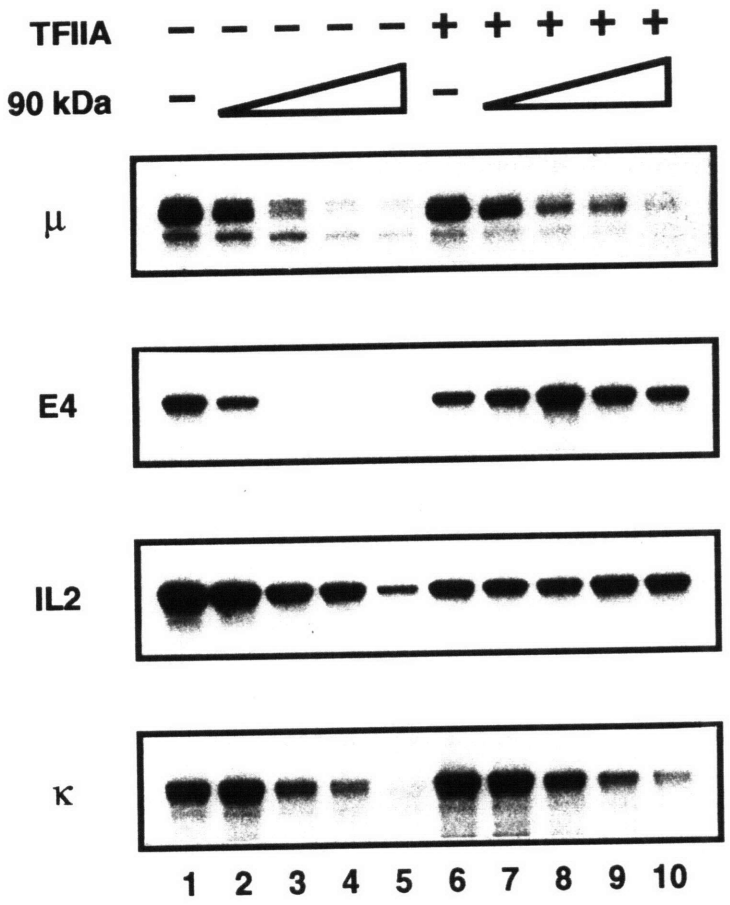
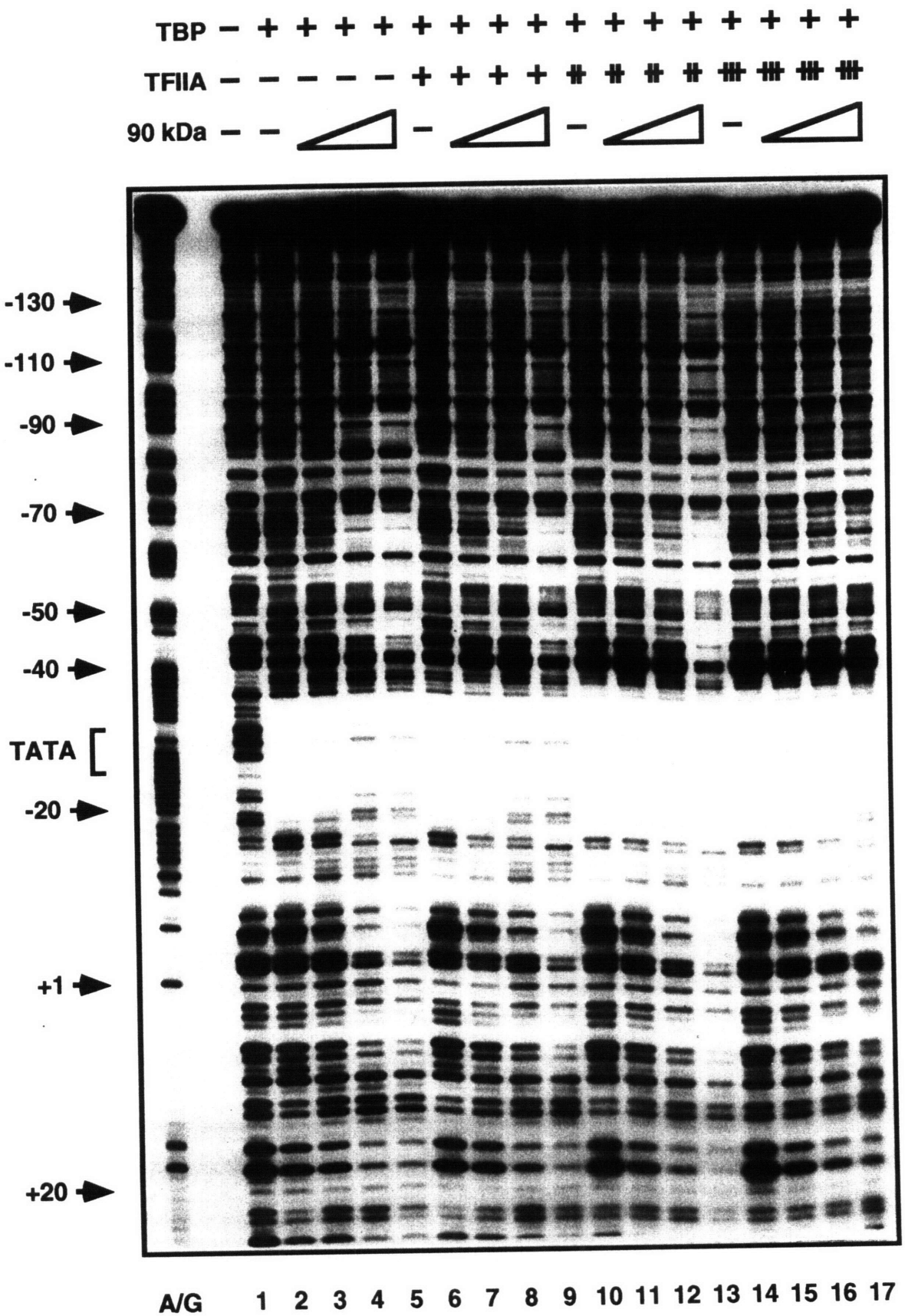
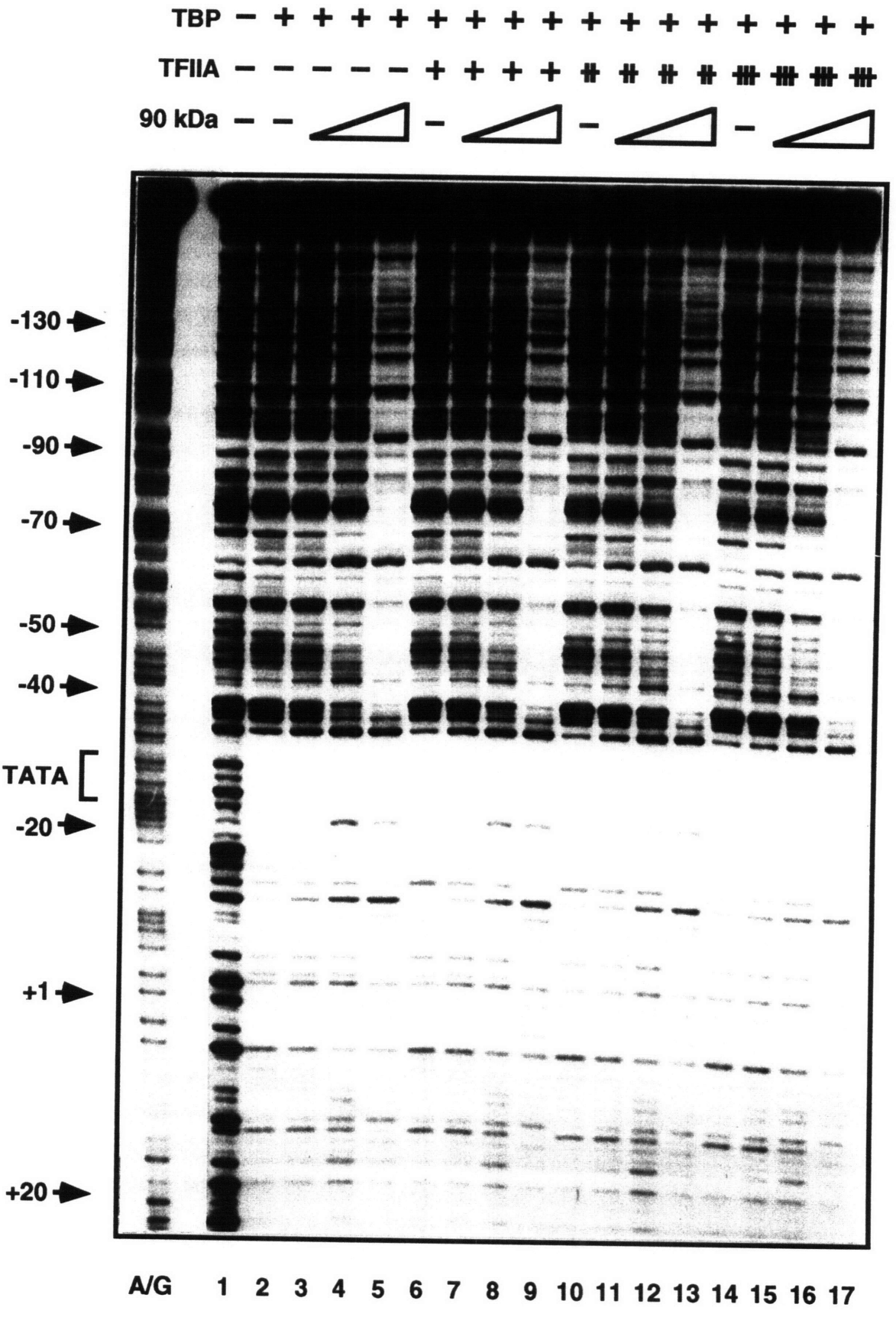

TFIIA
– – – – – + + + + +
90 kDa
– –
μ
E4
IL2
κ
1 2 3 4 5 6 7 8 9 10

**Figure 6. DNase I footprinting assay.** (A) The interaction of TBP, TFIIA, and the 90 kDa factor on MLP was analyzed by DNase I footprinting assay. The reaction included 80 ng of human recombinant TBP (lanes 2-17). The 90 kDa factor was titrated (0, 0.4, 1.2, 4 ng) in the absence (lanes 2-5) or in the presence of 0.2 ng (lanes 6-9), 0.8 ng (lanes 10-13), and 2 ng (lanes 14-17) of TFIIA. (B) The interaction of TBP, TFIIA, and the 90 kDa factor on μ promoter was analyzed by DNase I footprinting assay.

A


TBP – + + + + + + + + + + + + + + + +
TFIIA – – – – – + + + + + + + + + + +
90 kDa – – – –
-130
-110
-90
-70
-50
-40
TATA
-20
+1
+20
A/G 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

B


TBP
TFIIA
90 kDa
-130
-110
-90
-70
-50
-40
TATA
-20
+1
+20
A/G
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

cleavage pattern also changed; bases at -31, -29, -27, -22 and -20 showed slightly increased sensitivity (figure 6A: compare lanes 2 and 5). As anticipated, the addition of TFIIA in combination with TBP induced a slight extension of the protection from -35 to -38 followed by hypersensitive sites at -42 and -43 (figure 6A: compare lanes 2 and 14, for example). Adding TFIIA and the 90 kDa factor together showed that the pattern induced by the 90 kDa factor was modulated by TFIIA. At low and intermediate concentrations of TFIIA, high concentrations of the 90 kDa factor generated its typical pattern (figure 6A: lanes 9 and 13). However, at the highest concentration of TFIIA, the 90 kDa factor induced protection between the hypersensitive sites diminished and the cleavage pattern within the TATA region was restored to the TBP-TFIIA induced pattern (figure 6A: lanes 14 to 17). It should be noted however that even at the highest concentration of TFIIA and the lowest concentration of the 90 kDa factor, there were detectable alterations of the digestion pattern outside TATA element (figure 6A: compare lanes 14 and 15). This raised the possibility that on a given molecule of DNA, both the 90 kDa factor and TFIIA can coexist. It should also be added that the 90 kDa factor and TFIIA in the absence of TBP show no alterations on the footprint pattern either individually or together at the level assayed (data not shown).

The extension of footprints outside TATA box required a higher level of TBP than necessary to protect the TATA box. Specifically, although 10 ng of TBP was sufficient to protect the TATA region completely, adding a high level of the 90 kDa factor resulted in no extended footprint alterations beyond the TATA region (data not shown). Under such conditions, the 90 kDa factor still induced its typical pattern within the TATA region that was reversible by TFIIA. These data indicate that the 90 kDa factor probably forms oligomeric structures by association with multiple TBP molecules. Further confirmation was obtained from EMSA (see below). Also of interest, the addition of non-specific competitor DNA such as poly(dGdC) deprotected the TATA site from TBP in the presence but not in the absence of the 90 kDa factor (data not shown). It is likely that the 90 kDa

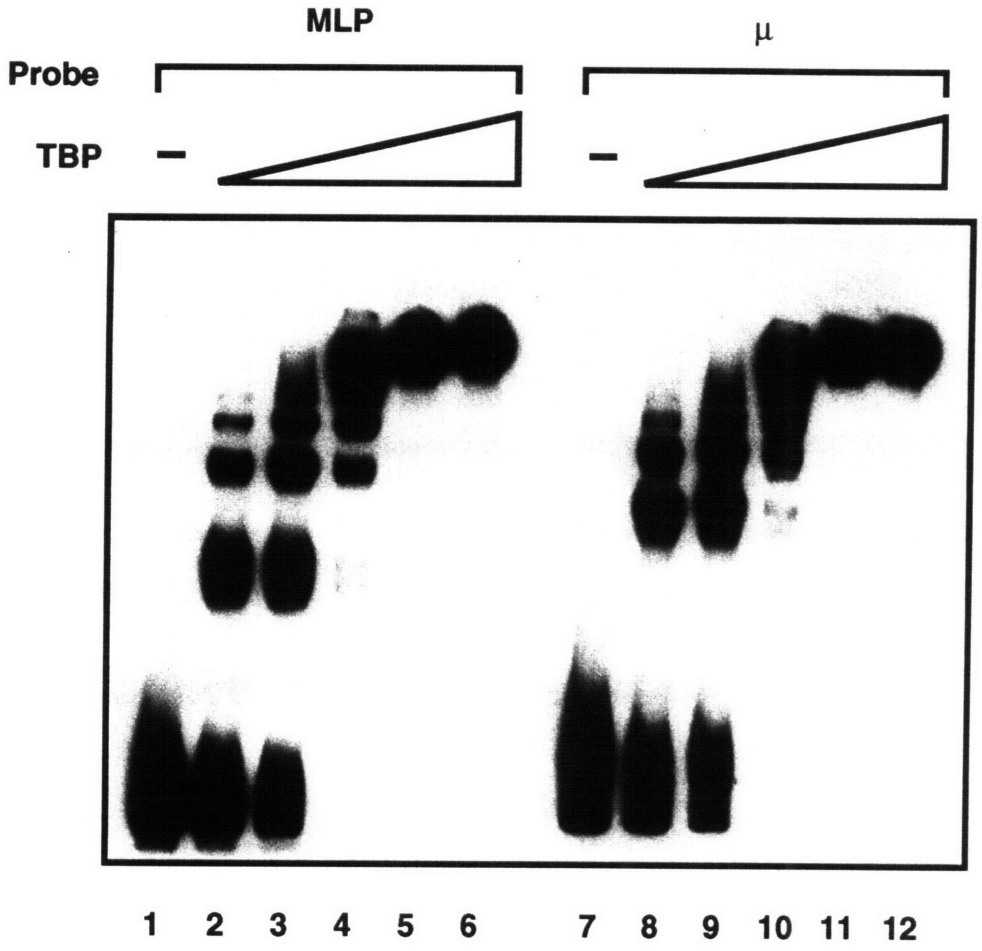
factor weakly binds to DNA as well as to TBP and under these conditions sequesters TBP away from the promoter onto the hetero-polymer DNA.

DNase I footprinting was repeated with μ promoter using identical levels of the proteins. As in the case of the MLP, TBP protected the TATA region from -18 to -35 (figure 6B: compare lanes 1 and 2). Titration of the 90 kDa factor induced alterations that were qualitatively similar to those with the MLP. At the highest level of the 90 kDa factor (figure 6B: lane 5), hypersensitive sites (-125, -118, -110, -95, -65, -21, -11, and +13) were generated and the regions in between were protected from the cleavage (figure 6B: lanes 2 to 5). Addition of TFIIA to intermediate levels of the 90 kDa factor partially restored the footprint pattern around the TATA region to the TBP-TFIIA induced pattern in that sensitivity of the base -39 increased and that of the base -21 decreased (figure 6B: compare lanes 4, 8, 12 and 16). However, unlike in the case of MLP, the extended footprint as well as the alterations within the TATA region induced by the highest level of the 90 kDa factor were resistant to TFIIA (figure 6B: lanes 5, 9, 13, and 17) indicating a significant difference between MLP and μ in their interaction with the 90 kDa factor. As in the case with the MLP, low levels of TBP and high levels of the 90 kDa factor induced a TATA-region contained alteration of the footprint. However, unlike in the case of the MLP, TFIIA could not restore the footprint to the TBP-TFIIA induced state (data not shown).

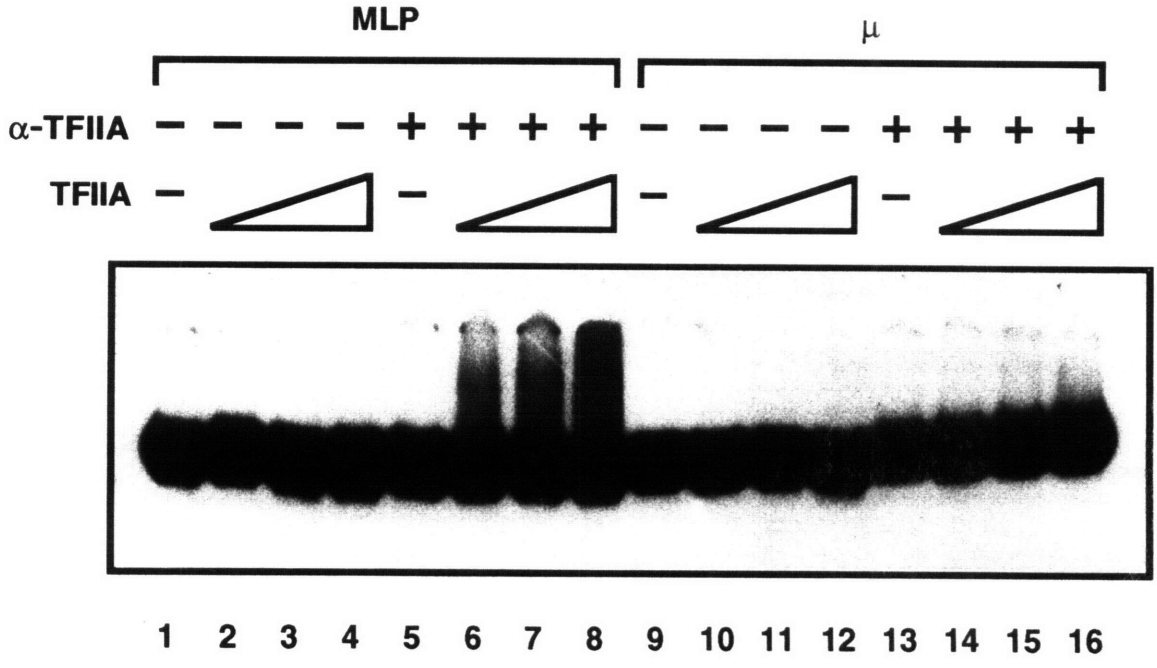
The interactions of the factors with the MLP and μ promoters were also analyzed by EMSA. Titration of increasing levels of TBP in the presence of the 90 kDa factor resulted in a series of protein-DNA complexes with increasingly lower mobilities (figure 7A). At the highest concentration of TBP assayed, a single low mobility complex was formed with both the MLP and the μ. This was not a non-specific complex that could not enter the gel matrix as prolonged electrophoresis resulted in a further migration (figure 7B). Titration of the 90 kDa factor with the high levels of TBP showed that formation of this low mobility complex also requires high levels of the 90 kDa factor (data not shown). Under the EMSA

**Figure 7. Electrophoretic mobility shift assay.** (A) TBP was titrated (0, 5, 10, 20, 40, 80 ng) into the reactions in the presence of 4 ng of the 90 kDa factor. Protein-DNA complexes on both MLP (lanes 1-6) and μ (lanes 7-12) were analyzed on a native gel. (B) TFIIA was titrated (0, 2, 4, 8 ng) in the absence (lanes 1-4 and lanes 9-12) and in the presence (lanes 5-8 and lanes 13-16) of anti-TFIIA antiserum (1 μg in PBS; kind gift of H. Handa). To lanes 1-4 and 9-12, equal volume of PBS was added. All lanes include 80 ng of TBP and 4 ng of the 90 kDa factor. Supershift due to antibody was tested on both MLP (lanes 1-8) and μ (lanes 9-16). Complexes were subjected to prolonged (4 hours instead of 2 hours) electrophoresis at 160 volts (lanes 1 and 9 are equivalent to lanes 6 and 12 of figure 7A respectively).

A


MLP
μ
Probe
TBP
–
–
1 2 3 4 5 6 7 8 9 10 11 12

B


MLP
μ
α-TFIIA – – – – + + + + – – – – + + + +
TFIIA –
–
–
–
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

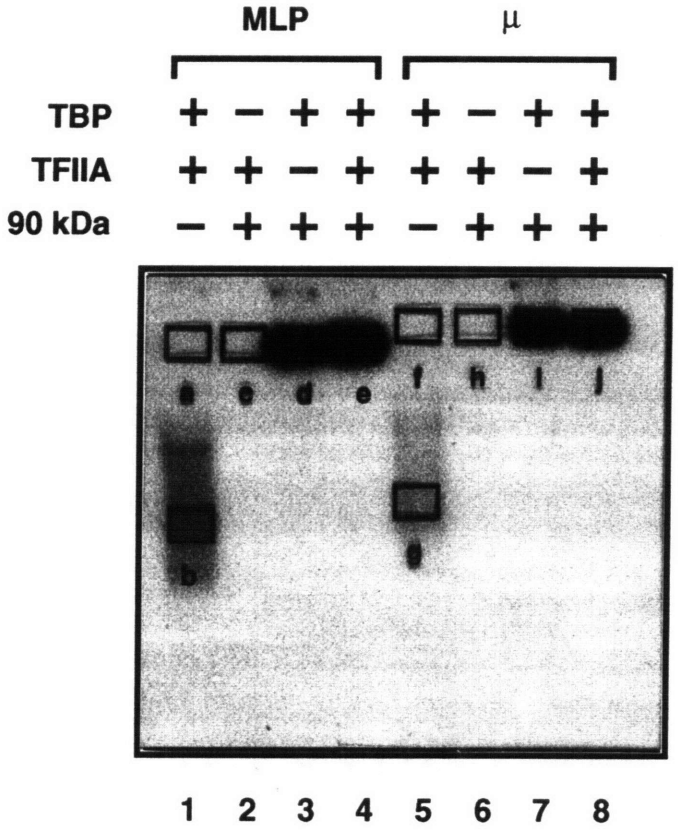
conditions used, TBP alone did not form a stable protein-DNA complex. Therefore, it is likely that this complex represents an oligomeric structure including multiple TBP's and the 90 kDa factors as suggested by the footprinting assay. As in the case of the footprinting assays, the EMSA reactions did not contain poly(dGdC). The 90 kDa factor alone produced a smearing of the probe DNA consistent with the proposed interactions with DNA (figure 7A: lanes 1 and 7).

Surprisingly, the mobilities and number of protein-DNA complexes produced by TBP and the 90 kDa factor were unaffected by TFIIA under these conditions. This was true with both the MLP and the μ promoter. Since addition of TFIIA under similar binding conditions altered the DNase I footprint patterns of MLP, this result was unexpected. One possibility was that TFIIA was part of the protein-DNA complexes but had no effect on the mobilities of the complexes. This possibility was examined on the low mobility complexes by addition of anti-TFIIA antiserum (gift of H. Handa) to the EMSA reaction. The presence of the antiserum caused a smearing of the protein-DNA complexes only if TFIIA was added to the reaction (figure 7B lanes 1 to 8). This likely reflects the binding of the anti-TFIIA antibodies to TFIIA in the protein-DNA complex. Interestingly, there was a difference in the response between the MLP and the μ promoter. The smearing effect was readily visible at the lowest level of TFIIA in the reactions containing the MLP (figure 7B: lane 6), but not until the highest level of TFIIA was there a hint of a smear in the reactions containing the μ promoter (figure 7B: lane 16). These data suggest that the alterations observed with the addition of TFIIA to the MLP footprinting assays were largely conformational and that TFIIA and the 90 kDa factor coexist on a single DNA molecule under these conditions (see discussion).

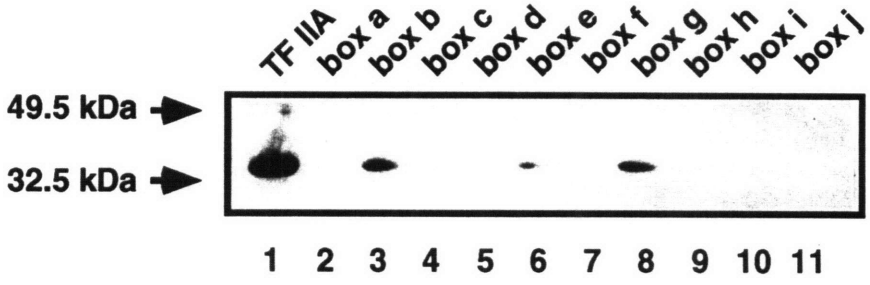
The presence of TFIIA in the low mobility complex was further tested by isolating the complex and assaying for TFIIA by Western blotting. Gel slices corresponding to the low mobility complexes were excised (figure 8A), and proteins contained in these bands were resolved by SDS-PAGE. The presence of TFIIA in the complexes were detected by

**Figure 8. Western assay of EMSA complexes for TFIIA** (A) EMSA complexes were formed using various combinations of TBP (10 ng in lanes 1 and 5; 80 ng in lanes 3, 4, 7, and 8), TFIIA (8 ng), and the 90 kDa factor (4 ng). The gel was exposed to an X-ray film without drying, and boxed regions (designated **a** to **j**) were excised. (B) The excised gel fragment was soaked in SDS buffer and then was subjected to SDS-PAGE. A standard Western assay with anti-TFIIA antiserum was run subsequently. Lane 1 contains 8 ng of TFIIA.

A


MLP
μ
TBP + − + + + − + +
TFIIA + + − + + + − +
90 kDa − + + + − + + +
a c d e f h i j
b
1 2 3 4 5 6 7 8

B


TF IIA
box a
box b
box c
box d
box e
box f
box g
box h
box i
box j
49.5 kDa
32.5 kDa
1 2 3 4 5 6 7 8 9 10 11

Western blotting with anti-TFIIA antiserum (figure 8B). Consistent with the supershifting seen in EMSA (figure 7B), TFIIA was detected only in the MLP containing low mobility complex (figure 8B: compare lanes 6 to 11). Corresponding regions of the gel lanes where reactions without the 90 kDa factor (figure 8A: lanes 1 and 5 ) or without TBP (figure 8A: lanes 2 and 6) had been run contained no TFIIA confirming that TFIIA is found in the low mobility complex in lane 4 by virtue of being a part of the complex. TBP and TFIIA made a diffuse complex in the absence of poly(dGdC) (figure 8A: lanes 1 and 5), but TFIIA was easily detected from these complexes (figure 8B: lanes 3 and 8).

## Discussion

We have identified and purified a cofactor of transcription that consists of Dr1/ p19 (p20) and a novel protein of molecular weight of 30 kDa (p30). The heteromeric composition of the complex has been suggested by co-association with TBP on the MLP as well as copurification over several chromatographic steps. The heteromeric complex has a molecular weight of 90 kDa and is probably composed of two subunits of Dr1/p19 (p20) and two subunits of p30. The formation of heteromeric structure by Dr1/p19 (p20) and p30 is also supported by their homology to the HAP3 and HAP5 proteins respectively. These two yeast proteins associate into a CAATT binding regulatory complex, and the peptide sequences necessary for their interaction is conserved in the Dr1/p19 (p20) and p30 polypeptides (McNabb et al., 1995; Xing, et al., 1993; D. McNabb and L. Guarente: personal communication).

Reinberg and coworkers have reported two separate forms of Dr1 (Inostroza et al., 1992). The first form was described as a homotetramer of phosphorylated Dr1/p19 protein with a molecular mass of 90 kDa which elutes in the 0.5 M KCl fraction from a phosphocellulose column. The second form is unphosphorylated and eluted in the 1.0 M KCl fraction from the same column. The 90 kDa factor eluted from a phosphocellulose column in the 0.6 M KCl fraction, and comparison of the mobility with the Dr1 found in the 1.0 M KCl fraction by Western blotting indicated that Dr1/p19 (p20) subunit of the 90 kDa factor was likely phosphorylated (data not shown). In addition, NC2, one of the negative cofactors reported by Roeder and coworkers has been shown to contain a phosphorylated form of Dr1/p19 although the subunit composition of the purified NC2 and its molecular size have not been described (Kim et al., 1995). We suggest that all three factors, the 90 kDa factor, the native Dr1 complex, and NC2 are the same activity and are a heterotetramer of p20 and p30 polypeptides. This proposal is consistent with the repression of transcription and interaction with TBP reported for all three factors.

The 90 kDa factor is a repressor of basal transcription. Although it will probably repress transcription from all promoters, the efficiency of repression varies depending on the identity of the core promoter. The mechanism of repression appears to be formation of transcriptionally inert complex with TBP on promoters. Interestingly, the 90 kDa factor and TBP can form a series of extended protein-DNA complexes. Dr1/p19 has been shown to bind to TBP directly. Since the 90 kDa factor is probably a heterotetramer of two Dr1/p19 (p20) subunits and two p30 subunits, each complex is likely bivalent for binding to TBP. Furthermore, the purified Dr1/p19 (p20) subunit oligomerizes into a tetramer when prepared separately from p30 (Inostroza et al., 1992) indicating the potential to form higher order structures of the 90 kDa factor under appropriate conditions. It is not surprising then that a series of TBP-90 kDa factor complexes can form on a promoter probe. The formation of the more extended protein-DNA complex is nucleated by TBP binding to TATA element and subsequent binding of the 90 kDa factor. Addition of higher amounts of TBP results in the formation of a complex which migrates slowly during the gel electrophoresis and has an extended footprint spanning over 150 base pairs of the probe. Thus, most likely, this complex is composed of multiple 90 kDa factors and TBP's, and the promoter is inaccessible to the other basal factors necessary to mediate transcription. It should be noted however that formation of the more extended structure is probably not required for the inhibition of transcription by the 90 kDa factor or for the promoter specific derepression by TFIIA since both phenomena occurs at low levels of TBP.

An intriguing aspect of transcriptional regulation by the 90 kDa factor is the promoter-specific modulation by TFIIA. In the presence of high levels of TFIIA, transcription from MLP is refractory to inhibition by the 90 kDa factor. In contrast, under the same condition, transcription from the immunoglobulin μ promoter is strongly inhibited. Correlatable differences between these two promoters were observed in the footprint assays. High levels of TFIIA restored the footprint within the TATA region to the TBP-TFIIA induced pattern from the 90 kDa factor-TBP induced pattern. Under conditions where the extended

footprint was observed, TFIIA reduced the protection outside the TATA region indicating that the stability or the conformation of the total complex was being affected. In contrast, on the μ promoter, the addition of high levels of TFIIA did not alter the footprint induced by the 90 kDa factor as effectively. Corresponding EMSA of the complexes on the two promoters suggests that in neither case did TFIIA dissociate the complex. Undoubtedly, TFIIA is part of the protein-DNA complex on the MLP in the presence of the 90 kDa factor as TFIIA is detected by supershifting of the complex by anti-TFIIA antiserum and by Western blotting of the proteins contained within the gel-shift complex. The difference between the MLP and the μ indicates that TFIIA is more readily incorporated into the 90 kDa factor-TBP-DNA complexes on the MLP than into complexes on the μ promoter. Thus, it is likely that TFIIA joins the promoter in the context of the 90 kDa factor-TBP-promoter complex, induces local reversion of protein-DNA conformation around the core promoter element, and promotes transcription from the MLP.

The extent of promoter sequences contributing to the promoter specific regulation by the 90 kDa factor and TFIIA is not clear. Transcriptional activation by TFIIA is weakly promoter specific indicating that either TFIIA binds to TBP in a promoter specific manner or the dependence of the initiation process on this factor varies between promoters. The latter is likely to be the case since TFIIA binds readily to TBP bound to the MLP. The 90 kDa factor binds to TBP and can form oligomeric structures on both the MLP and the μ promoters without detectable differences. However, significant differences must exist as TFIIA is incorporated readily into the 90 kDa factor-TBP complex on the MLP but not on the μ promoter. Since the gross composition of oligomeric complexes appears to be similar, the difference is likely to be conformational and most pronounced around the TATA elements of the two promoters. This is supported by the observation that both footprinting and transcription assays indicate that promoter specific interaction between TFIIA and the 90 kDa factor occurs at low levels of TBP as well as at high levels of TBP. However, as the oligomeric structure is propagated outside the TATA region, the flanking

sequences, where additional protein-DNA contacts occur, also may be sites of conformational differences in protein-DNA complexes.

It is now well-accepted that the regulation of pol II transcription involves negative modulation of basal and activated transcription. The 90 kDa factor is probably one of these negative components and suppresses transcription *in vivo* as well as *in vitro*. Cotransfection assays indicate that an overexpression of Dr1/p19 subunit represses transcription *in vivo*, and a subset of activation domains including VP16 and E1A-CR3 can derepress transcription (Kraus et al., 1994; Yeung et al., 1994). Comparison of sequences of the p30 subunit of the 90 kDa factor allowed identification of a related protein from *S. cerevisiae* encoded by the Yer159c gene. A mutation in Yer159c can suppress a conditional loss of function mutation in SRB4, a component of the yeast pol II holoenzyme complex (Thompson and Young 1995; R. Young: personal communications). Shifting the SRB4 mutant strain to restrictive temperature results in rapid cessation of mRNA synthesis from many pol II promoters indicating that SRB4 is an important component of transcriptional activation (Thompson and Young, 1995). That a mutation in the p30 homologue suppresses the loss of function mutation of SRB4 is consistent with the proposal that the p30 homologue is a component of a general repressor activity such as the 90 kDa factor. Since the 90 kDa factor forms repressed complexes with TBP on promoters, the pol II holoenzyme containing SRB4 and other basal factors may be prevented from entering the initiation complex by such a repressor. Furthermore, the results presented here indicate that transcriptional regulation by the repressors such as the 90 kDa factor may have a measure of promoter specificity dictated by the content of the core promoter and the flanking sequences.

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# Chapter III

# Activation of the TFIID-TFIIA Complex with HMG-2

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## Summary

The non-histone chromosomal protein HMG-2 was identified as a factor necessary for activation in a defined transcription reaction *in vitro* containing RNA polymerase II and purified factors. Activation occurred on all promoters assayed except that of the immunoglobulin IgH gene. TFIIA was required for stimulated levels of transcription. The activation process depended on the presence of TAFs in the TFIID complex and generated a preinitiation complex from which TFIIB more slowly dissociated. However, titration of TFIIB over three orders of magnitude did not obviate the requirement of activator and HMG-2 to achieve stimulated levels of transcription. Analysis of the activated reaction identified the TFIID-TFIIA complex as the first stage of modification during activation. These results suggest that activation can occur solely in the presence of the basal factors, activator protein, and an "architectural" HMG factor which probably stabilizes an activated conformation of the TFIID-TFIIA-promoter complex.

## Introduction

The basal reaction for initiation of transcription by RNA Polymerase II (pol II) can be reconstituted with distinct protein factors that assemble on the promoter in an ordered fashion (reviewed by Buratowski 1994). This process is nucleated by recognition of the TATA element by TFIID (or the TATA binding protein, TBP) and culminates, after assembly, with conversion of the initiation complex into the elongation complex. The basal factors required for this reaction have been purified to homogeneity, and the genes for many have been cloned. On promoters such as the Adenovirus Major Late (MLP), this set of factors is comprised of TFIID, TFIIB, TFIIE, TFIIF, TFIIH, and pol II. The factors TFIIA and TFIIJ can stimulate the basal reaction (Samuels et al. 1982; Cortes et al. 1992).

Positive modulation of transcription requires additional activities, as reactions reconstituted with purified basal factors typically do not respond to activators such as SP1 and Gal4-VP16. Coactivating factors enable the basal factors to respond to sequence specific activators. Stimulation of transcription *in vitro* is dependent upon the TAF polypeptides which are associated with the TBP in TFIID (Dynlacht et al. 1991). Coactivators such as USA (Meisterernst et al. 1991b) and ACF (Merino et al. 1993) are not associated with TBP. These coactivator fractions potentiate the stimulated reaction. Some of the components of these complex activities have been purified. The gene encoding PC4, a positive acting component of USA, has recently been cloned. PC4 is thought to interact with both basal factors and sequence-specific activators (Ge and Roeder 1994a; Kretzschmar et al. 1994a). Topoisomerase I also coactivates stimulation both by repression of basal and potentiation of stimulated transcription (Merino et al. 1993; Kretzschmar et al. 1993). It is possible that the high levels of activation *in vivo* are the result of combinations of potentiating and repressing factors (Meisterernst and Roeder 1991).

The degree of stimulation of transcription by activators is modest in reactions of defined basal factors and this has made it difficult to determine the functional interactions of factors necessary for stimulation. In less defined systems, several of the basal factors have been implicated as targets of activation. Interactions of activator and TFIID fraction have been shown to yield both quantitative and qualitative effects in the binding of TFIID to the promoter (Sawadogo and Roeder 1985). The interaction of the TBP component of TFIID with an acidic activation domain (Stringer et al. 1990) has been proposed as a link in activation, as mutants in the VP16 activation domain that reduce function *in vivo* also reduce TBP association *in vitro* (Ingles et al. 1991). Functional assays using partially fractionated components have suggested TFIIB as a candidate target of activation (Lin and Green 1991; Choy and Green 1993). Recently, the activator Zta, in the presence of TFIIA, has been shown to enhance the rate of binding of TFIID to the promoter (Lieberman and Berk 1994). Additionally, the formation of the TFIID-TFIIA complex has been suggested to be a rate limiting step in transcription facilitated by activator (Wang et al. 1992). Genetic and biochemical analysis in yeast suggest a different important step in activation. Stimulation of transcription *in vitro* depends on the presence of a large RNA polymerase II complex composed of general transcription factors, SRB proteins and polymerase (Koleske et al. 1994; Kim et al. 1994).

Here we report the characterization and identification of a coactivator that functions in a defined transcription reaction, otherwise unable to respond to sequence-specific activators. The identification of the coactivator as HMG-2, and the subsequent demonstration that the HMG-box alone has the same function, suggest a structural role for these coactivators in establishing the activated initiation complex. The activation requires TFIIA, and an immobilized template transcription assay defines the TFIID-TFIIA complex as the first stage of initiation altered during activation.

## Materials and Methods

**Purification of Co-B-** The preparation of crude MLTF fraction, HBrBC, which contained HMG-2, was described in detail previously (Chodosh, 1988) and is briefly summarized here. Nuclear extract was made from homogenized calf brain with minor modifications to the Dignam method (Dignam et al., 1983). The extract was equilibrated in buffer A (20 mM Hepes-NaOH [pH 7.9], 20% glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) + 100 mM KCl + 0.03% Nonidet P-40 and heated at 80°C for 35 minutes with continuous stirring. After cooling to room temperature and centrifugation, the supernatant was loaded onto a DEAE-Sepharose column equilibrated in buffer A + 100 mM KCl + 0.03 % NP-40. The column was sequentially washed with 3 column volumes of buffer A + 100 mM KCl, 3 column volumes of buffer A + 250 mM KCl, and 3 column volumes of buffer A + 500 mM KCl. MLTF activity, assayed by EMSA, was found in the 250 mM step (HBrB). Heparin-Sepharose (Pharmacia) was prepared according to the manufacturer's instructions and was equilibrated in buffer A + 100 mM KCl. After loading the HBrB, the column was washed sequentially with 3 column volumes of buffer A + 100 mM KCl, 3 column volumes of buffer A + 250 mM KCl, and 3 column volumes of buffer A + 500 mM KCl. MLTF was found in the 500 mM KCl step (HBrBC).

Phosphocellulose (Whatman P11) matrix (1.3 ml) was prepared according to the manufacturer's instructions and equilibrated with buffer A + 200 mM KCl. HBrBC (7 mgs in 7.5 mls) was batch absorbed and sequentially washed with 4 column volumes of buffer A + 200 mM KCl, 4 column volumes of buffer A + 350 mM KCl, 4 column volumes of buffer A + 600 mM KCl, and 4 column volumes of buffer A + 1 M KCl. The MLTF was found in the supernatant of binding and 350 mM KCl step. The Co-B activity, found in the 600 mM KCl step, was dialyzed to buffer A + 100 mM KCl and loaded onto a

Mono Q HR 5/5 column (FPLC, Pharmacia ). The bound material was eluted with a 12 ml linear gradient of 100 to 400 mM KCl in buffer A. The activity was eluted near 300 mM KCl. The active fractions were dialyzed to buffer A + 1.2 M ammonium sulfate and loaded onto a phenyl sepharose HR 5/5 column (FPLC, Pharmacia). The loaded material was eluted with a 12 ml linear gradient of 1.2 M to 0 M ammonium sulfate in buffer A. The activity, found in the flowthrough, was dialyzed to buffer A + 100 mM KCl and loaded onto a Mono S HR 5/5 column (FPLC, Pharmacia). From a 12 ml linear gradient of 100 mM to 1M, the peak of the activity was found near 200 mM KCl. Further processing of the purified material for renaturation and amino acid sequencing was done using a C4 reverse phase column ( HPLC system, HP Model 1090M, part #214TP52 )

**Renaturation of HPLC Fractions-** Each of the fractions from the C4 column was brought to 0.1M Hepes [pH 7.9], frozen, and then lyophilized. To each fraction, 15 µl of denaturation buffer ( 6 M Guanidine-HCl, 50 mM Tris [pH 7.9], 0.1 mM EDTA, 10% glycerol, 50 mM NaCl, 2 mM DTT) was added to thoroughly solubilize the dried material. After incubating at room temperature for 15 minutes, 105 µl of dilution buffer ( 50 mM Tris [pH7.9], 5% glycerol, 150 mM KCl, 0.1 mg/ml Bovine Serum Albumin, 0.1 mM EDTA, 2 mM DTT) was added. Fractions were dialyzed overnight to buffer A + 100 mM KCl at 4$^{o}$C.

**Amino Acid Sequencing of Co-B-** N-terminal sequencing of the sole polypeptide in the active renatured fraction (fraction C) was carried out on an Applied Biosystems Model 477A Protein Sequencer with on-line Model 120 PTH Amino Acid Analyzer. The N-terminal 21 amino acids revealed a sequence which matched that of the High Mobility Group 2 (HMG-2) protein from several species including bovine HMG-2.

**Expression of HMG-2 in *E. coli*-** A pair of primers bearing the N-terminal and C-terminal ends of HMG-2 coding sequence flanked by Bam H1 restriction sites were generated (N-terminus, 5'-dGCACGGATCCAGGTAAAGGAGACCCCAACAAGC CG-3'; C-terminus, 5'-dGCACGGATCCTCATTATTCTTCATCTTCATCCTCTTC

CT-3'). These oligonucleotides were used to generate a PCR product containing the complete coding sequence of human HMG-2 using a cDNA clone (a gift of M. Seidman of Otsuka Pharmaceuticals; Majumdar et al., 1991) as the template. The PCR product was digested with Bam H1 and cloned into pET-15b (Novagene). *E. coli* strain BL21DEpLysS (Novagen) containing pHMG-2 was grown in LB medium supplemented with 150 μg/ml of ampicillin. Upon reaching an O.D. 600 of 0.6, HMG-2 was induced with 1 mM IPTG for 3 hours. The harvested cells were resuspended in buffer L ( 20 mM Tris [pH 7.9], 200 mM NaCl, 20 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride; 20 mls per liter of culture) and lysed by sonication. Induction and subsequent purification of HMG-2 were followed by western assay with polyclonal antibody against rat HMG-1 (gift of S. Lippard). The supernatant of the lysis was sequentially precipitated with 45% and 75% ammonium sulfate. HMG-2, found mostly in the 75% ammonium sulfate precipitate, was dialyzed to buffer N (20 mM Hepes [pH7.9], 20% glycerol, 7 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride + 0.1% Nonidet P-40) + 5 mM imidazole + 1M KCl. This was batch absorbed onto a 1 ml of Ni-NTA resin (Qiagen). The matrix was poured into a small column and washed with 10 column volumes of buffer N + 100 mM KCl. HMG-2 was subsequently eluted with buffer N + 250 mM imidazole +100 mM KCl. The peak of activity was dialyzed to buffer A + 500 mM KCl and loaded onto a Superdex 200 16/60 column (FPLC, Pharmacia). The active fractions were dialyzed to buffer A +100 mM KCl and loaded onto a Mono Q HR 10/10 column (FPLC, Pharmacia). A linear gradient elution from 100 mM to 1M KCl resulted in a homogenous recombinant HMG-2 preparation.

**In vitro transcription-** Preparation of the recombinant transcription factors, TBP, IIB, IIE, and IIF has been described (Parvin et al. 1994). Purification of holo-TFIID was done essentially as described (Zhou et al. 1992). RNA polymerase II was the highly purified preparation from a CHO cell line (Carthew et al. 1988). The source of TFIIA was either A/J fraction (Parvin et al. 1994) or a homogenous preparation of TFIIA. The latter

was prepared following the steps of a previously published purification through the single-strand-DNA agarose chromatography step (Cortes et al. 1992). The active fractions were purified to homogeneity with Ni-NTA resin (Qiagen). TFIIH was purified from HeLa-nuclear-extract-derived fraction C1 (Parvin et al. 1992). The purification was followed by western assay using an antibody to the p62 subunit (gift of J. Egly; Fischer et al. 1992). C1 fraction (80 mg in 50 ml) in buffer A + 100 mM KCl was loaded onto a Mono Q HR 10/10 column (FPLC, Pharmacia). An 80 ml linear gradient from 100 mM to 600 mM KCl was used to elute the TFIIH complex. The peak fractions, near 230 mM KCl, were dialyzed to 1.0 M ammonium sulfate in buffer A and loaded onto a phenyl sepharose HR 5/5 column (FPLC, Pharmacia). The loaded material was eluted with a linear gradient from 1.0 M to 0 M ammonium sulfate in buffer A. The activity, found near 300 mM ammonium sulfate, was dialyzed to buffer A + 500 mM KCl and loaded onto a Superdex 200 16/60 column(FPLC, Pharmacia). TFIIH, by western assay and transcription, was found to have an apparent size of 700 kDa. Gal4-VP16 was prepared as described (Chasman et al. 1989). Rat HMG-1 and rat HMG1-boxB were kind gifts of S. Lippard (Pil and Lippard 1992). MLTF used in the transcription assay was the Phosphocellulose flowthrough fraction described above.

DNA templates for transcription *in vitro*, Gal4(3)-MLP, and MLTF(5)-MLP, were gifts of H. Timmers and S. Harper, respectively. Gal4(3)-μ and Gal4(5)-HSP were gifts of J. Parvin. The control template MLP has been described (Timmers and Sharp 1991). The conditions for the assay and typical amounts of basal transcription factors used were as previously described (Parvin et al., 1994). Where indicated, 10 ng of Gal4-VP16, 1 μl of MLTF fraction, and 100 ng to 500 ng of purified or cloned HMG-1/2 preparations were added.

The immobilized templates were generated by cutting the appropriate templates with Xmn I and Nde I followed by Klenow end-filling with biotin-16-dUTP (Boehringer-Mannheim) and dATP (100 μM each). The gel-purified 2500 bp long fragment was

incubated with streptavidin-coupled M-280 Dynabeads (Dynal Inc., 10 µl of slurry per 100 ng of DNA) for 60 minutes. The DNA coupled resin was washed and stored in buffer A + 100 mM KCl + 0.1 % Nonidet P-40 in the original volume.

Staged transcription assays were typically done as follows. The DNA coupled resin (10 µl) was incubated with the "pre-inc" factors for 60 minutes at 30°C in buffer A + 100 mM KCl + 0.1 % Nonidet P-40 + 5 mM $MgCl_2$ ( total volume, 20 µl). The protein-DNA complexes were washed with 300 µl of the incubation buffer. The "chase" factors and nucleotides were added subsequently, and the reaction was brought to the transcription condition (see above). The incubation was continued for 90 minutes at 30°C.

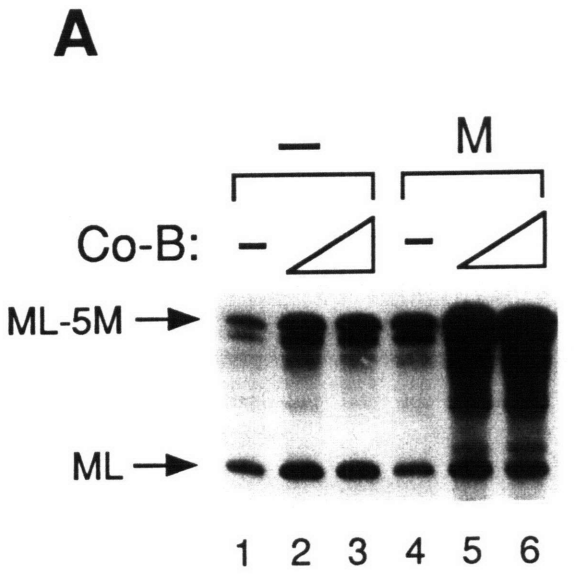
**EMSA and DNase I Footprinting-** A pair of primers were generated to produce a 260 bp PCR product from a Gal4(3)-MLP transcription template (5'-dGACCATGATTAC GCCAAGCTTGCATGCCTGC-3'; 5'-CGCTACTCGAGAGGAATAATGAGGAAAGG AGAGTAG-3'). The PCR product, covering the regions -180 to +65 of the promoter region, were digested with Hind III and Xho I and inserted into pBSII-SK+. The probe for EMSA and DNase I footprinting was generated by cutting the plasmid with Hind III, Klenow-end filling with [$^{32}$P] dATP, and then cutting with Xho I. Protein-DNA-binding reactions were done following the published protocol (Lieberman and Berk 1994) with several modifications. Binding was done in 16 µl of binding buffer (12.5 mM Hepes [pH7.9], 12.5 % Glycerol, 6.25 mM $MgCl_2$, 70 mM KCl, 10.8 mM β-mercaptoethanol, 0.5 mg/ml BSA, 5 µg/ml poly(dGdC), and 1 ng of probe) at 30°C for 30 min. Gels for EMSA were cast with 1.6 % agarose in 0.5X TBE with 6.25 mM $MgCl_2$. The gel was run in 0.5X TBE with 6.25 mM $MgCl_2$ at 70 volts for 5 hours and then dried on DE81 paper. For the DNase I footprinting assay, 16 µl of DNase I solution containing 1 ng of DNase I (Worthington Enzymes; DPFF grade) and 5 mM $CaCl_2$ were added to the binding reaction above. After 1 minute of incubation at room temperature, 90 µl of stop solution (20 mM EDTA [pH8.0], 1% SDS, 200 mM NaCl, 250µg/ml yeast tRNA) was added. The DNase I digestion was analyzed by sequencing gel electrophoresis.

## Results

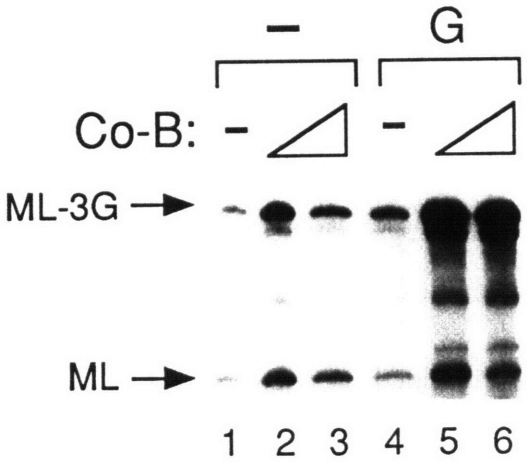
**An activity potentiates activation: Coactivator-B-** Reconstitution of transcription with a defined set of basal factors (general transcription factors, GTFs) resulted in reactions which were not stimulated by the addition of sequence-specific activators. In these reactions, factors derived from cDNA clones were used where possible and the remaining purified components were defined reagents (see Experimental Procedures). Addition of each factor was absolutely required for transcription (Parvin and Sharp 1993; and data not shown). The degree of stimulation was determined in reactions that contained both templates with and without binding sites for activators. Templates of the major late promoter (MLP) with the activator binding sites produced a 390nt transcript while the basal control template which did not contain such binding sites produced a 180nt message. This reconstituted reaction was active in the basal reaction but did not show significant stimulation upon addition of Gal4-VP16. However, we observed that addition of a MLTF (Major Late Transcription Factor) preparation derived from a heat-treated nuclear extract (Chodosh 1988) dramatically boosted transcription from templates containing five MLTF binding sites (ML-5M), but not from basal control templates (ML) lacking these sites (data not shown). To determine whether the stimulation of transcription observed was mediated solely by MLTF or was the result of MLTF plus another activity, the MLTF preparation was subjected to conventional chromatography and fractions were assayed by transcription for MLTF stimulatory activity. MLTF separated from a coactivating activity which was subsequently purified to near homogeneity (see Experimental Procedures). This activity, called coactivator-B (Co-B), coeluted with a protein doublet of about 30 kDa. To determine the generality of Co-B as a coactivator, it was tested with different sequence-specific activators and on different promoters.

Coactivator-B functioned as a potentiator of stimulation with different classes of transcriptional activators. Addition of either the major late transcription factor (MLTF),

**Figure 1. Ability of coactivator-B to stimulate transcription with different activators.** (A) Activity with the cellular activator MLTF (M). ML transcript (180nt) was generated by 25 ng supercoiled MLP basal control promoter. ML-5M transcript (390nt) was generated by 25 ng supercoiled MLP with five MLTF binding sites fused upstream. Transcripts were separated by urea-PAGE. Lanes 1-3: titration of Co-B (0, 1, 5μl) into the reaction in the absence of activator. Lanes 4-6: titration of Co-B (0, 1, 5μl) into the reaction in the presence of saturating amounts of MLTF (M). (B) Quantitation of stimulation from reactions shown in figure 1A was by Phosphorimager (Molecular Dynamics, Inc.). Fold stimulation is expressed as the ratio of message generated by ML-5M template versus that generated by ML template. (C) Effect with the synthetic activator Gal4-VP16. ML transcript (180nt) was generated by 25 ng supercoiled MLP basal control promoter. ML-3G transcript (390nt) was generated by 25 ng supercoiled MLP with three Gal4 binding sites fused upstream. Lanes 1-3: titration of Co-B (0, 1, 5μl) into the reaction in the absence of Gal4-VP16. Lanes 4-6: titration of Co-B (0, 1, 5μl) into the reaction in the presence of saturating amounts of Gal4-VP16 (G).


A
—
M
Co-B:
–
–
ML-5M
ML
1 2 3 4 5 6
B
Fold stimulation
15
10
5
1 2 3 4 5 6

**C**



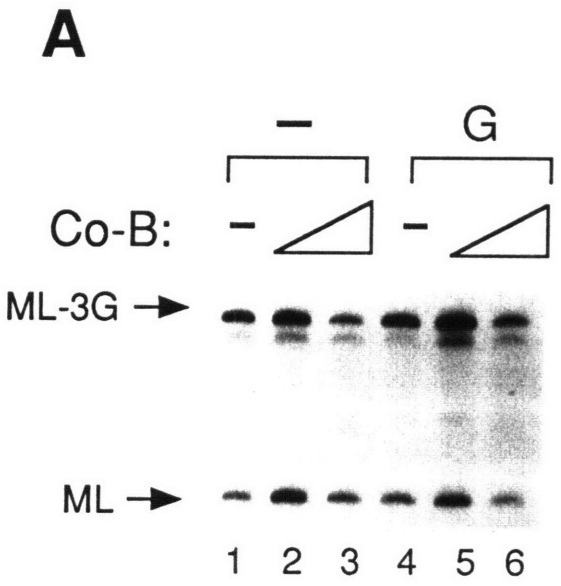
(Figure 1A) or Gal4-VP16 (Figure 1C) stimulated transcription from the appropriate template in the presence of Co-B (ML-5M and ML-3G respectively). Furthermore, the levels of stimulation with Co-B approached those observed in reactions containing nuclear extract (data not shown and Figure 1B). Stimulation by SP1 was similarly affected by Co-B (data not shown). Co-B preparations were not contaminated with any basal factors since they were unable to substitute for any of the these factors (data not shown), although titration of Co-B gave modest augmentation and then slight repression of the basal reaction (Figure 1C, lanes 1-3). Co-B required the presence of both activator and activator binding sites to exert its potentiation of stimulation. The activation observed in these reactions was due to a net increase in transcription from the specific template and was not due to a selective derepression of the stimulated template nor to a selective repression of the basal template (compare Figure 1A lanes 4-6).

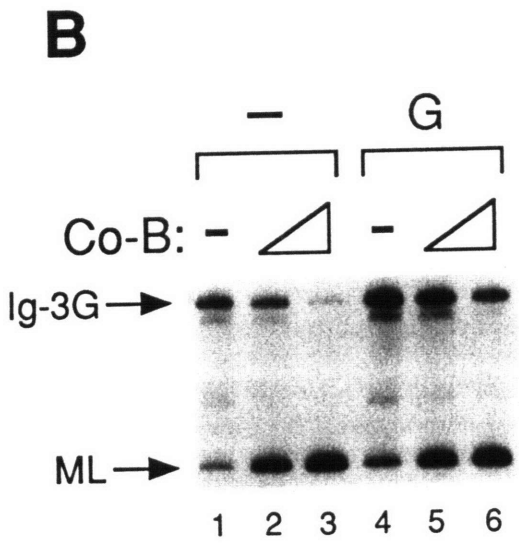
To characterize the basal machinery requirements for activation, the necessity of TAFs in the TFIID complex was examined. In almost all *in vitro* systems tested, stimulation by activators is dependent upon the presence of the holo-TFIID complex which is composed of TBP and TAFs (Dynlacht et al. 1991; Zhou et al. 1992; reviewed in Tjian and Maniatis 1994). This was also the case with Co-B; recombinant human TBP supported basal transcription but did not respond to Gal4-VP16 when Co-B was titrated into the reaction (Figure 2A). The effect of Co-B on the basal reaction in the presence of TBP was similar to that observed with holo-TFIID: transcription slightly increased and then similarly decreased as Co-B was titrated into the reaction. Thus, the effect of Co-B on the basal reaction is TAF independent whereas its effect on the stimulated reaction is critically dependent on TAFs. This implies that Co-B cannot obviate the need for TAFs in the stimulated reaction but rather functions as a complementing factor enabling the stimulatory effect of the activator on the basal machine.

The ability of Co-B to potentiate stimulation in the context of other promoters was tested. The μ-promoter of the heavy chain immunoglobulin gene and the human heat-

**Figure 2A. Stimulation of activation by coactivator-B requires TAFs in the holo-TFIID complex.** Titration of Co-B (0, 1, 5μl) into transcription reactions with TBP in the absence (lanes 1-3) and presence of Gal4-VP16 (G), (lanes 4-6). ML transcript (180nt) was generated by 25 ng supercoiled MLP basal control promoter. ML-3G transcript (390nt) was generated by 25 ng supercoiled MLP with three Gal4 binding sites fused upstream.

**Figure 2B. Template specificity of coactivator-B.** Titration of Co-B (0, 1, 2μl) into transcription reactions in the absence (lanes 1-3) and presence (lanes 4-6) of Gal4-VP16 (G). ML transcript (180nt) was generated by 25 ng of supercoiled MLP basal control template. Ig-3G transcript (390nt) was generated by 25 ng supercoiled Ig heavy-chain μ-promoter with three Gal4 sites fused upstream.


A
–
G
Co-B:
–
–
ML-3G
ML
1 2 3 4 5 6


B
–
G
Co-B: - - -
Ig-3G
ML
1 2 3 4 5 6

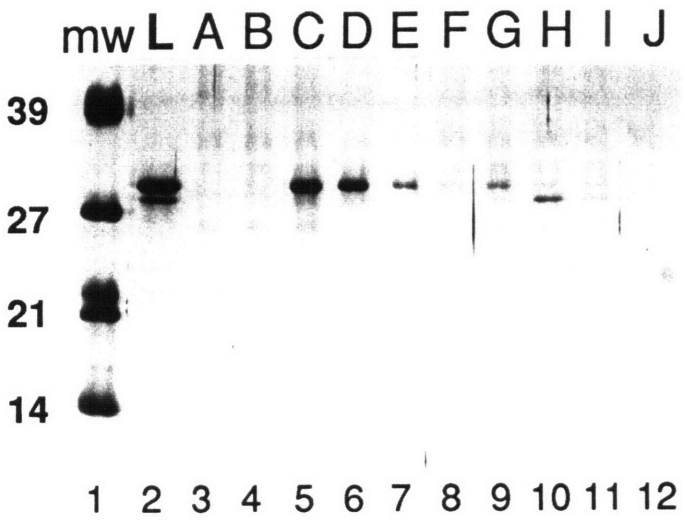
shock promoter were substituted for the MLP upstream of the 390nt G-less cassette. Gal4 binding sites were inserted upstream of the promoters (gifts of J. Parvin) and the templates were tested in the basal and stimulated reactions with the coactivator. The MLP without binding sites was included as a control promoter. The human heat-shock promoter (HSP) responded to activation potentiated by Co-B similarly to the MLP (data not shown). Surprisingly, Co-B exhibited a different effect on the μ-promoter (Figure 2B). In the absence of Gal4-VP16, Co-B had opposing effects on the two promoters: transcription from the MLP increased slightly, as was observed in Figure 1, while transcription mediated by the μ-promoter decreased (lanes 1-3). When Gal4-VP16 was included in the titration, no activation was observed from the μ-promoter with its three Gal4 binding sites. However, at high levels of Co-B, the activator derepressed the μ-promoter and brought the transcription up almost to the basal level (compare lanes 1, 3 and 6). This is in striking contrast to the MLP and HSP templates with Gal4-VP16 binding sites which were strongly stimulated by activator as Co-B levels were increased, and suggests that the activation process potentiated by Co-B involves promoter specific interactions of the initiation complex, coactivator, and the upstream activator (see Discussion).

**Coactivator B is HMG-2-** Reverse phase HPLC was used to separate the two polypeptides which comigrated as approximately 30 kDa bands in the Co-B preparation (Figure 3A, lane 2). The HPLC fractions were resolved by SDS-PAGE and silver stained (Figure 3A), and a portion of each fraction (A-J) was denatured with 6M Guanidine-HCl, renatured (see Experimental Procedures), and assayed by transcription in the presence of Gal4-VP16 (Figure 3B). Fraction C, which contained the peak concentration of the larger polypeptide (Figure 3A, lane 5) also contained the peak level of coactivator activity. (Figure 3B, lane 5).

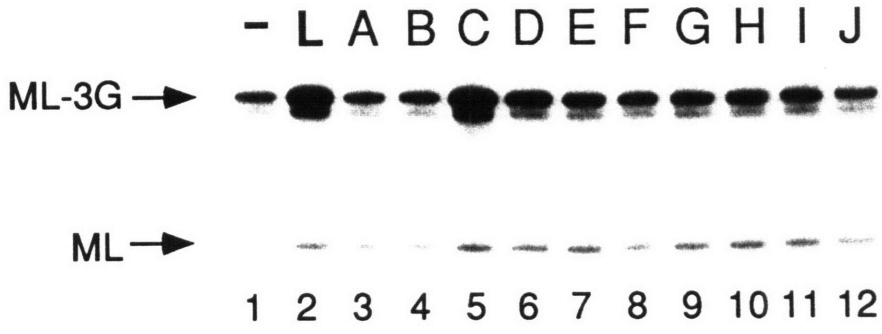
Amino-terminal protein sequencing of the HPLC purified Co-B polypeptide generated a 21 amino acid sequence which was identical to the amino-terminal sequence of the non-histone chromosomal protein HMG-2. This nuclear protein also has a molecular mass of

**Figure 3. HPLC purification of Co-B and transcriptional activity of renatured fractions.** (A) Purified Co-B preparation was subjected to reverse phase HPLC using a C4 column and fractions were resolved in a 14% SDS-PAGE and silver stained. Lane 2: load material and lanes 3-12 : HPLC fractions A-J respectively. (B) HPLC fractions and load were denatured with 6M guanidium-HCl, renatured by dialysis and assayed by transcription in the presence of Gal4-VP16. Lanes 3-12: HPLC fractions A-J, with load material (L) assayed in lane 2 and buffer added to reaction in lane 1. ML transcript (180nt) was generated by 25 ng of supercoiled MLP basal control template. ML-3G transcript (390nt) was generated by 25 ng of supercoiled MLP with three Gal4 binding sites fused upstream.

**A**


mw L A B C D E F G H I J
39
27
21
14
1 2 3 4 5 6 7 8 9 10 11 12

**B**

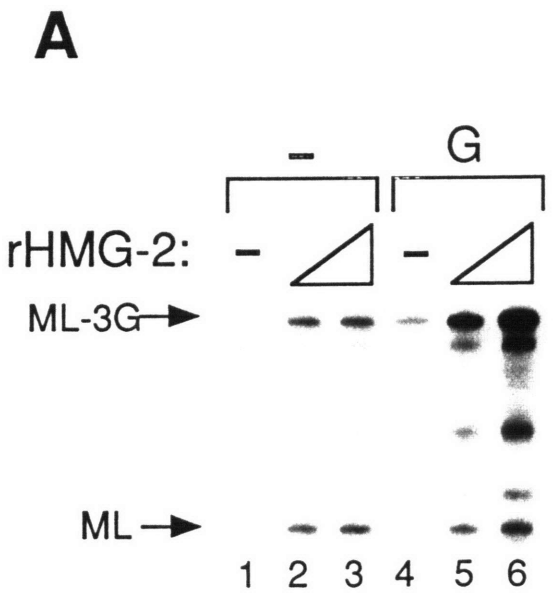

- L A B C D E F G H I J
ML-3G
ML
1 2 3 4 5 6 7 8 9 10 11 12

30 kDa in SDS-PAGE. To demonstrate that HMG-2 contained the Co-B activity, recombinant HMG-2 was produced in bacteria from a human cDNA clone (Majumdar et al. 1991) and the protein was purified to homogeneity (see Materials and Methods). The recombinant protein potentiated activation by Gal4-VP16 as efficiently as the original Co-B preparation (Figure 4A). Typically, purified HMG-2 levels of 100 ng and levels of recombinant HMG-2 of 200 ng to 400 ng in the transcription reactions gave saturating amounts of activation, which suggests that only a few molecules of HMG-2 per template are required for activation (see Discussion). The modest stimulatory activity for basal transcription observed with the purified, cellular preparation was also observed with rHMG-2 indicating that this activity was not due to a contaminant.

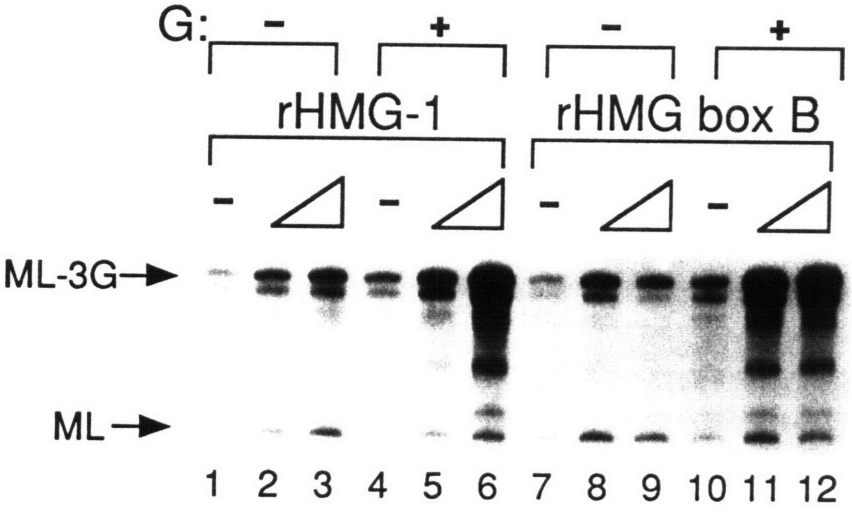
HMG-1, the cellular homologue of HMG-2, is functionally equivalent in many assays *in vitro* (reviewed by Grosschedl et al. 1994). This functional equivalence was also observed in the activation assay. Recombinant HMG-1 (generous gift of S. Lippard) was titrated into basal (Figure 4B, lanes 1-3) and Gal4-VP16 stimulated (lanes 4-6) transcription reactions. The coactivation by rHMG-1 suggested that a common feature of the HMG-1/2 family was responsible for such activity. The most striking common features of this family are subdomains which non-specifically bind DNA in the minor groove. These sub-domains, called HMG-boxes, are repeated twice in HMG-1/2. The HMG-box from the B-domain of HMG-1 is a folded polypeptide of about 80 amino acids that binds and dramatically bends DNA (Pil et al. 1993). A purified recombinant peptide corresponding to this domain was assayed in the reconstituted reaction and was functionally equivalent to HMG-1 (Figure 4B, lanes 7-12). Thus the sequences necessary for DNA binding by HMG-1/2 are also sufficient for coactivation of transcription.

The identification of HMG-1/2 as a coactivator of pol II transcription in a defined *in vitro* system containing purified general transcription factors made it possible to study the mechanism of activation. The stimulated reaction was analyzed by titration of basal factors, and by characterization of the effects of activation on individual steps in initiation.

**Figure 4. Recombinant HMG-1 and HMG-2 proteins coactivate stimulation.** (A) Recombinant human HMG-2 protein was titrated into transcription reactions at 0 ng, 100 ng 500 ng, in the absence (lanes 1-3) and the presence (lanes 4-6) of Gal4-VP16 (G). ML transcript (180nt) was generated by 25 ng of supercoiled MLP basal control template. ML-3G transcript (390nt) was generated by 25 ng supercoiled MLP with three Gal4 binding sites fused upstream. (B) Recombinant rat HMG-1 and recombinant HMG-1 B-subdomain were titrated into reactions in the absence (lanes 1-3 and 7-9) and the presence (lanes 4-6 and 10-12) of saturating amounts of Gal4-VP16 (G), at 0 ng, 100 ng, and 500 ng of HMG-1 and 0 ng, 50 ng, and 250 ng of the B-domain polypeptide. ML transcript (180nt) was generated by 25 ng of supercoiled MLP basal control template. ML-3G transcript (390nt) was generated by 25 ng supercoiled MLP with three Gal4 binding sites fused upstream.


A
–
G
rHMG-2:
–
–
ML-3G
ML
1 2 3 4 5 6

**B**

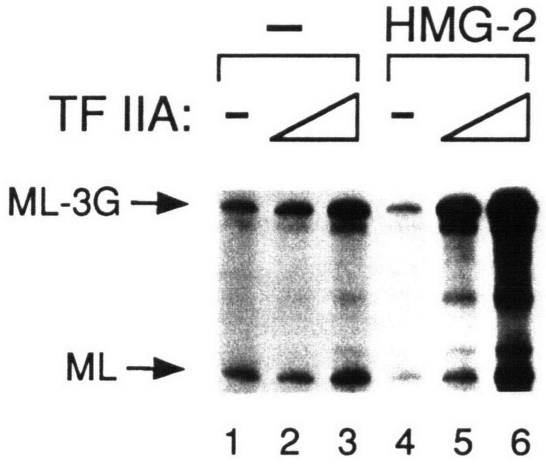


**TFIIA and TFIIB in activation-** Consistent with previous observations (Ma et al. 1993), TFIIA only slightly augmented the basal reaction in the purified system used in these experiments. More recently, TFIIA was shown to be required, or to enhance *in vitro* activation in systems of transcription factors of varying purity (Ozer et al. 1994; Yokomori et al. 1994; Sun et al., 1994). The requirement for TFIIA in activation potentiated by HMG-1/2 was tested (Figure 5A). Titration of TFIIA in the absence of HMG-1/2 but in the presence of activator slightly increased the level of transcription both from templates with and without Gal4 sites (lanes 1-3). More interestingly, no stimulation was observed in the absence of TFIIA when HMG-1/2 and Gal4-VP16 were present (compare lanes 1 and 4). To achieve significant levels of stimulation both HMG-1/2 and TFIIA were required in the presence of activator (lanes 4-6). Thus TFIIA is essential for stimulation of transcription by Gal4-VP16 in the presence of HMG-1/2.

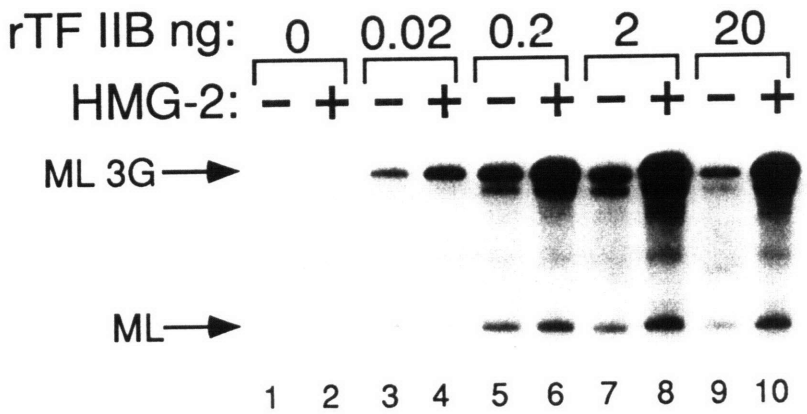
The potential importance of TFIIB binding in activation has been described (Lin and Green 1991; Choy and Green 1993). However, some experiments suggest that at least part of the activation process precedes the interaction of TFIIB with the template bound TFIID complex (White et al. 1992). If the function of activators and coactivators were to increase a rate limiting TFIIB binding step during initiation, then increasing the concentration of TFIIB should reduce the level of stimulated versus basal transcription. The concentration of TFIIB was titrated over three orders of magnitude (Figure 5B). High levels of TFIIB did not obviate the need for activator or HMG-1/2 to attain significant levels of stimulation. In fact, as the concentration of TFIIB increased, transcription from the ML-3G template in the absence of HMG-1/2 saturated at lower concentrations of TFIIB than in the presence of HMG-1/2. This increase in the degree of stimulation with increased TFIIB concentration was also observed by Choy and Green (1993) in a TFIIB-depleted nuclear extract and suggests that activation of transcription is not equivalent to an increased affinity of the initiation complex for TFIIB.

**Figure 5. Roles of TFIIA and TFIIB in activation.** (A) TFIIA is required for activation by coactivator B. Homogeneous preparation of purified human TFIIA was titrated into transcription reactions at 0, 0.5, and 5μl, in the absence (lane 1-3) and the presence (lanes 4-6) of purified HMG-2 (pHMG-2). Gal4-VP16 was added to each reaction. ML transcript (180nt) was generated by 25 ng of supercoiled MLP basal control template. ML-3G transcript (390nt) was generated by 25 ng supercoiled MLP with three Gal4 binding sites fused upstream. (B) Titration of rTFIIB. Recombinant human TFIIB was titrated into transcription reactions in the absence (odd numbered lanes) and the presence (even numbered lanes) of pHMG-2. Gal4-VP16 was added to each reaction. ML transcript (180nt) was generated by 25 ng of supercoiled MLP basal control template. ML-3G transcript (390nt) was generated by 25 ng supercoiled MLP with three Gal4 binding sites fused upstream.

**A**



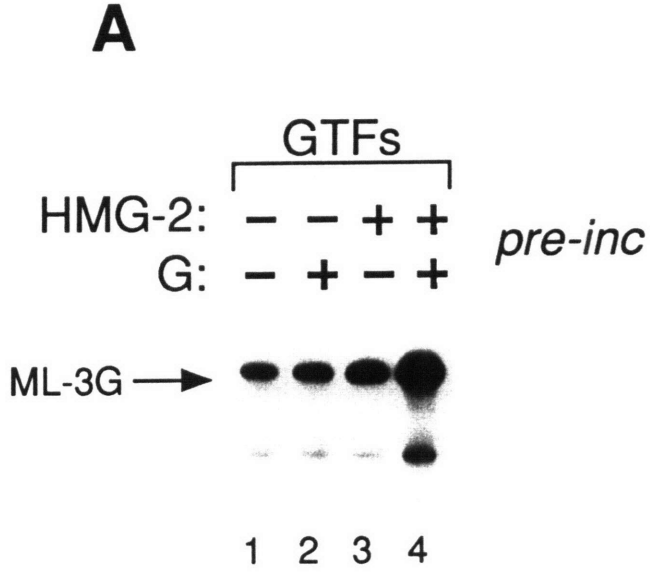
**B**



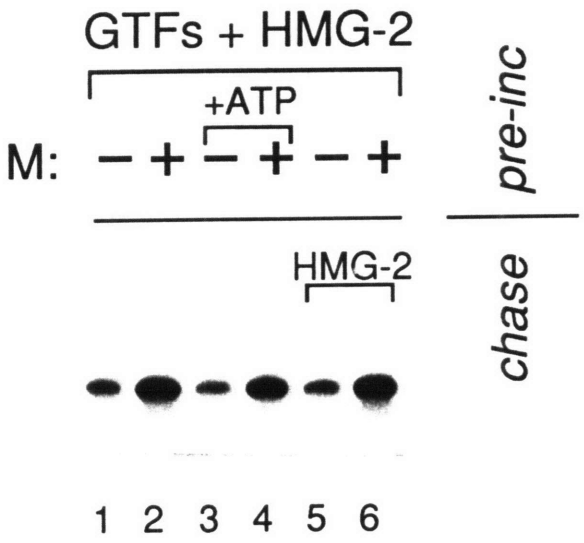
**Staged reactions on immobilized templates-** The step in the assembly of the initiation complex affected by activation can be partially characterized by an immobilized template system (Lin and Green 1991). By tethering the templates to magnetic beads, distinct complexes formed on the template can be purified from unbound or loosely associated factors and tested for transcriptional activity with the subsequent addition of other factors and nucleotides. Template DNA was end-labeled with a single biotinylated deoxynucleotide and then bound to streptavidin covalently linked to paramagnetic beads. Typically two incubations are done. The first incubation, the "pre-inc," allows the formation of a distinct preinitiation complex on the promoter. The complexes formed in the pre-inc are then washed to remove uncommitted factors. In the second incubation, the "chase," the remaining transcription factors are added along with nucleotides. Thus the effect of stimulation on the activity of basal factors in the formation of stable preinitiation complexes can be determined by preincubation of different combinations of factors.

In the presence of both activator and HMG-1/2, stimulation of transcription resulted in an increased formation of active preinitiation complex (Figure 6A). In lanes 1 and 2, all the general transcription factors (GTFs) were pre-incubated with and without Gal4-VP16 (G) respectively, washed, and chased with nucleotide triphosphate under transcription conditions. Gal4-VP16 had no effect on the formation of initiation complexes until HMG-1/2 was added during the pre-inc (compare lanes 2 and 4). In figure 6B, the basal factors and HMG-1/2 were pre-incubated in the presence and absence of the activator MLTF. As was observed with Gal4-VP16, the amount of active preinitiation complex was increased only if the activator was present in the pre-inc. Additionally, lanes 3 and 4 show that the addition of ATP during the pre-inc did not alter the amount of stimulation. If HMG-1/2 was added both in the pre-inc and afterwards in the chase no change in the level of stimulation was observed. This strongly argues that the positive effect of HMG-1/2 does not require ATP hydrolysis and acts during preinitiation and not at a post-initiation step.

**Figure 6. Preinitiation complex formation is increased in the presence of activator and HMG-2.** (A) Linear MLP template containing the 390nt G-less cassette with three Gal4 binding sites fused upstream was biotinylated at one end and bound to magnetic streptavidin beads. The general transcription factors were incubated with template bound beads alone (lane 1), with Gal4-VP16 (G), (lane 2), with pHMG-2 (lane 3), or with both Gal4-VP16 (G) and pHMG-2 (lane 4), for 60 min. at 30°C. Complexes formed on the template-beads were washed and nucleotides were added under transcription conditions. (B) Template-beads of the MLP with five MLTF binding sites fused upstream of the 390nt G-less cassette were prepared as described above. General transcription factors and pHMG-2 were incubated in the absence and presence of saturating amounts of purified MLTF (M) as described above. ATP was added in this preincubation to 0.1 mM in lanes 3 and 4. Complexes formed were washed and nucleotides were added under transcription conditions in the chase step. Additionally, pHMG-2 was added again in the *chase* , in lanes 5 and 6.


A
GTFs
HMG-2: – – + +
G: – + – +
pre-inc
ML-3G
1 2 3 4

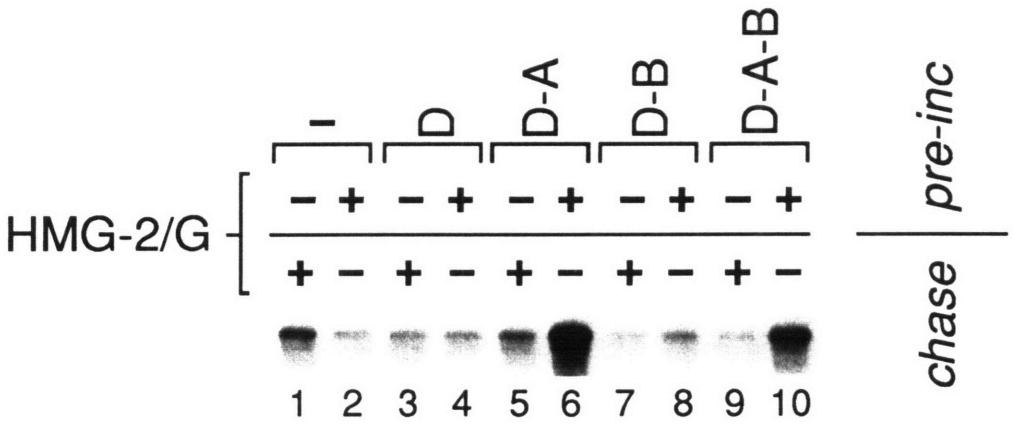
**B**



**Activation occurs at the TFIID-TFIIA complex stage-** To determine the first step of initiation which is altered by activation, activator and HMG-1/2 were added together at distinct steps in the assembly of the initiation complex (Figure 7). Activation was observed when all factors including activator and HMG-1/2 were added together in the chase , but was not observed if the activator and HMG-1/2 were pre-incubated with template (compare lanes 1 and 2). This indicates that HMG-1/2 is probably not simply stabilizing the binding of activator on the template. When TFIID was pre-incubated on the template with HMG-1/2 and Gal4-VP16 (lane 4) or committed alone and then chased with both (lane 3), no activation was observed. This indicates both that TFIID alone is not recruited to the template during activation and that template committed TFIID does not subsequently support activation. However, when TFIIA and TFIID were pre-incubated in the presence of activator and HMG-1/2, stimulation was observed (compare lanes 5 and 6). These results are consistent with the previous finding that TFIIA was essential for activation (Figure 5A).

Lin and Green (1991) have reported that TFIIB more stably associates with TFIID in the presence of activators. Further, they have suggested that the direct interaction of TFIIB with activator promotes the association of this protein with TFIID (Choy and Green 1993). As shown by a comparison of lanes 7 and 8 (and data not shown), preincubation of the activator, HMG-1/2, and TFIID did not significantly increase the stable association of TFIIB. However, consistent with the phenomenology of the Lin and Green (1991) result, preincubation of TFIIB with TFIID and TFIIA under activating conditions resulted in a more stable association of TFIIB (compare lanes 9 and 10). This enhanced association clearly occurs either after or simultaneously with the formation of the activated TFIID-TFIIA complex and may reflect a difference in the conformation of the activated versus basal complexes. These data suggest that retention of TFIIB on the TFIID complex is not the first step in activation but that activation precedes this step, at the stage of interaction of activator, coactivator, TFIID, TFIIA and template.

**Figure 7. Activation occurs at the TFIID-TFIIA complex.** Linear MLP template with three Gal4 binding sites was biotinylated and bound to magnetic beads as described (Experimental Procedures, Figure 6 legend). General transcription factors including homogeneously purified human TFIIA were used to form the preinitiation complexes: TFIID (lanes 3-4), TFIID-TFIIA (lane 5-6), TFIID-TFIIB (lanes 7-8) and TFIID-TFIIA-TFIIB (lanes 9-10). Gal4-VP16 (G) and pHMG-2 were incubate with the particular factors in the *pre-inc* (even numbered lanes) or added after complex formation in the *chase* along with the remaining general factors and nucleotides (odd numbered lanes).


–
D
D-A
D-B
D-A-B
HMG-2/G
– + – + – + – + – +
+ – + – + – + – + –
pre-inc
chase
1 2 3 4 5 6 7 8 9 10

Electrophoretic Mobility Shift Assay (EMSA) was used to investigate the stability of TFIID-TFIIA complexes after activation by activator and HMG-2. Interestingly, no quantitative differences were observed in the formation of TFIID-TFIIA complex under activation conditions in the presence of HMG-2 and Gal4-VP16 (Figure 8). Holo-TFIID and radio-labeled MLP with three Gal4 sites fused upstream were coincubated with combinations of TFIIA, HMG-2 and Gal4-VP16 and then subjected to EMSA on agarose TBE gels. Essentially no TFIID complex was detected in the absence of TFIIA even in the presence of activator and HMG-2 (lanes 1-4). In the presence of TFIIA a stable TFIID-TFIIA complex formed. This complex was slightly altered in mobility when coincubated with Gal4-VP16, however, no significant change was observed in the TFIID-TFIIA complex if HMG-2 was included with Gal4-VP16 (lanes 5-8). These last conditions generated activation in the previous transcription assays. These results suggest that during the activation process no quantitative change occurs in the formation of the TFIID-TFIIA complex.

The state of the TFIID-TFIIA complex on the promoter under basal and activating conditions was also examined using DNase I footprint analysis. No significant changes in either the extent of the footprint or its strength were observed when HMG-2 and activator were coincubated with TFIID and TFIIA on the template (Figure 9). As expected, the TBP-TFIIA complex protected the TATA region from -35 to -20 (lane 3). Both the TBP-TFIIA and the TFIID-TFIIA complexes stabilized Gal4-VP16 binding, increasing the strength of the footprint over the Gal4 binding sites. HMG-2 did not alter the TBP-TFIIA footprint (compare lanes 3 and 5). As has been reported (Sawadogo and Roeder 1985; Lieberman and Berk 1994), the TFIID-TFIIA complex gave an extended footprint of protected and hypersensitive sites downstream of the TATA box from -35 to +40 (compare lanes 3 and 4 with 7 and 8). However, no alteration of the footprint was observed when HMG-2 was added, either alone or with activator, in the TFIID-TFIIA binding reaction (compare lanes 7 and 8 with 9 and 10). Thus, under activation conditions, the presence of

**Figure 8. Formation of the TFIID-TFIIA complex.** Radio-labeled MLP probe was incubated with TFIID and various combinations of homogeneously purified TFIIA, rHMG-2 and Gal4-VP16 under conditions consistent with activated transcription and analyzed by electrophoresis in 1.6% agarose-TBE gels (Lieberman and Berk, 1994).

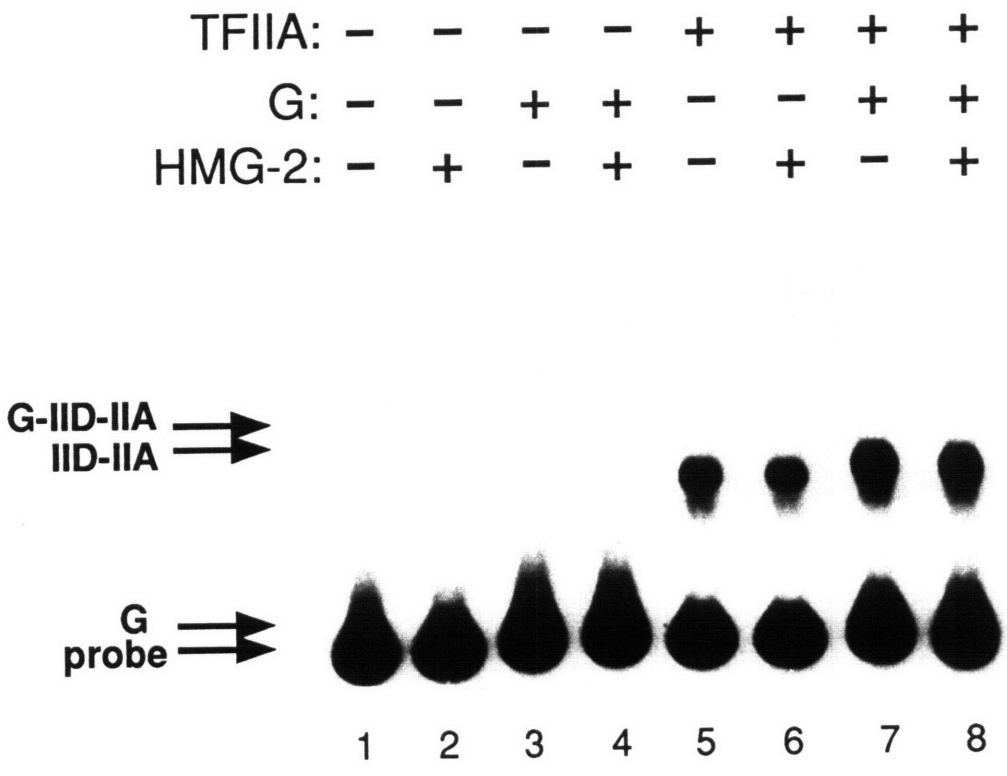
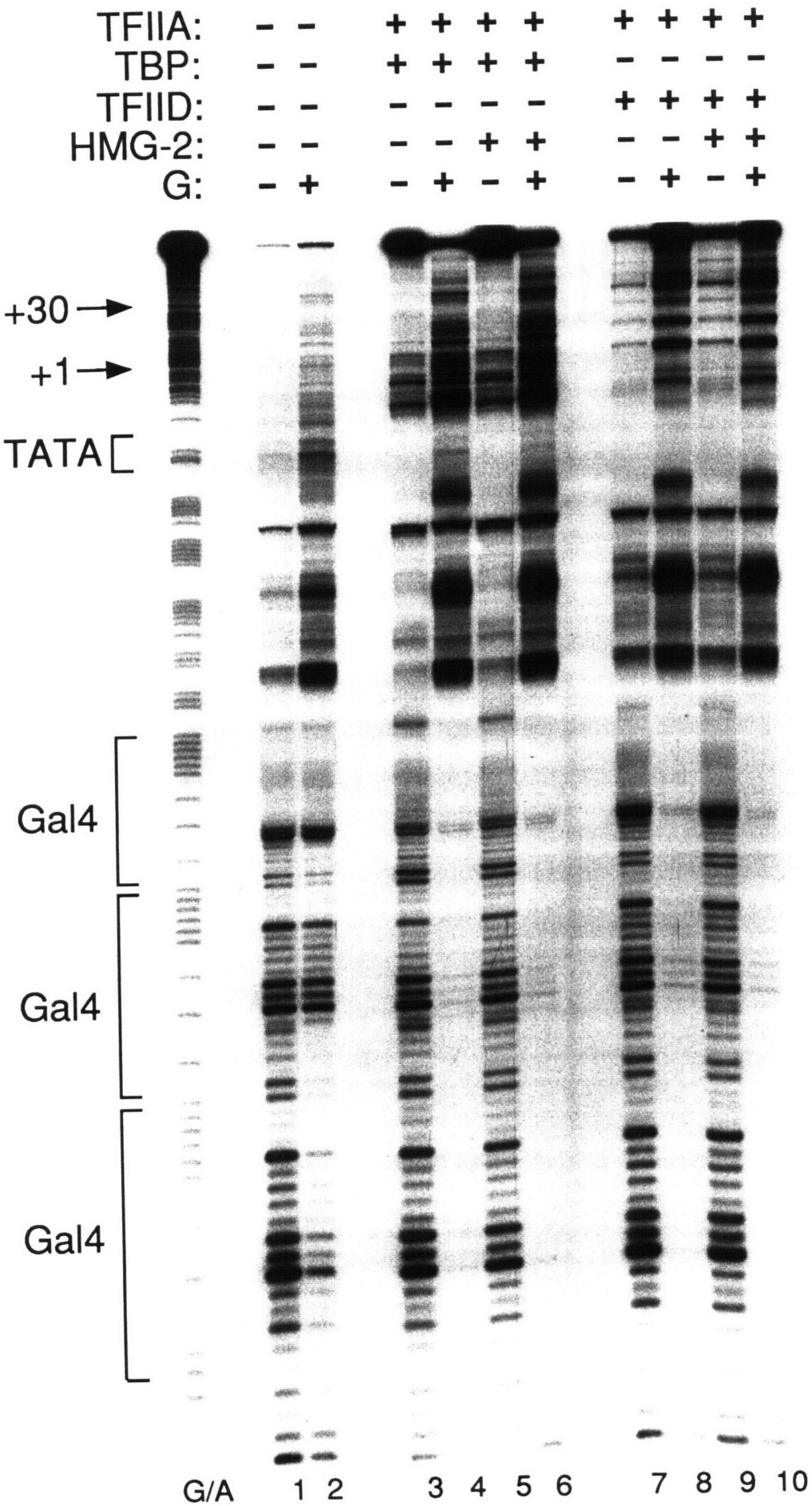

TFIIA: – – – – + + + +
G: – – + + – – + +
HMG-2: – + – + – + – +
G-IID-IIA
IID-IIA
G
probe
1 2 3 4 5 6 7 8

**Figure 9. Footprint of the TFIID-TFIIA complex.** The DNase I footprint pattern of TBP-TFIIA and TFIID-TFIIA complexes were analyzed with various combinations of pHMG-2 and Gal4-VP16. Binding reactions were identical to those used in figure 8.


TFIIA: - - + + + + + + + +
TBP: - - + + + + - - - -
TFIID: - - - - - - + + + +
HMG-2: - - - - + + - - + +
G: - + - + - + - + - +
+30
+1
TATA
Gal4
Gal4
Gal4
G/A 1 2 3 4 5 6 7 8 9 10

activator and HMG-2 did not alter the footprint of the TFIID-TFIIA complex on the promoter.

## Discussion

We have purified a factor important for activation and identified it as HMG-2. Interestingly, the activity of HMG-2 is specific for some promoters. Activation of transcription requires the presence of TFIIA, and the early initiation complex composed of TFIID and TFIIA is modified by interaction with activator and HMG-1/2. Similar to activation in total extracts and in partially fractionated systems, stimulation of transcription in this system depends upon the presence of TAFs in the holo-TFIID complex (Dynlacht et al. 1991; Zhou et al. 1992) and results in a change in the nature of the interaction of TFIIB with the activated preinitiation complex. We propose that this activation step involves the induction of a structure in the TFIID-TFIIA-promoter complex which facilitates initiation.

HMG-1 and HMG-2 function interchangeably in reactions containing the major late promoter. In fact, the HMG-box from the B-subdomain of HMG-1 function indistinguishably from the full-length protein. This subdomain consists of 80 amino acids folded into three α-helices arranged roughly into an L-shape (Weir et al. 1993; Read et al. 1993). The HMG-box, as well as HMG-1 and 2 bind DNA in the minor groove and bend the helix toward the major groove (reviewed by Grosschedl et al. 1994). Since the HMG-box subdomain alone enables activation in the system, it is likely that these proteins function by stabilizing conformations of DNA bound to the basal factors and activator. These critical conformations could involve looping of the activator to the basal complex, or altering of the local topology of the DNA around the basal factors (Bazett-Jones et al. 1994; Giese et al. 1992; Paull et al. 1993).

The HMG-1/2 proteins dramatically enhanced activation in reactions containing the MLP or the HSP but suppressed transcription in reactions containing the IgH μ-promoter. Maximal activation was observed at a HMG-2 to template ratio of approximately 100 to 1, an absolute concentration of HMG-2 of 150 nM. Based on the reported affinity of HMG-1/2 for dsDNA (Pil and Lippard 1992), this ratio represents only a few molecules of bound

HMG-1/2 per template DNA. The template specificity of HMG-1/2 must reflect a difference in the sequence of these promoters and consequently a difference in the nature of their interactions with protein factors. For example, such specificity could be due to a sequence preference for the binding of the HMG-box to the MLP or HSP templates. However, HMG-1/2 binds DNA with little or no direct sequence specificity other than binding to DNA with irregular or bent structures (reviewed by Bustin et al. 1990; Grosschedl et al. 1994 and references therein). More likely, the template specificity reflects the dependence of a critical activation complex on the enhanced stability conferred by the HMG protein. For example, a hypothetical activated conformation of the TFIID-TFIIA complex could be dependent upon the topology of the promoter stabilized by HMG-1/2. We favor this latter possibility since the IgH μ-promoter was not activated at any concentration of HMG-2 and its basal activity was in fact repressed by low concentrations of HMG-1/2.

Activation was first detected at the stage of the interaction of TFIID, TFIIA, HMG-2, and activator in the presence of template. This activated complex is distinguishable by a decrease in the rate of dissociation of TFIIB and a more efficient assembly of a transcriptionally active polymerase complex. However, activation is not due to a limitation in the availability of TFIIB for binding, as the difference in activated versus basal activity could not be reduced by addition of high concentrations of TFIIB. Both results are consistent with the proposal that the conformation of the activated TFIID-TFIIA-HMG-2 complex is different than that of the equivalent basal complex. The former is conjectured to interact with TFIIB and probably other basal factors in a different fashion such that the rate of initiation is increased. The observations in these defined reactions are remarkably consistent with previous work, in which partially purified fractions were used, where TFIIB was more stably bound to the activated template than the control promoter, but high concentrations of TFIIB did not decrease the fold activation (White et al. 1992; Choy and Green 1993).

The dependence of activation in the presence of HMG-1/2 on TFIIA agrees with previous suggestions that the TFIID-TFIIA binding step is important for stimulation (Wang et al. 1992; Ma et al. 1993) and with results demonstrating the importance of TFIIA in stimulation in various less well defined in vitro systems (Ozer et al. 1994; Yokomori et al. 1994; Sun et al. 1994). Recently, the viral activator Zta was shown to increase the rate of formation of a TFIID-TFIIA complex (Lieberman and Berk 1994). However, this enhanced rate of formation of TFIID-TFIIA complex did not directly correlate with enhanced transcription. Thus it was not possible to equate formation of the TFIID-TFIIA -Zta complex with the activation event. The kinetic analysis of initiation complex formation has also shown that in the presence of partially fractionated TFIID, TFIIA and activator, the lag before open complex formation was shortened (Wang et al. 1992). However, in the present study, it is unlikely that an acceleration in the rate of formation of the TFIID-TFIIA-template complex is the critical aspect in activation since prolonged incubations did not suppress the degree of activation (data not shown). Furthermore, activation at the TFIID-TFIIA stage was not manifested by an increase in complex formation as assayed either by footprint analysis or resolution of complexes by gel electrophoresis.

In the defined reconstituted reaction, association of TFIID and template in the absence of TFIIA rendered this complex refractory to subsequent activation, but still able to support the basal reaction. The refractory nature of the template committed TFIID suggests that the conformation of TFIID-promoter complexes can vary. This observation is similar to previous results where template committed TFIID complexes were unable to respond to the addition of an activator (Wang et al. 1992). Those complexes formed in the absence of activator, which were referred to as "inactive" by Wang et al., may in fact be locked in a conformation that only supports basal levels of transcription.

Consistent with all of the above results, the activation event probably involves a conformational change in the TFIID-TFIIA complex, possibly involving an alternate arrangement of the TAFs on the template. The activation event may expose multiple or

altered binding sites for TFIIB that in turn stabilize a specific conformation of TFIIB. This conformation of TFIIB could more efficiently recruit the other proximal basal factors for initiation. Such a conformational change in TFIIB has been postulated (Choy and Green 1993; Roberts and Green 1994), and mutants in TFIIB exist that support basal but not stimulated transcription (Roberts et al. 1993). It is also likely that the activated TFIID-TFIIA-template conformation preferentially interacts with basal factors beyond TFIIB in initiation. In fact, a polymerase II holoenzyme complex, such as that defined in yeast (Koleske and Young 1994), could be recruited through multiple interactions with the activated TFIID-TFIIA complex.

In this system, HMG-1/2 allows the basal reaction to respond to activators although it is probably acting by a different mechanism than the factors previously defined as coactivators. The soluble, non-TAF, coactivators described to date, while not essential for the basal reaction, can modulate this reaction in the absence of activators, possibly altering the TFIID-TFIIA interaction. The defined transcription reaction described here which mediates activation with HMG-1/2 does not contain any of the known soluble coactivators. The USA component, PC4 has been shown to interact with the basal factors and with the activation domains of activators and thus is probably acting as an "adapter" molecule, physically linking the activator and the basal machine. HMG-1 does not interact with activators (Ge and Roeder 1994b; our unpublished data), but has been reported to form a complex with TBP on the promoter (Ge and Roeder 1994b). Interestingly HMG-1/2 has been reported to repress both the basal and stimulated reactions in vitro (Ge and Roeder 1994b; Stelzer et al. 1994). We have found that the presence of either topoisomerase I or the USA component PC4 alter reactions such that HMG-1/2 represses transcription upon titration (manuscript in preparation).Topoisomerase I/PC3 antagonizes the formation of the TBP-TFIIA complex on the promoter (Merino et al. 1993), and thus could alter the nature of the interaction of activators with the TFIID-TFIIA complex. The enzymatic activity of topoisomerase I is dispensable for its repression of transcription (Merino et al. 1993).

HMG-1 and HMG-2 are the founding members of a family of DNA binding proteins involved in transcription including LEF-1 (Giese et al. 1992), SRY (Ferrari et al. 1992), hUBF (Jantzen et al. 1992) and DSP-1 (Lehming et al. 1994), all of which contain one or more HMG-boxes (reviewed by Grosschedl et al. 1994). The ability of the HMG-box containing factors to modulate DNA conformation has led to the description of this family as "architectural" transcription factors (Wolffe 1994). That HMG-1/2 potentiates stimulation in the presence of purified basal factors and activator suggests that the activation step does not require components beyond the basal machinery, activator, and an "architectural" factor. We propose that this activation step involves the stabilization by the HMG-1/2 proteins of a specific conformation of the TFIID-TFIIA-activator complex that facilitates enhanced rates of initiation.

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# Appendix A

## Analysis of HMG-1/2 Induced Activated Conformation of the TFIID-TFIIA Complex: Linking Number Change Assay

## Introduction

In Chapter 3, it was proposed that the mechanism of coactivation by HMG-1/2 proteins involves an induction or stabilization of the otherwise unstable activated complex formed by TFIID, TFIIA, and Gal4-VP16. HMG-1/2 proteins bind to DNA non-specifically and have been shown to reduce the linking number if present during a covalent closure of DNA (Javaherian et al., 1978). Therefore, participation of HMG-1/2 proteins in the formation of the initiation complex may result in a change of linking numbers in a TATA- or enhancer-dependent manner. This appendix describes the experimental design and results of linking number change assays aimed at detecting the altered conformation of TFIID-TFIIA-DNA (DA) complex induced by HMG-1/2.

## Materials and Methods

**Template plasmids: mutagenesis** - The pBSII-SK+ derived construct containing the region -180 to +65 of the Gal4(3)-MLP transcription template has been described (Shykind et al., 1995). A single transformant DH5α clone was grown for 1 hour at 37°C in a 20 ml of LB with 100 μg/ml ampicillin. VCS M13 helper virus was used to infect the culture which was then further incubated for 1 hour. Subsequently, kanamycin was added (70 μg/ml), and the culture was grown for an additional 12 hours at 37°C. Following centrifugation at 10000g for 10 minutes, the supernatant was mixed with 0.25 volume of 20 % PEG and 3.5 M $NH_4Ac$. After incubating on ice for 30 minutes, single stranded DNA was obtained by centrifugation at 10000g for 20 minutes at 4°C. The pellet was resuspended in TE, extracted five times with phenol/chloroform, precipitated with EtOH, and resuspended in TE again for use as the substrate for mutagenesis. Mutagenesis was performed using the Sculptor *in vitro* mutagenesis system (Amersham) according to the manufacturer's instructions. Oligonucleotides used for the mutagenesis were designed so

as to contain mutated TATA sites flanked by approximately 10 bases of MLP sequence on each side: 5'-CCACCCCCTTCTCTAGCCCCCTTCAGG-3' (T1); 5'-CCACCCCCTC CATGGGCCCCCCTTCAGG-3' (T2). The sequences of T1 and T2 TATA mutants were confirmed by sequencing.

**Template Plasmids: multimerization-** In order to amplify the promoter-dependent linking number change, the promoter sites were multimerized following these steps. From each of the plasmids containing the wild type (WT), T1, and T2 TATA sites, an Xmn I-Hind III fragment containing the promoter region and an Xmn I-Xho I fragment also containing the promoter region were obtained from separate digestions. The fragments were treated with Klenow and dNTP's so as to blunt the Hind III and Xho I cleavage ends and then were ligated to each other. Constructs thus obtained contained two direct repeats of the Gal4(3)-MLP region and were designated WTx2, T1x2, and T2x2. From each of these plasmids, an Xmn I-Eco RV fragment containing two direct repeats of the promoter and an Xmn I-Xho I fragment containing two direct repeats of the promoter were obtained from separate digestions. The Xho I end was filled by treatment with Klenow and dNTP's, and the two fragments from each clone were ligated to each other. The constructs thus obtained contained four direct repeats of the promoter region and were designated WTx4, T1x4, and T2x4.

**Linking number change assay-** Vaccinia virus topoisomerase was a kind gift of J. Wang (Shuman et al., 1988; Hanai and Wang, 1994). Plasmid templates (10 μg) were incubated at 37°C for 30 minutes with 10 units of enzyme in the relaxation buffer (40 mm Tris pH 7.5, 100 mM NaCl, 5 mM EDTA), phenol/chloroform extracted, EtOH precipitated, and resuspended in TE. The preparation of TFIID, TFIIA, Gal4-VP16, and homogeneous purified and recombinant HMG-2 have been described (Shykind et al., 1995). The binding reactions (20 μl) were done under essentially transcription conditions (12.5 mM Hepes[pH7.9], 12.5 % glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 60 mM KCl, 5 mM β-mercaptoethanol) with 25 ng of plasmid template. After 30 minute incubation at

30°C, 1 unit of topoisomerase was added, and the incubation was continued at 37°C for 20 minutes. Subsequently, 1 µl of the stop solution (8 mg/ml proteinase K, 4% SDS, 10 mM $CaCl_2$) was added, and the incubation was continued for another 15 minutes at 37°C. The reaction mixture was extracted with phenol/chloroform and loaded directly onto the gel. For the electrophoresis, vertical gels were cast with 1.2 % SeaKem LE agarose (FMC Bioproducts) in 1X TBE with 1.5 µg/ml of chloroquine and were run in 1X TBE with 1.5 µg/ml chloroquine typically at 35 volts for approximately 18 hours. The visualization of the topoisomers was achieved by ethidium bromide (EtBr) staining.

### Results and Discussion

The linking number change upon binding of protein was assessed by treatment of the protein-DNA complexes with topoisomerase I followed by deproteination and electrophoresis in a chloroquine containing agarose gel. The topoisomer distribution was visualized by EtBr staining. As previously reported, the presence of HMG-2 resulted in a reduction of the linking number of the plasmid DNA (Javaherian et al., 1978; figure 1). In the absence of HMG-2, 1.5 µg/ml of chloroquine renders the majority of the relaxed plasmids to be distributed among five or six topoisomers with supercoils ranging from +11 to +15 (figure 1; lane 1). The number of supercoils is determined by comparison to the nicked plasmid with no supercoil. A reduction of the linking number by HMG-2 is indicated by the shifting of the topoisomer distribution toward topoisomers with less electrophoretic mobility or less supercoils (figure 1: lanes 2 to 6). Interestingly, binding of HMG-2 and the consequent linking number change appear to be synergistic. This is evidenced by the fact that rather than inducing the initial five or six topoisomers to undergo an even reduction of linking number and thus to maintain the same distribution, HMG-2 appears to spread out the distribution by preferentially binding to the already HMG-2 bound plasmids.

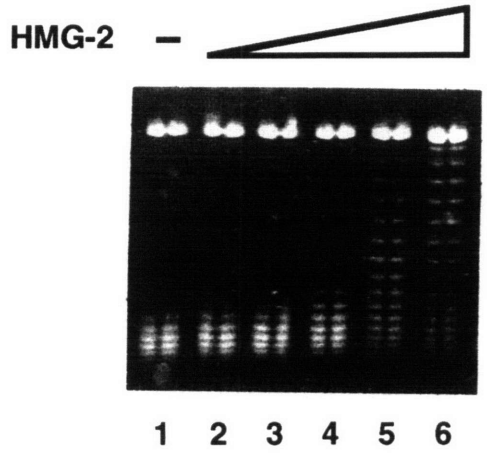


**Figure 1.** HMG-2 changes the linking number. Increasing amounts (0, 5, 10, 25, 50 and 100 ng) of homogeneous prepartion of bovine HMG-2 were incubated with 25 ng of WTx4 plasmid. Topoisomerase I treatment, deproteination, and electrophoresis were carried out as described in the Materials and Methods section.

The cooperative change of the helical structure by HMG-2 and transcription factors was examined using the WTx4 plasmid. An intermediate level of HMG-2 was used and a minor but visible reduction in linking number was observed (figure 2: compare lanes 1 and 2). TFIID had no effect by itself (figure 2: compare lanes 1 and 3). Remarkably, coincubation of HMG-2 and TFIID resulted in a dramatic reduction of the linking number indicating a cooperative interaction by these two factors (figure 2: compare lanes 2 and 4). In a similar assay, TBP was examined for its ability to interact with HMG-2 for a linking number change. As in the case with TFIID, TBP by itself had no effect on linking number (data not shown). This is in agreement with a published report (Lorch and Kornberg; 1993). However, unlike TFIID, TBP did not mediate a cooperative linking number change in association with HMG-2 (data not shown). TFIIA and Gal4-VP16 are additional components of the proposed activated complex. However, TFIIA either by itself (figure 2: lanes 5 and 6) or in combination with Gal4-VP16 (figure 2: lanes 7 and 8) did not further alter the distribution of the topoisomers.

The TATA dependence of the cooperative interaction between HMG-2 and TFIID were examined with TATA element mutants. As expected, TFIID cooperatively changed linking number with HMG-2 in plasmids WTx2 and WTx4 (figure 3: lanes 5 and 6 and lanes 9 and 10). Identical results were obtained with the T1 mutant derived templates (data not shown) and with the T2 mutant derived templates (figure 3: lanes 7 and 8 and lanes 11 and 12). In fact, even with the pBS II-SK+ plasmid, containing no promoter elements, an effective linking number change is seen (figure 3: lane 4), indicating that the phenomenon is not TATA specific or dependent on other promoter elements. Consistently, titration of non-specific DNA carrier in the reaction resulted in identical levels of inhibition of the linking number change in wild type and mutant templates (data not shown).

In conclusion, the cooperative change of linking number by TFIID and HMG-2 falls short of being a signature of the activated complex. Most importantly, it is not dependent

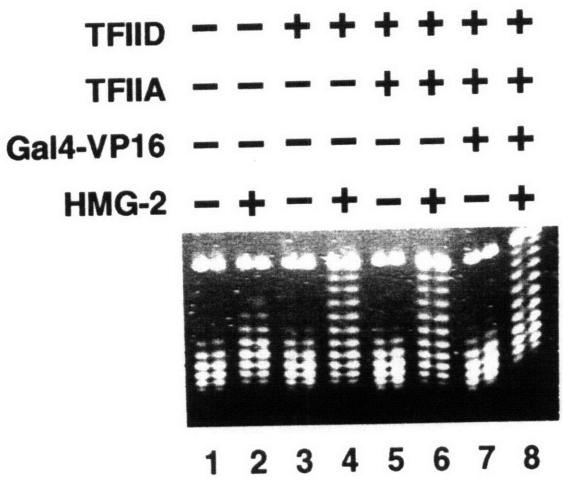


**Figure 2.** TFIID and HMG-2 cooperatively change the linking number. Various combinations of recombinant human HMG-2 (30 ng) and TFIID were incubated with 25 ng of WTx4 plasmid. The effects of TFIIA and Gal4-VP16 were examined by coincubation with TFIID in the absence and presence of HMG-2.

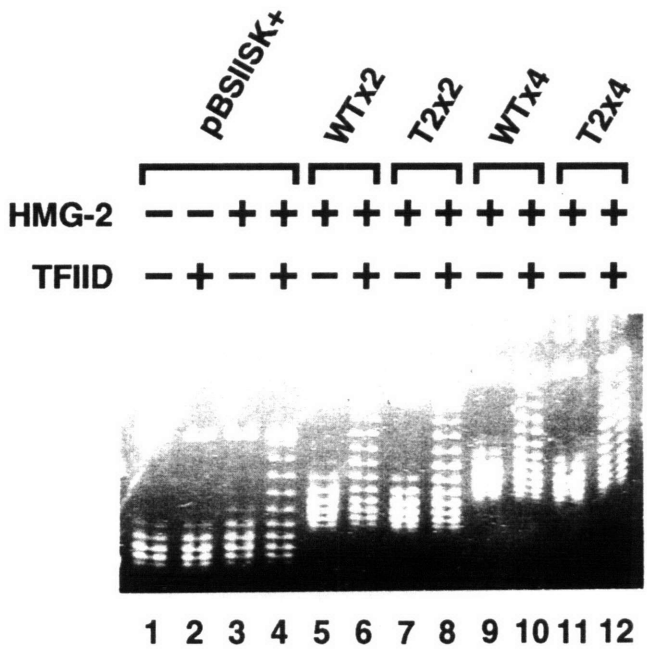


**Figure 3.** Linking number change is sequence independent. The cooperativity between TFIID and HMG-2 is tested on TATA-mutant plasmids (T2x2 and T2x4) and pBSII-SK+.

on promoter elements. In addition, the phenomenon is not modulated by Gal4-VP16 and TFIIA, obligatory components of the activation process. The linking number change may be a result of interaction between HMG-2 and one of the TATA-associated factors. One candidate is human homologue of TAF 150 which was shown to interact with the initiation site of the promoter (Verrijzer et al., 1994). Others include TAF's 80, 31, and 20 which show sequence similarities to histones H4, H3, and H2B thus suggesting a possible reconfiguring interaction with DNA (Hisatake, et al., 1995). Still, even in considering these possibilities, the fact that the TATA element, where the primary interaction between TFIID and promoter occurs, does not contribute to specificity or efficiency of the linking number change suggests a limitation of this assay in assessing the conformation of activated complex forming at the promoter.

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# Appendix B

## Analysis of HMG-1/2-induced Activated Conformation of the TFIID-TFIIA Complex: DNase I Footprinting Assay

## Introduction

In Chapter 3, the identification and characterization of the non-histone chromosomal high mobility group protein-2 (HMG-2) as a coactivator of transcription were presented (Shykind et al., 1995). It was proposed that HMG-2 functions at the TFIID-TFIIA (DA) complex stage which represents the first intermediate preinitiation complex stage modifiable by activation. Further, HMG-2 was proposed to play an architectural role of stabilizing the activated conformation of the DA complex. Preliminary DNase I footprinting analyses of the activated complex failed to reveal a HMG-2 dependent conformational alteration of the DA complex. This appendix describes a more detailed analysis of the conformation of the activated complex using DNase I footprinting assay. The results indicate that HMG-1/2 induce an alteration of DA complex conformation that is further modulatable by Gal4-VP16.

## Materials and Methods

The preparations of TBP, TFIID, TFIIA, and Gal4-VP16 have been described (Parvin et al., 1994; Shykind et al., 1995). Rat HMG-1 box B was a kind gift of S. Lippard (Pil et al., 1993). The DNA probe, representing regions from -180 to +65 of Gal4(3)-MLP (for the major late promoter of adenovirus) transcription template has been described (Shykind et al., 1995). It was prepared by cleavage of the plasmid with Xho I followed by Klenow-end filling with [$^{32}$P] dCTP, Hind III digestion, and gel purification. The binding reaction prior to DNase I digestion was performed under essentially transcription conditions [12 mM Hepes (pH 7.9), 12% glycerol, 60 mM KCl, 5 mM $MgCl_2$, 0.6 mM EDTA] supplemented with 5 mM β-mercaptoethanol and 0.5 mg/ml of Bovine Serum Albumin (BSA) in a total volume of 15 μl. Each reaction, containing 0.5 ng of the labeled probe and 15 ng of poly(dGdC), was incubated at 30$^{o}$C for 30 minutes followed by an 1 minute digestion at room temperature with a 15 μl of 5 mM $CaCl_2$ solution containing 1 ng of

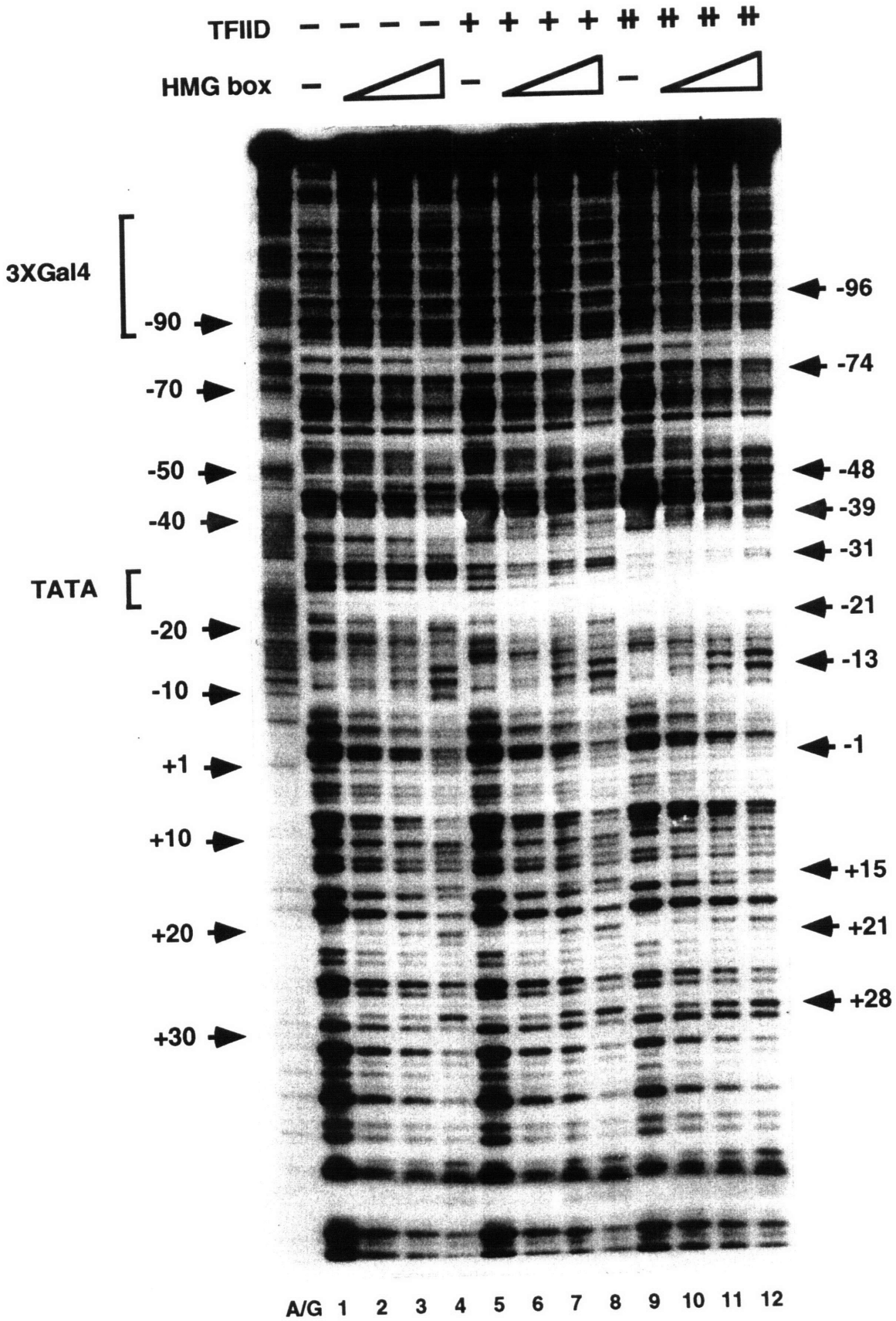
DNase I (Worthington Enzymes: DPFF grade). The reaction was stopped with 90 µl of the stop solution [20 mM EDTA (pH 8.0), 1% SDS, 200 mM NaCl, 100 µg/ml of yeast tRNA], phenol/chloroform extracted, EtOH precipitated, and then analyzed by sequencing gel electrophoresis [6% acrylamide, 0.75X TBE].

**Results and Discussion**

Rat HMG-1 box B (henceforth HMG box) has been shown to bind to DNA and induce a sharp bend (Pil et al., 1993). In a coactivator dependent activated transcription assays, this 80 amino acid long polypeptide was sufficient as the coactivator (Shykind et al., 1995). This fact, together with the lack of quantitative increase of DA complex mediated by Gal4-VP16 and HMG-2, led to the proposal that HMG-1/2 proteins function as an architectural element stabilizing the activated DA complex. Several modifications have been made to the DNase I footprinting assay which initially failed to show any HMG-2 dependent conformational alterations. First, the HMG box rather than the full length protein was used as the coactivator. The advantage of using a smaller coactivator is the higher accessibility of DNA to DNase I which in turn would allow a more detailed probing of the conformation of protein-DNA complexes. Secondly, the concentration of the non-specific DNA carrier, poly(dGdC), was adjusted to approximate the activated transcription more closely. HMG-1/2 proteins bind to DNA non-specifically, and too high or too low a concentration of the carrier would preclude the examination of the specific interaction of HMG-1/2 with the activated complex.

The protein-DNA complexes formed by various combinations of TFIID, TFIIA, Gal4-VP16, and HMG box were analyzed by DNase I footprinting. HMG box bound to DNA at several sites as evidenced by its effect throughout the length of the probe (figure 1: lanes 1-4). As expected for a non-specific DNA binding protein, HMG box lowered the overall level of cleavage. However, as it was titrated, several hypersensitive sites (+28, +21, +20,

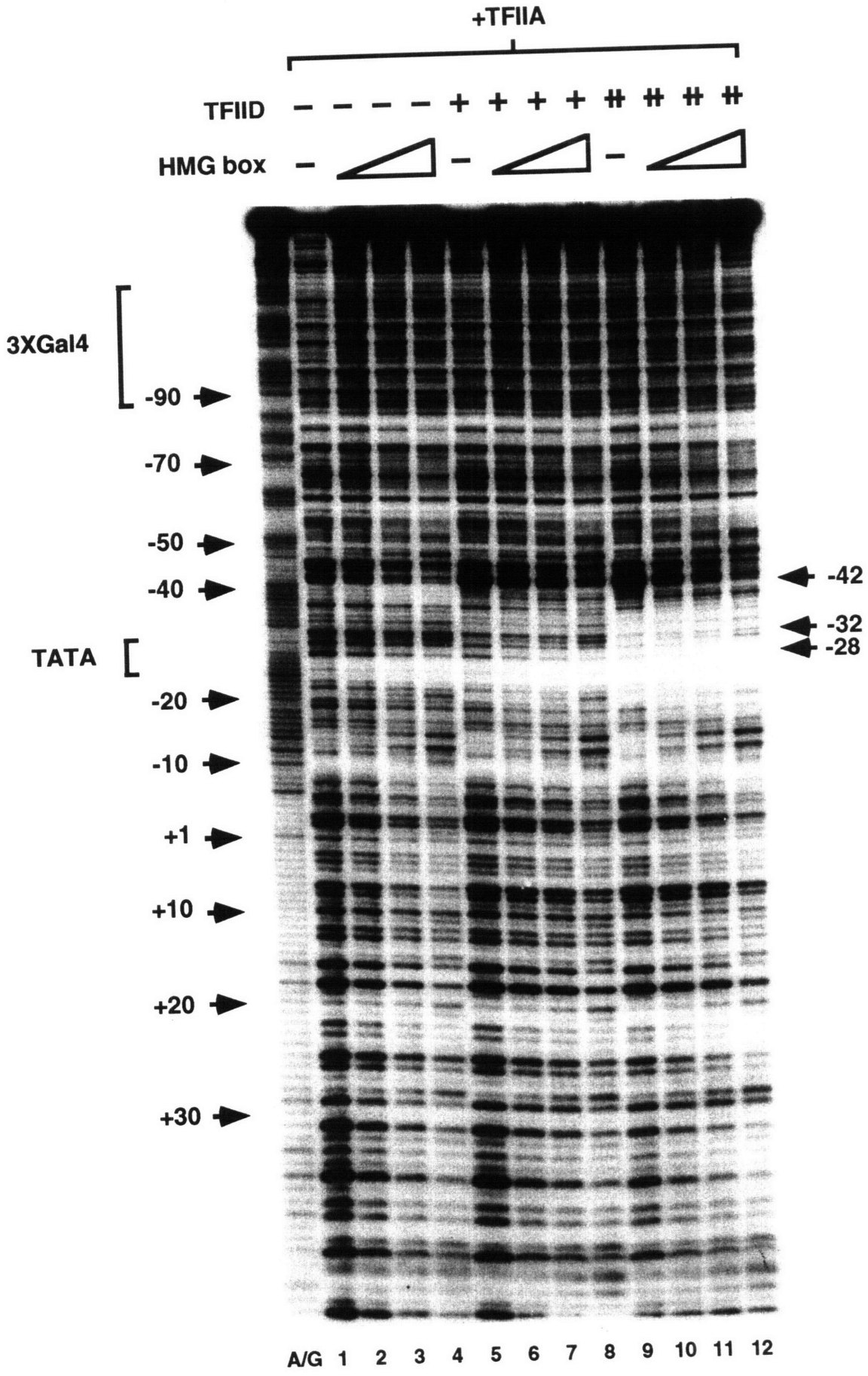
**Figure 1.** The interaction of TFIID and HMG box was analyzed by a DNase I footprinting assay. HMG box was titrated (0, 90, 180, 360 ng) in the absence (lanes 1-4) or in presence (0.75 μl: lanes 5-8; 2 μl: lanes 9-12) of TFIID.


TFIID
HMG box
3XGal4
TATA
-90
-70
-50
-40
-20
-10
+1
+10
+20
+30
-96
-74
-48
-39
-31
-21
-13
-1
+15
+21
+28
A/G 1 2 3 4 5 6 7 8 9 10 11 12

+15, -1, -11, -13, -15, -21, -31, -34, -48, -74, and -96) emerged (figure 1: lane 4). This observation suggested that HMG box organized the DNA into a protein-DNA complex with a specific and stable conformation rather than into a non-specific oligomeric complex by randomly coating the DNA. TFIID protected the TATA region (-20 to -36) as expected and induced hypersensitive sites at bases -42 and -43 (figure 1: compare lanes 1, 5, and 9). At the highest level of TFIID assayed, the majority of the probe was bound by TFIID as indicated by a dramatic reduction of the cleavage at bases -28, -30 and -32. As HMG box was titrated into the reactions containing this level of TFIID, the overall pattern of cleavage was similar to that seen in the titration of HMG box alone (figure 1: compare lanes 4 and 12). These alterations included a hypersensitive site induction at base -21, reversing the protection of this base by TFIID. Also, HMG box resulted in a protection of the bases at -42 and -43 also reversing the effect of TFIID at these bases (figure 1: compare lanes 9 and 12). However, the protection level of the bases representing the core TATA element remained similar to that induced by TFIID alone (figure 1: compare lanes 9 and 12). These observations indicated that TFIID and HMG box were co-occupying most of the probes under these conditions and that HMG box alters the nature of TFIID binding with the TATA region of MLP. Further support for the altered conformation came from the induction of hypersensitivity for the base -39 by co-occupation of the DNA by TFIID and HMG-box (figure 1: compare lanes 1, 4, 9 and 12). Thus, HMG box altered the nature of TFIID binding to the TATA element of the MLP.

To reactions containing identical concentrations of TFIID and HMG box, TFIIA was added to the level that was previously shown to be adequate for an activated transcription (figure 2). TFIIA did not interact with DNA by itself or change the DNase I cleavage pattern of HMG box (compare lanes 1-4 of figure 2 to lanes 1-4 of figure 1). Surprisingly, TFIIA had no visible effect on the interaction between TFIID and the TATA element in the absence of HMG box (compare lanes 5 and 9 of figure 2 to lanes 5 and 9 of figure 1) or in the presence of HMG box (compare lanes 8 and 12 of figure 2 to lanes 8 and 12 of figure

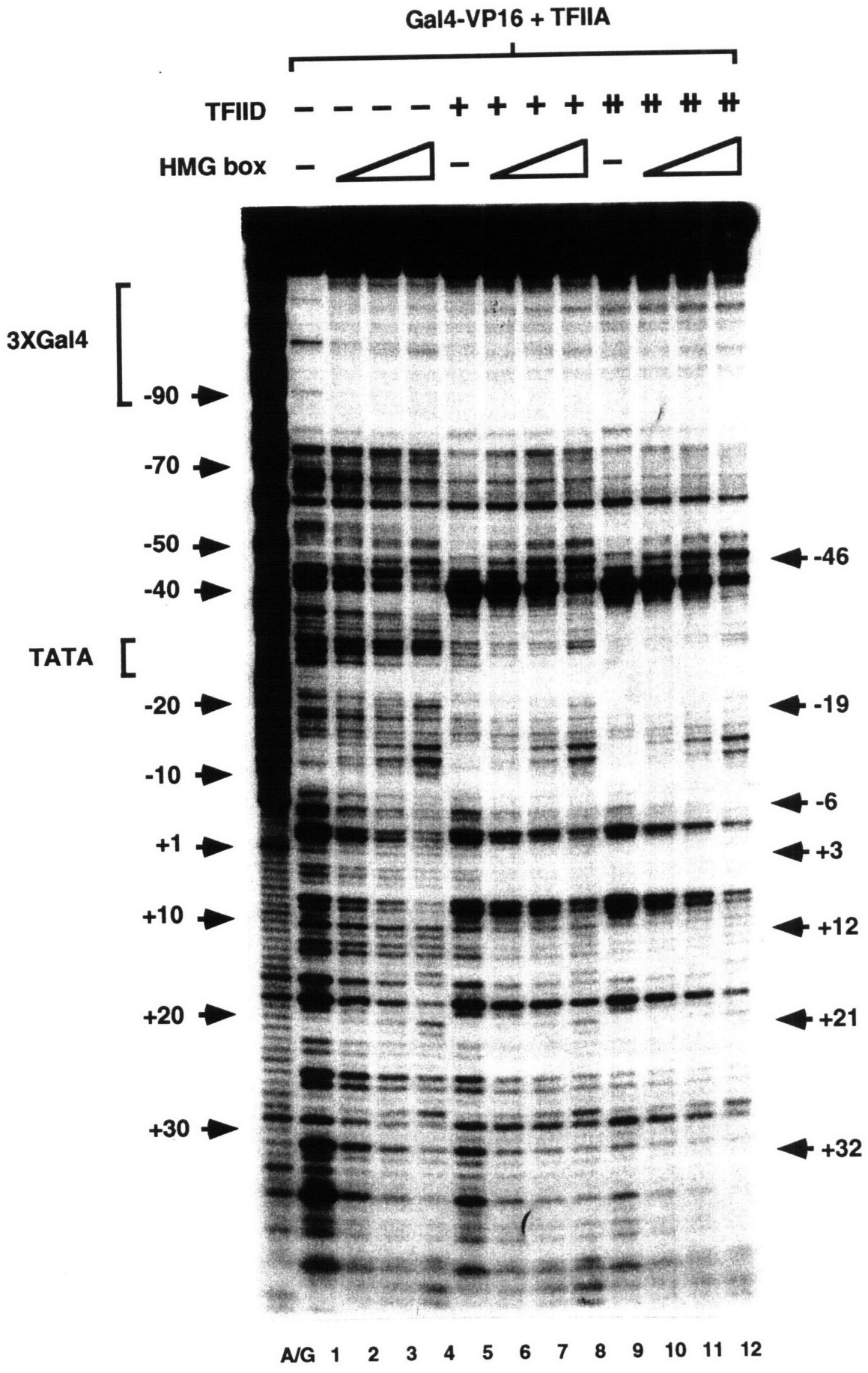
**Figure 2.** The interaction of TFIIA, TFIID, and HMG box was analyzed by DNase I footprinting analysis. HMG box was titrated (0, 90, 180, 360 ng) in the absence (lanes 1-4) or in presence (0.75 µl: lanes 5-8; 2 µl: lanes 9-12) of TFIID. All reactions contained 2.5 µl of purified TFIIA.


+TFIIA
TFIID
HMG box
3XGal4
-90
-70
-50
-40
TATA
-20
-10
+1
+10
+20
+30
-42
-32
-28
A/G 1 2 3 4 5 6 7 8 9 10 11 12

1). One possible explanation was that the TFIIA preparation was inactive. This possibility was ruled out based on the observation that the same preparation of TFIIA made expected alterations on the DNase I cleavage pattern in combination with TBP (data not shown). Another possibility was that the TFIID preparation used in these assays contained TFIIA activity. This was unlikely since phosphocellulose chromatography separates these two factors effectively and similarly produced preparations of TFIID could not mediate an activated transcription without addition of TFIIA. Thus, it is most likely that TFIIA makes no conformational change on the MLP detectable by DNase I cleavage in combination with TFIID.

Gal4-VP16 was added together with TFIIA to the identical set of reactions containing TFIID and HMG box (figure 3). As expected, Gal4-VP16 protected the three Gal4 dimer binding sites located upstream of the TATA element (figure 3: lane 1). When added to the reaction containing TFIID and TFIIA, Gal4-VP16 resulted in additional protections in the regions surrounding the TATA element (e. g. bases -46, -19, and -16) and in the downstream region past the initiation site (bases -6, +1, +3, +4, +10, +12, +13, +16, +22, +23, +25, +26, +31, +32, and +33: compare lane 9 of figure 3 and lane 9 of figure 2). The addition of HMG box resulted in its typical protection of bases -42 and -43 and elevated cleavage of bases -13 and -15 indicating that HMG box occupation of the regions around the TATA element was occurring under these conditions in a similar manner to the binding in the absence of Gal4-VP16 (compare lane 12 of figure 3 to lane 12 of figure 2). Likewise, in the downstream region, the HMG-box induced hypersensitive sites (e.g. bases +15, +20, and +28: compare lane 12 of figure 3 and lane 12 of figure 2) were still seen. Importantly, base -39, which became hypersensitive in the presence of TFIID and HMG box (and TFIIA), was protected by the addition of Gal4-VP16 (compare lane 12 of figure 3 and lane 12 of figure 2). Taken together, these data indicated that Gal4-VP16 induced a further rearrangement of the TFIID-TFIIA-HMG box-DNA complex, especially around the TATA region.

**Figure 3.** The interaction of Gal4-VP16, TFIIA, TFIID and HMG box was analyzed by DNase I footprinting assay. HMG box was titrated (0, 90, 180, 360 ng) in the absence (lanes 1-4) or in presence (0.75 μl: lanes 5-8; 2 μl: lanes 9-12) of TFIID. All reactions contained 2.5 μl of purified TFIIA and 10 ng of Gal4-VP16


Gal4-VP16 + TFIIA
TFIID
HMG box
3XGal4
-90
-70
-50
-40
TATA
-20
-10
+1
+10
+20
+30
-46
-19
-6
+3
+12
+21
+32
A/G 1 2 3 4 5 6 7 8 9 10 11 12

The series of DNase I cleavage pattern analyses presented here showed that conformational alterations of the DA complex occur as the consequence of interaction with the activator Gal4-VP16 and the coactivator HMG box. Future efforts should be directed to linking the specific conformational change with the activation of transcription. One line of investigation may take advantage of the promoter specificity of coactivation by HMG-1/2 proteins. Under conditions where activated transcription from MLP is mediated, HMG-1/2 proteins do not coactivate transcription from the immunoglobulin μ promoter (Shykind et al., 1995). Qualitative or quantitative differences in the conformation of the preinitiation complexes assembled on the μ and MLP may be detectable by DNase I cleavages. This may in turn lead to identifying specific candidate DNA elements responsible for the differential effect of HMG-1/2 proteins in the coactivation of transcription from these two promoters. Mutations or DNA element swappings would provide transcription templates and DNase I footprinting probes that could be used to link protein-DNA interactions to the activation of transcription. Another informative line of investigation may be the examination of the interaction of other basal factors with the DA complex. For example, it should be straight-forward to test if the stability of association of TFIIB with the DA complex or the conformation of TFIIB itself alters in the presence of activators and HMG-1/2 proteins. Finally, a direct visualization of the activated complex may be possible through electron microscopy. Using this technique, Moss and colleagues studied the structure of the protein-DNA complex formed by *xenopus* UBF, an HMG box containing protein, in association with a ribosomal promoter (Bazett-Jones et al., 1994). Their study showed that an in-phasing bending by UBF loops the DNA into a 180 base turn. Similar studies with HMG-1/2 proteins in the context of activated complex formation may reveal the exact configuration of the complex thereby elaborating on the architectural role of HMG-1/2 proteins in activated transcription.

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# Biographical Note

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