Influence of HMG-1 And Adenovirus Oncoprotein E1A on Early Stages of Transcriptional Preinitiation Complex Assembly

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Running title: Impact of transcriptional cofactors on PIC assembly
Summary

The TATA-binding protein (TBP) in the TFIID complex binds specifically to the TATA-box to initiate the step-wise assembly of the preinitiation complex (PIC) for RNA polymerase II transcription. Transcriptional activators and repressors compete with general transcription factors at each step to influence the course of the assembly. To investigate this process, the TBP/TATA complex was titrated with HMG-1 and the interaction monitored by electrophoretic mobility shift assays (EMSA). The titration produced a ternary HMG-1/TBP/TATA complex, which exhibits increased mobility relative to the TBP/TATA complex. The addition of increasing levels of TFIIB to this complex results in the formation of the TFIIB/TBP/TATA complex. However, in the reverse titration, with very high molar ratios of HMG-1 present, TFIIB is not dissociated off and a complex is formed that contains all factors. The simultaneous addition of E1A to a mixture of TBP, TATA; or HMG-1, TBP, TATA; or TFIIB, TBP, TATA inhibits complex formation. On the other hand, E1A added to the pre-established complexes shows a significantly reduced capability to disrupt the complex. In add-back experiments with all complexes, increased levels of TBP reestablished the complexes, indicating that the primary target for E1A in all complexes is TBP.
Introduction

The assembly of the transcriptional preinitiation complex (PIC)* on a promoter is the pivotal event in the regulation of gene expression (reviewed in 1-4). The successful completion of the step-wise formation of PIC is prerequisite to the initiation of RNA polymerase II transcription. The initial steps in assembly involve the binding of TFIID to the TATA element in the promoter, in which recognition and binding occurs through the sequence-specific subunit, the TATA-binding protein (TBP). This is followed by the binding of TFIIB to form the TFIIB/TBP/TATA complex, which represents the molecular platform for the subsequent complexation with RNA polymerase II/TFIIF and other general transcription factors essential for basal level transcription.

However, there are a multitude of regulatory proteins - activator and repressor proteins and cofactors - that may actively impinge on the assembly process, leading to either an enhancement or inhibition of the level of transcription (reviewed in 1, 3, 5-7). Two major targets for many regulatory proteins appear to be TBP (8, 9) and TFIIB (10, 11). Cellular and viral proteins which interact directly with TBP and TFIIB include the TAFs (12-14), TFIIA (15-17), c-myc (18), HMG-1 (19), p53 (20, 21), NC1 (22, 23), human Dr1-DRAP complex (identical to NC2) (24-26), c-rel (27), adenovirus E1A (17, 28-32) and VP16 (33, 34).

Repressors exhibit a number of mechanisms to effect their action (6, 35). HMG-1 and NC2 represent general repressors, in that they both interact with TBP to block PIC formation (19, 25, 26). The ubiquitous, abundant and highly conserved HMG-1 protein has been further implicated in the regulation of transcription, exhibiting both positive and negative effects on transcription (9, 19, 36-39). HMG-1 has been reported to bind to the TBP/TATA complex, which inhibits subsequent
TFIIB binding, resulting in incomplete PIC assembly and thereby inhibiting transcription. Interestingly, the addition of increasing levels of TFIIB in in vitro transcription assays was unable to restore activity (19). The multifunctional adenovirus E1A oncoprotein, which like HMG-1, exhibits no sequence-specific DNA binding activity (40-42), has been shown to serve as an activator of viral gene expression (5), while exhibiting inhibitor or activator activities with specific cellular promoters (43). The E1A product has been reported to effect its action in some cases by binding to TBP (28, 29, 31), while the 12S E1A product is reported to bind Dr1, thereby facilitating its dissociation from the TBP/TATA complex (26).

In this work, we use a sensitive gel shift assay and provide direct evidence that HMG-1 forms a stable EMSA complex with TBP/TATA. The complex exhibits an increased mobility relative to the TBP/TATA complex, contrary to EMSA findings with other reported DNA/protein complexes. The TFIIB/TBP/TATA complex is stable in the presence of low levels of HMG-1, but conditions in which there are high excesses of HMG-1 produce a complex that contains both HMG-1 and TFIIB. Furthermore, adenovirus E1A inhibits the formation of the TBP/TATA, HMG-1/TBP/TATA and TFIIB/TBP/TATA complexes, while the pre-established complexes resist dissociation by E1A. The addition of excess TBP reestablishes the complexes, indicating that TBP is the primary target for E1A action in all complexes.
Experimental Procedures

Isolation, purification and characterization of proteins

Calf thymus HMG-1 was purified in non-denaturing conditions using salt extraction, differential ammonium sulfate precipitation and fractionation by HPLC using a MonoQ column as outlined previously (44). The expression vector, pET-His6-hTBP, provided by F. Pugh, was transfected into E. coli BL21(DE3) cells and the protein purified by PEI fractionation, phosphocellulose chromatography and ammonium sulfate precipitation as described by Pugh (45). The expression vector, pHIIIB, obtained from D. Reinberg, was similarly used to obtain TFIIB, which was purified using phosphocellulose chromatography (46). The GST-E1A (13S; 289 amino acid residues) fusion protein was expressed in BL21 cells containing the pGEX-E1A (obtained from T. Shenk) and was purified by binding to glutathione agarose, elution by glutathione, followed by dialysis into reaction buffer (47). All proteins were greater than 80% pure as evidenced by Coomassie staining of gels run on SDS-PAGE.

EMSA studies

Oligonucleotides that make up the adenovirus major late promoter (Ad MLP; -40 to –1) were purchased from National Biosystems and ³²P-end-labeled. The DNA (ca. 0.4 ng) was reacted with human TBP (ca. 1 nM unless indicated otherwise), with and without other transcription factors, in binding buffer (24 mM Tris-acetate, pH 8.0, 10% glycerol, 4 mM magnesium acetate, 50 mM potassium glutamate, 0.1 mM EDTA, 1 mM DTT, 0.01% NP-40, 4 mM spermidine, 5 ug/mL poly (dG-dC) and 50 ug/mL BSA) for 30 mins at 30° C or 4° C, as indicated. The reaction
mixture was loaded on a 4% polyacrylamide gel in 0.35xTBE buffer containing 0.05% NP-40 and the electrophoresis was carried out at 4°C. The gels were then dried and exposed to X-ray film at –80°C. Oligonucleotides (Santa Cruz Biotech) used in competition studies for TBP binding with the Ad MLP DNA contained either the wild-type or mutated TATA motif. Binding studies were carried out by either simultaneous addition of all components, or alternatively, the final transcription factor was added 30 minutes after the initial complex was established and reaction was continued for an additional 30 minutes.

For supershift experiments, the reaction mixtures were incubated for 30 mins at 30°C and then the antibody (polyclonal α-HMG-1 from R. Roeder or affinity purified α-TFIIB from Santa Cruz Biotechnology) was added at 4°C for 10 mins. prior to loading the gel.

**Results**

HMG-1 was reported to bind to TBP both as a GST-TBP fusion protein and when TBP was complexed with the TATA element in the adenovirus major late promoter (Ad MLP)(19). To further characterize this interaction, the titration of the TBP/TATA complex with HMG-1 was followed by EMSA. Preliminary experiments showed that the glutamate buffer system consistently produced an EMSA-stable TBP/TATA complex and provided an essential “window” to determine if competing factors affected the TBP/TATA interaction. Figure 1A shows that the addition of HMG-1 produces a concentration-dependent decrease in the original band for the TBP/TATA complex, with the concomitant increase in a single band for an HMG-1/TBP/TATA complex. Interestingly, the new band exhibits an increased mobility relative to that for the TBP/TATA complex. Figure 1B shows that this
complex requires the presence of both TBP and HMG-1 and can be
titrated away with cold oligonucleotide containing the TATA sequence,
while it is unaffected by the addition of an oligonucleotide containing
the GCTA sequence in lieu of TATA. Figure 1C confirms the presence
of HMG-1 in the new complex, since the band is supershifted by anti-
HMG-1, while the anti-TFIIB control produced no effect. These data
indicate that the HMG-1 binding is dependent on both the TATA
sequence and TBP and the HMG-1/TBP/TATA complex exhibits an
anomalous increased mobility. The unusual mobility is not unique to this
electrophoretic buffer system, since the increased mobility of the
complex is also observed using other buffers (data not shown). Therefore,
this electrophoretic behavior is a characteristic of this
complex under a variety of conditions.

Insert Figure 1

Figure 2 compares the relative stability of the TBP/TATA and
HMG-1/TBP/TATA complexes at a number of TBP levels. At equal
levels of TBP, the band intensity for the HMG-1/TBP/TATA complex is
significantly greater than that for the corresponding TBP/TATA (and
also in relation to the free DNA band intensity) at all levels of TBP
examined. This indicates that HMG-1 binding enhances complex
formation, by at least 10-fold, leading to an increased population of the
HMG-1/TBP/TATA complex. In similar experiments in which
individual structural domains of HMG-1 (the A-box and B-box domains;
residues 1-89 and 86-165, respectively) were reacted with the
TBP/TATA complex, the mobility and the band intensity of the
TBP/TATA complex was unchanged, even at [HMG-1 box/TBP] mole
ratios as high as 2,000 (data not shown). The addition of the individual A- and B-boxes together also exhibited no changes (data not shown).

Insert Figure 2

The addition of TFIIB to the TBP/TATA complex forms the TFIIB/TBP/TATA complex, which, like the HMG-1/TBP/TATA complex, also exhibits a greater stability in gel shift experiments than does the TBP/TATA complex (48, unpublished data). However, unlike the HMG-1/TBP/TATA complex, this ternary complex exhibits a reduced mobility relative to the TBP/TATA complex.

It was of interest to determine if TFIIB and HMG-1 compete for overlapping sites on the TBP/TATA complex or whether these two factors bind simultaneously to TBP/TATA and form a stable complex. This electrophoretic buffer system provides a convenient means to resolve this question since the two complexes exhibit opposite mobilities in the EMSA system. Figure 3A shows that the addition of increasing amounts of TFIIB to the TBP, TATA and limiting HMG-1 (sufficient for complex formation) mixture results in the formation of the TFIIB/TBP/TATA complex. At a [TFIIB/HMG-1] mole ratio of 0.2 (lane 3), there is a 50/50 mixture of the two complexes, while only the TFIIB/TBP/TATA complex is detectable at or above a mole ratio 0.6 (lane 5). This indicates that TFIIB binds much more effectively than HMG-1 to the TBP/TATA complex.

In the reverse titration (lanes 8-14), limiting amounts of TFIIB were used to permit TFIIB/TBP/TATA complex formation, with the addition of increasing levels of HMG-1. The addition of HMG-1, up to mole ratios of less than 0.4 (lane 10) had no effect on the complex,
consistent with the data in lanes 1-7. However, as the levels of HMG-1 were increased enormously, with the [TFIIB/HMG-1] mole ratio in the range of 0.3-0.03 (lanes 12-14), a band of intermediate mobility was produced, indicating that a simple HMG-1/TBP/TATA complex was not formed.

Figure 3B shows that the band in lane 7 in 3A is not supershifted by anti-HMG-1 (lane 2), but is supershifted by the addition of anti-TFIIB (lane 3), indicating that the complex contains TFIIB/TBP/TATA. Figure 3C shows similar results that examined whether TFIIB remained in the complex after TFIIB/TBP/TATA was reacted with large excesses of HMG-1. Lane 2 shows that anti-TFIIB supershifts this complex (lane 1 is the same complex from lane 14 in 3A). Under these conditions of limiting TFIIB and huge excesses of HMG-1, TFIIB remains in this complex, with the resultant formation of a TFIIB/HMG-1/TBP/TATA complex.

The adenovirus E1A protein can either activate or repress transcription presumably by interacting with coactivators, the PIC or components at different stages in the assembling preinitiation complex. Figure 4 shows the effect of the GST-E1A fusion protein on disrupting the 4A) TBP/TATA; 4B) TFIIB/TBP/TATA; and the 4C) HMG-1/TBP/TATA complexes. This experiment was carried out by either a) the addition of GST-E1A after the complexes were established (lanes 1-6) or b) by simultaneous addition of all proteins (lanes 7-12). Figure 4 reveals that similar results were observed for all complexes examined. The presence of E1A was able to completely inhibit complex formation.
if E1A was added simultaneously with all components. On the other hand, E1A was unable to effect complete complex dissociation of the established complexes, but did partially disrupt them at the highest GST-E1A levels (ca. 80 ng). The control reaction in which GST was added alone exhibited no effect.

Insert Figure 4

To begin to understand the mechanism of E1A action and investigate the primary target of E1A, add-back experiments were carried out. In these experiments, sufficient E1A was added to just obviate the formation of the original three complexes, followed by addition of increasing amounts of TBP, HMG-1 or TFIIB to the respective complexes, in an attempt to compete with E1A and as a result, reestablish the complexes. Figures 5A, B and D show that the addition of an increasing levels of TBP to the mixture of E1A, DNA and A) TBP or B) TFIIB and TBP or C) HMG-1 and TBP reestablishes each of the three complexes at about the same level. In each case, it requires an 8-fold increase of TBP. Of importance is that the [TBP/E1A] mole ratio at the point of the reestablished complexes is about 0.3 in all cases. In the case in which increasing levels of TFIIB is added back to the TBP, TFIIB, E1A, oligonucleotide mixture (Figure 5C), a [TFIIB/E1A] mole ratio of greater than 60 (last lane) has very little effect. In the case of adding increasing levels of HMG-1 back to the corresponding HMG-1, TBP, E1A, oligonucleotide mixture (Figure 5E), a [HMG-1/E1A] mole ratio of greater than 50 (last lane) is shown to have no significant effect. These results indicate that the primary interaction of E1A is with TBP in all the complexes examined.
Insert Figure 5

Discussion

The action of regulatory proteins on the formation of the transcriptional preinitiation complex determines the rate at which transcriptional initiation will ensue, and indeed, the fate of the committed complex. The regulation of PIC assembly itself, by a dynamic balance in the binding of general factors and activator and repressor proteins, is an important element in transcriptional control. We have examined the early stages of PIC assembly, with the focus on the ternary complexes containing TBP/TATA and either HMG-1 or TFIIB and the effect of E1A on the course of the assembly process.

Reaction of TBP/TATA with increasing concentrations of HMG-1 produced a distinct EMSA complex, which is dependent on the presence of both TBP and a TATA-containing oligonucleotide, with the band being supershifted by anti-HMG-1. Identification of this complex as HMG-1/TBP/TATA complex is in accord with a previous report (19). HMG-1 occurs as a monomer in solution and although the stoichiometry of the complex is not known, HMG-1 is presumed to bind as a monomer. Interestingly, the mobility of the HMG-1/TBP/TATA complex is increased relative to that for TBP/TATA complex and differs from the mobility of the complex in the previous study (19). As far as we know, the increased mobility observed, as a result of another protein complexing with TBP/TATA, has not been observed previously for any other EMSA complexes. This unexpected mobility may be influenced by the electrophoretic buffer, but is fundamentally a result of either the large net negative charge on HMG-1 (-9), the ability of HMG-1 to alter the bend angle of DNA in the complex and/or shape of the complex (49), or contributions from both.
Previous studies have shown that the sequence-specific binding affinity of a number of regulatory factors, including steroid receptor proteins (50, 51), HOX9 protein (52), p53 (53) and the Oct-POU domains of Oct-1, 2 and 6 (54), was stimulated in the presence of HMG-1. In these cases, although the original EMSA band for the complex did increase in intensity, its position did not shift in the presence of HMG-1. The behavior of HMG-1 in these systems contrasts with our findings that indicate the formation of an EMSA-stable HMG-1/TBP/TATA complex.

The stability of the HMG-1/TBP/TATA complex is greater than that for the TBP/TATA complex as evidenced that at equivalent amounts of TBP, there is significantly more HMG-1/TBP/TATA complex than TBP/TATA complex. This enhancement of complexation is similar to that observed for both TFIIB and/or TFIIA bound to the TBP/TATA complex. In addition, we find no evidence that the A- and B-boxes of HMG-1 bind with the TBP/TATA complex (data not shown), suggesting that the stable HMG-1 binding may involve multiple-site interactions between HMG-1 and the TBP/TATA complex. The requirement for multiple-site interactions is quite common in stable protein-protein interactions and has been proposed, for example, in the binding of E7 protein to TBP and for E1A protein binding to the retinoblastoma protein (55, 56). Added support for this proposal comes from competition experiments in which HMG-1 is not competed off the HMG-1/TBP/TATA complex by high levels (600-fold excess) of A- and B-boxes, either individually or together (data not shown). These findings may also suggest that the C-terminal domain of HMG-1, which contains a highly acidic tail, may take part in the stable interaction with TBP. This possibility has been suggested previously (19).
It was reported that addition of increasing levels of TFIIB could not restore basal level transcription in an HMG-1-inhibited *in vitro* assay (19). However, as shown in Figure 3A (lanes 1-7), increasing levels of TFIIB, in the context of limiting HMG-1, does effectively prevent HMG-1 binding in the complex, resulting in the formation of TFIIB/TBP/TATA. Figure 3B shows that this band is supershifted by anti-TFIIB, but not by anti-HMG-1, indicating that the band corresponds to the TFIIB/TBP/TATA complex. The reverse titration, done in conditions of limiting TFIIB, showed that low levels of HMG-1 had no effect on TFIIB/TBP/TATA complex formation (in agreement with the previous titration). The addition of very high levels of HMG-1, however, produced a band, intermediate in mobility to the TFIIB/TBP/TATA and the HMG-1/TBP/TATA complexes. The presence of TFIIB in this complex was confirmed since the addition of anti-TFIIB produced a supershift of the band (Figure 3C). These data indicate that the band represents a complex that contains both TFIIB and HMG-1 and, under our conditions, this complex is observed only at high HMG-1 levels that were used in an attempt to compete off TFIIB. An EMSA complex assumed to be HMG-1/TFIIB/TBP/TATA was reported previously, but no evidence for the presence of TFIIB in the complex was presented (57). This finding indicates that there are conditions in which an intermediate complex can be formed which contains TBP/TATA, with the simultaneous and stable binding of both HMG-1 and TFIIB. It should be pointed out that the transcription factor, TFIIB, is conformationally pliable as indicated by both structural and biochemical studies. It has been shown that transcriptional activators, such as VP16 and Pho4 (58, 59), induce a conformational change in TFIIB and this
behavior is consistent with what is observed in conditions of very different levels of HMG-1 in our experiments.

The finding that TFIIB is very effective in competing limiting amounts of the transcriptional inhibitor, HMG-1, from the HMG-1/TBP/TATA complex is consistent with both factors competing for the same or overlapping binding sites on the TBP/TATA complex. The crystal structure for the TFIIB/TBP/TATA complex shows that TFIIB binds to residue 289 at the junction of S2’-S3’ in the second stirrup of TBP (26), suggesting this region as a potential target. In addition, TFIIB interaction also involves binding to DNA, both upstream and downstream of the TATA element (58, 59, 60), which HMG-1 binding may also include. Another aspect of this, that may not be mutually exclusive, is that HMG-1 binding to TBP may lead to an alteration of the bend angle of the DNA.

The presence of the E1A protein is devastating to the assembly of all complexes investigated. It does not just inhibit HMG-1 or TFIIB from associating with TBP on the TATA element, it completely inhibits the formation of the TBP/TATA, HMG-1/TBP/TATA and the TFIIB/TBP/TATA complexes (when all factors are added simultaneously). Since E1A is known to bind to TBP (17, 28-32), this is consistent with E1A/TBP complexation in solution, resulting in the inhibition of TBP binding to the TATA sequence. This may also suggest that TBP is a highly specific target for E1A, with binding to the other factors being less significant in this context. Consistent with this proposal, approximately the same level of E1A was effective in inhibiting formation of all three complexes. On the other hand, if these three complexes are established prior to E1A addition, E1A exhibits only a weakly disruptive effect. Although there is some reduction in the
level of complex, a significant amount of complex remains even at the highest levels of E1A. These data indicate that E1A is significantly less effective in dissociating or disrupting the preformed complex than it is in inhibiting the assembly of the factors prior to complexation.

HMG-1 shares some common features with the general transcriptional repressor, Dr1 (24-26). HMG-1 and Dr1 repressor proteins bind directly to TBP, both in solution and in the TBP/TATA complex, leading to a reduced transcriptional activity due to inhibition of PIC formation. However, although Dr1 inhibition cannot be overcome by increasing the concentrations of TFIIA, RNA pol II or the other general transcription factors (25), inhibition of transcription by HMG-1 can be reversed by increased levels of TFIIA, but not TFIIB (19). Future studies must be done to reveal further the extent of similarity of these two repressors.

To gain some initial insight into the mechanism of action and the primary targets for E1A in these complexes, add-back experiments were performed. The addition of increased levels of TBP to a solution with sufficient E1A to inhibit TBP/TATA formation (Figure 5A) led to the reestablishment of the TBP/TATA complex. The complex was completely reestablished at about an 8-fold increase in TBP. This was consistent with previous findings that TBP is a target for E1A (24, 25) and that the addition of excess TBP in these experiments overcomes TBP/E1A complexation and permits productive TBP/TATA complex formation. This effect of TBP is a strong one in that it occurs at low mole ratio (0.3) of [TBP/E1A]. TBP is likewise able to reestablish the TFIIB/TBP/TATA and the HMG-1/TBP/TATA complexes (Figure 5B and D) at approximately this same mole ratio of [TBP/E1A]. On the other hand, the addition of comparable mole ratios of TFIIB/E1A was
ineffective in reestablishing the TFIIB/TBP/TATA complex (Figure 5C). In fact, a [TFIIB/E1A] mole ratio of greater than 60 produced no significant TFIIB/TBP/TATA complex formation. The same was true for attempts to reestablish the HMG-1/TBP/TATA complex (Figure 5E), in which a [HMG-1/E1A] mole ratio of greater than 50 had only a small effect. These collective findings indicate that E1A is specific for targeting TBP, with neither TFIIB nor HMG-1 being targets for E1A binding. In comparison with similar competition studies in which the 12S E1A product was used with Dr1, TBP and a TATA-containing DNA, enormous excesses (ug quantities) of E1A were required to inhibit assembly of the Dr1/TBP/TATA complex (26). Nonetheless, our results indicate that E1A has the capability of preventing the initial stages of PIC formation and therefore may lead to inhibition of transcription.

Acknowledgments

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References
The abbreviations used are: PIC, preinitiation complex; TBP, TATA-binding protein; TAFs, TATA-binding protein associated factors; HMG-1, high mobility protein-1; EMSA, electrophoretic mobility shift assay; HPLC, high pressure liquid chromatography; PEI, polyethyleneimine; his-tagged hTBP, amino-terminally hexahistidine-tagged human TBP; GST-E1A, glutathione S-transferase-E1A fusion protein; Ad MLP, adenovirus major late promoter; S2’-S3’, segment containing beta-sheets 2’-3’.
Figure Captions

1. HMG-1 interaction with the TBP/TATA complex.
   A. HMG-1 forms a stable ternary complex with TBP bound to the Ad MLP. Increasing amounts of HMG-1 were added to the TBP/TATA complex. HMG-1 concentrations in lanes 1-6 are 0, 0.6, 1.7, 5.0, 15 and 45 nM.
   B. Sequence specificity of TBP/TATA and HMG-1/TBP/TATA complexes. Unlabeled oligonucleotides were used to compete in complex formation with $^{32}$P-labeled Ad MLP. All lanes contain mixtures of TBP and $^{32}$P-labeled Ad MLP, with lanes 4-6 also containing ca. 40 nM HMG-1. No competitor oligonucleotide was added in lanes 1 and 4. A 25-fold molar excess of unlabeled oligonucleotide with a TATA sequence (lanes 2 and 5) or with a GCTA sequence in lieu of TATA (lanes 3 and 6) was added in competition. The TATA-containing oligonucleotide is GCAGAGCATATAAAATGAGGTTAGGA.
   C. HMG-1/TBP/TATA complex is supershifted by $\alpha$-HMG-1. The HMG-1/TBP/TATA complex (lane 1) was treated with two levels of $\alpha$-HMG-1 (lanes 2 & 3) or with $\alpha$-TFIIB control (lane 4). The (*) indicates the band position for the TBP/TATA complex, while the arrow points to the supershifted band.

2. HMG-1 binding to the TBP/TATA complex enhances complex formation. Increasing levels of TBP were incubated without HMG-1 (lanes 1, 3, 5, 7) or with 40 nM HMG-1 (lanes 2, 4, 6, 8). TBP levels (nM) were 0 (lanes 1, 2); 0.15 (lanes 3, 4); 0.31 (lanes 5, 6) and 0.62 (lanes 7, 8).
3A. Competitive binding of TFIIB and HMG-1 for the TBP/TATA complex. Increasing levels of TFIIB (lanes 2-7) were added to the TBP, TATA and limiting amounts of HMG-1, or increasing levels of HMG-1 (lanes 9-14) were added to TBP, TATA and limiting amounts of TFIIB. All mixtures contained 1.2 nM TBP. Lanes 1-7 contained 45 nM HMG-1 and increasing levels of TFIIB were 0, 3, 9, 27, 80, 160 and 480 nM, respectively. Lanes 8-14 contained 82 nM TFIIB and 0, 10, 31, 91, 274, 820 and 2500 nM HMG-1.

B. TFIIB/TBP/TATA complex is formed at high TFIIB and limiting HMG-1. Lane 1 contains the complex from lane 7 in 3A and is treated with α-HMG-1 (lane 2) or α-TFIIB (lane 3). Lane 4 contains the TFIIB/TBP/TATA complex (same as lane 8 in 3A), treated with α-HMG-1 as a control. The (*) indicates the position of the TBP/TATA complex and the arrow points to the supershifted band.

C. TFIIB/HMG-1/TBP/TATA complex is formed at high HMG-1 levels and limiting TFIIB. Lane 1 is the complex in lane 14 in 3A. Lane 2 is the complex treated with α-TFIIB. The (*) indicates the position of TFIIB/TBP/TATA complex and the arrow points to the supershifted band.

4. The effect of 13S E1A protein on the dissociation or formation of the a) TBP/TATA; b) TFIIB/TBP/TATA; and c) HMG-1/TBP/TATA complexes. GST-E1A was added to mixtures containing Ad MLP oligonucleotide and (A) TBP; (B) TBP and TFIIB (82 nM); (C) TBP and HMG-1 (34 nM). GST-E1A was added subsequent to complex formation (lanes 2-5) or simultaneously with all proteins (lanes 8-11). TBP was at 0.5 nM in all lanes and GST-E1A concentrations
were 7 nM, 21 nM, 42 nM and 84 nM. GST controls (lanes 6 and 12) were at 80 nM.

5. **Add-back experiments: The effect of excess transcription factor on reversing E1A inhibition of complex formation.** The Ad MLP oligonucleotide was incubated with 1.2 nM TBP in (A); TBP and TFIIB (B and C); or TBP and HMG-1 (D and E) and sufficient GST-E1A (30 nM with A, B, C and D; 50 nM in E) to just inhibit complex formation. Increasing amounts of TBP (A, B and D), or TFIIB (C), or HMG-1 (E) were subsequently added in an attempt to reestablish the complex. Lane 1 in each case is the complex formed in the absence of E1A. Lane 2 includes sufficient E1A to inhibit complex formation. **A, B and D.** Lanes 2-5 contain 1.2, 2.5, 5.0 and 10 nM TBP. **C.** Lanes 1-6 contain 118, 118, 235, 472, 941 and 1880 nM of TFIIB, respectively. **E.** Lanes 1-7 contain 80, 80, 160, 320, 640, 1280 and 2560 nM HMG-1, respectively.
Figures

Figure 1A.
Figure 1B.
Figure 1C.
Figure 2
The following two figures should be aligned side-by-side to each other, using the identifying labels to the left for the figures and lanes 1-14.

**Figure 3A.**
3B.
Figure 3C
Figure 4A.
Figure 4B
Figure 4C.
Figure 5 A-E.
Influence of HMG-1 and Adenovirus Oncoprotein E1A on Early Stages of Transcriptional Preinitiation Complex Assembly
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