

TFIID (TBP) stabilizes the binding of MyoD to its DNA site at the promoter and MyoD facilitates the association of TFIIB with the preinitiation complex

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ABSTRACT

The myogenic determination factor MyoD activates the transcription of muscle-specific genes by binding to consensus DNA sites found in the regulatory sequences of these genes. The interaction of MyoD with the basal transcription machinery is not known. Several activators induce transcription by recruiting TFIID and/or TFIIB to the promoter. We asked whether MyoD interacted functionally with TFIID and TFIIB in transcription. We reconstituted *in vitro* DNA binding and transcription systems of MyoD and basal transcription factors, and found that MyoD function in transcription occurred during the assembly of the preinitiation complex. Interestingly, MyoD activated transcription without affecting the binding of TFIID to the promoter. However, TFIID or TBP dramatically stabilized the binding of MyoD to its recognition site. MyoD and TBP interacted in solution. Deletion analysis of MyoD suggested that interaction of MyoD with TBP is needed for its activity in transcription. At a later stage of assembly, MyoD stabilized the binding of TFIIB to the preinitiation complex. These findings suggest that MyoD is involved in two steps of preinitiation; first, TFIID stabilizes MyoD binding to its DNA recognition site and at a later stage MyoD facilitates the association of TFIIB with the preinitiation complex.

INTRODUCTION

Two distinct groups of protein factors are involved in regulated transcription by RNA polymerase II. The first group consists of the general basal transcription factors that are necessary for positioning the polymerase at the initiation site. Polymerase positioning involves binding of TFIID to the TATA element, which serves as a core for the sequential binding of basal transcription factors IIA, IIB, Pol II/IIF, IIE and IIH (1). The resulting complex, known as the preinitiation complex (PIC), can initiate transcription *in vitro* in the presence of nucleoside triphosphates. Stimulation of transcriptional activity requires a second class of factors known as promoter-specific activator

proteins (activators). The activators are sequence-specific DNA binding proteins and their binding in the vicinity of the promoter modulates the binding and the activity of the basal transcription factors in a way that is only partly understood. Multiple protein-protein interactions have been suggested to explain the activation process. Several activators were found to interact with TFIID either directly through TBP (TATA binding protein) or through the TAFs (TBP associated factors) (2–9). These interactions are usually thought to facilitate and stabilize TFIID interactions with DNA (4,8–13). TFIIB is also a target for activators. The association of TFIIB with the preinitiation complex is rate-limiting (14). The GAL4–VP16 chimeric activator interacts directly with TFIIB through its acidic activation domain and stabilizes its association with the PIC (14–16). TFIIB has been demonstrated to interact functionally with a growing number of activators such as a HeLa cell factor, LSF, p65 of NFκB, p53, the *Drosophila fushi tarazu*, CTF1 and several members of the nuclear hormone receptor superfamily (17–31).

The myogenic regulator MyoD is a transcriptional activator that belongs to the basic helix-loop-helix family (bHLH) (32–34). The basic region constitutes the DNA binding motif of these proteins, whereas the helix-loop-helix (HLH) region is a dimerization motif that allows interaction with other HLH proteins (35). Like other members of myogenic bHLH proteins (Myf5, Mrf-4 and myogenin), MyoD can form homodimers, but it prefers to heterodimerize with bHLH proteins of class A (E2A, E2-2 and HTF4) (35). Dimers of MyoD bind to specific DNA sequences known as the E box (CANNTG) of muscle-specific genes and activate their transcription (36). Another functional element of MyoD is an acidic activation domain located within a sequence of 53 amino acids at the N-terminus (37).

How does MyoD activate transcription? To investigate the manner in which MyoD affects the basal transcription machinery, we chose to study its activity in *in vitro* systems of DNA binding, protein association and transcription (38). We report here that MyoD affects transcription during the assembly of the basal transcription factors to form the PIC. MyoD is involved in transcription during two stages. In the first stage, it interacts with TFIID or TBP. This physical interaction results in the stabilization of MyoD binding to its own DNA binding site without affecting the binding of TFIID or TBP. A study with deletion mutants of

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MyoD suggests that the interaction with TBP is required for MyoD function in transcription. In the second stage, MyoD recruits TFIIB to the preinitiation complex.

MATERIALS AND METHODS

Plasmids

MyoD binding sites (MBS) were inserted into the pML-52/260 plasmid as described (38). The plasmid used in the transcription studies carried six MBS upstream to the major late promoter (MLP) of adenovirus type 2 (from -52 to +10 relative to transcription start site) and a G-less sequence of 260 nt (pML-52/260-6× MBS). In some transcription reactions a control template was used, pML(C₂AT)Δ50. This plasmid contains the same Ad2 MLP sequences, and a G-less sequence of 390 nt. The plasmid p110MCK-CAT (39) was used to generate enhancer-promoter fragment for the protein binding assays.

Antibodies

Polyclonal antibodies to TBP were purchased from Santa Cruz Biotechnology Inc. Polyclonal antibodies to MyoD were produced by injecting rabbits with full-length bacterial MyoD protein (38). Antibodies to MyoD were purified from serum over Affi-gel column (Bio-Rad) to which the full length MyoD was covalently bound.

Immobilized DNA templates

Immobilized DNA templates were prepared as described (40). For transcription studies, pML-52/260-6× MBS was cut with *EcoRI* that is located 3' to the G-less sequence and filled in with Biotin 14-dATP by DNA-polymerase (Klenow fragment). A second digest with *PvuII* followed, and a 600 bp fragment was gel-purified. The fragment, (~25 μg) which includes the promoter and G-less sequence, was then coupled to 1 ml of streptavidin-agarose beads (BRL) (1:1) and incubated 3 h at 4°C. The beads were washed five times to remove unbound DNA fragments and stored in TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) at 4°C for 2 months. In the transcription factors binding assay, p110-MCK-CAT was digested with *HindIII* and *BstEII* to recover a fragment of 200 bp that includes the MCK enhancer and promoter sequences. The fragment was filled in with Biotin 14-dATP and DNA polymerase (Klenow fragment) prior to the *BstEII* cut. About 10 μg of the fragment were mixed with streptavidin magnetic beads (Dynabeads) as described above.

Transcription extracts and partial purification of basal transcription factors

HeLa nuclear extracts (NE) and HeLa whole cell extracts (WCE) were prepared as described (41,42). Proteins of MyoD (38), TFIIB (43), TBP (44) and TFIIE (45) were purified from recombinant *Escherichia coli* cells. The His-TBP protein was a gift from Dr Yossef Shaul, the Weizmann Institute of Science, Rehovot. Other basal factors were purified from HeLa nuclear extracts as follows: phosphocellulose and DEAE-52 column chromatography steps used to generate the TFIID and TFIIA fractions were as described (46). TFIIF and TFIIH were purified as described previously (47) with the following changes: purification was performed up to the DEAE-Sephacel step as described, but the following DEAE 5PW and Mono S purification

steps were replaced by a Superdex 200 HR 16/60 (Pharmacia). The fractions that contained TFIIF/TFIIH activities were pooled and further fractionated on a phenyl-Superose column (HR5/5, Pharmacia) as described previously (47). TFIIF was eluted from this column with 0.7–0.5 M ammonium sulfate whereas TFIIH was eluted with 0.2–0.1 M ammonium sulfate. Salt was removed by dialysis against buffer C (20 mM Tris-HCl pH 7.9, 20% v/v glycerol, 0.2 mM EDTA, 10 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF). RNA polymerase II was purified as previously described (48).

In vitro transcription assays

Soluble transcription assays were described before (38). The assay of transcription on an immobilized template was performed as follows: transcription factors were incubated with 5–10 μl of DNA beads (50–100 ng of DNA) in a total volume of 35 μl transcription buffer (25 mM Tris-HCl pH 7.9, 50 mM KCl, 5 mM MgCl₂, 5% glycerol, 2 mM DTT) and 4 mM creatine phosphate, 2% polyethylene glycol (PEG 6000). Following incubation, beads were washed five times with 200 μl of transcription buffer. After washing, the beads were incubated with nucleoside triphosphate mixture {0.5 mM ATP, 0.5 mM CTP, 15 μM UTP and 10 μCi per reaction of [α-³²P]UTP (800 Ci/mmol)} and RNase T1 (Boehringer Mannheim) at 20 U per reaction to initiate transcription. Alternatively, the beads were incubated again with transcription factors as indicated in each experiment. Transcription was carried out at 30°C for 60 min. Transcripts were treated as previously described (38) and separated on sequencing gels.

Binding of transcription factors to the MCK fragment

Transcription factors were added to a reaction mixture that contained 5 μl of immobilized MCK fragment (~90 ng) and 1 μg of d(G-C)d(G-C) in buffer containing 20 mM HEPES pH 7.9, 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 4 mM MgCl₂ and 0.5 mg/ml BSA. After incubation at 30°C for 60 min with occasional stirring, the beads were washed four times in washing buffer (20 mM HEPES pH 7.9, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.1% Triton X-100). The beads were suspended in SDS sample buffer. Proteins were separated over 12.5% SDS-PAGE and analyzed by western blotting.

Western blotting

For analyzing basal transcription factors and MyoD, proteins were transferred from gels to reinforced cellulose nitrate membrane (Schleicher & Schuell). The membrane was blocked in PSB containing 2% milk powder and 0.1% Tween-20. Polyclonal antibodies to TBP or MyoD were added for 1 h at room temperature in a dilution of 1:500. The second antibody was horseradish peroxidase (HRP)-conjugated protein A (Sigma). Proteins were detected by enhanced chemiluminescence reactions (ECL, Pierce).

DNase footprinting analysis

Transcription factors were incubated in 4 mM MgCl₂, 5 mM (NH₄)₂SO₄, 1% PEG, 0.05% NP-40, 0.5 mg/ml BSA, 2 mM DTT, 10 mM HEPES pH 7.9, 60 mM KCl, 0.1 mM EDTA, 7.5% glycerol. The following recombinant purified factors were added: His-TBP at ~25 ng/μl and MyoD at 200 ng/μl. After 15 min incubation on ice, ³²P-end-labeled MCK fragment (*HindIII*-*BstEII*,

labelled at the *Hind*III (10 000 c.p.m. ~0.5 ng) was added together with 1 μ l of 1 mg/ml poly d(G-C)d(G-C) in a total volume of 10 μ l. DNA and proteins were incubated at 30°C for 60 min. Mixtures were treated with DNase (2 mg/ml, Wharthington) for 1 min. The DNase concentration was calibrated for each experiment separately. Samples of DNase solution (10 μ l) were added to a buffer containing 5 mM CaCl₂, 10 mM Tris 7.5, 10 mM MgCl₂. The activity of DNase was terminated by the addition of 430 μ l stop solution (0.5% SDS, 10 mM EDTA, 10 mM Tris pH 7.9 and proteinase K at 10 μ g/ml and 20 μ g tRNA). After phenol/chloroform extraction and ethanol precipitation, DNA fragments were separated over 8% denaturing polyacrylamide gels.

Interactions of MyoD with TBP

MyoD proteins (up to 100 ng) were incubated for 60 min with His-TBP (300 ng) in 50 μ l of a reaction mixture that contained 10 mM HEPES pH 7.9, 60 mM KCl, 0.1 mM EDTA, 7.5% glycerol, 0.5 mg/ml BSA, 5 mM imidazole, 4 mM MgCl₂, 5 mM (NH₄)₂SO₄, 10% PEG, 0.05% NP-40. To this solution 5 μ l of washed Ni⁺-beads (Qiagen) were added. Mixtures were rotated at 4°C for 60 min. Beads were washed three times with 0.5 ml 10 mM Tris pH 7.9, 5 mM MgCl₂, 0.4% NP-40, 0.1% Tween 20, 7.5% glycerol, 20 mM imidazole and twice with the same buffer but without imidazole and NaCl. Beads were suspended in 40 μ l of sample buffer and proteins were separated over 12.5% SDS-PAGE. Western blotting using anti-MyoD and anti-TBP was performed as described above.

RESULTS

MyoD functions during the assembly of preinitiation complexes

To study the relationship between MyoD and the basal transcription machinery, we utilized an *in vitro* transcription system in which MyoD activates transcription from templates that carry high-affinity MyoD binding sites (MBS) from the muscle creatine kinase (MCK) enhancer in proximity to minimal promoter sequences (38). For the sake of simplicity, only the experiments performed with homodimers of MyoD are described here. However, it should be mentioned that many of the experiments were repeated with heterodimers of MyoD and E47. Results with heterodimers were qualitatively similar to the results with homodimers of MyoD (data not shown) (38).

At first we analyzed at what stage of transcription MyoD is involved. In order to differentiate between the assembly of preinitiation complexes and the subsequent processes of initiation and elongation of transcription, we purified preinitiation complexes on DNA templates that were immobilized on agarose beads. The immobilized DNA template that contained six MBS in the promoter was incubated with HeLa whole cell extract in the presence or absence of MyoD protein. Factors that were stably associated with the DNA template were separated from unbound or unstable factors by washing the beads in transcription buffer (see Materials and Methods). When MyoD was not present with the basal transcription factors either weak or no transcription was evident (Fig. 1, lanes 3 and 5). The small differences in transcription were probably due to variations in the washing procedure. As seen in Figure 1, MyoD stimulated transcription significantly when present with the free basal transcription factors during their binding to the DNA template (lane 4). In contrast,

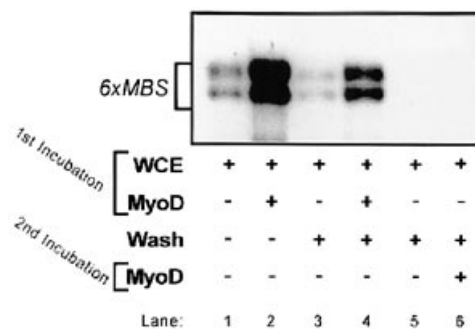


Figure 1. MyoD affects transcription during the assembly of the preinitiation complexes. Immobilized promoter template that contained 6 \times MBS (50 ng/reaction) was incubated with whole cell extract (WCE) (50 μ g/reaction) with and without 400 ng MyoD for 1 h at room temperature. Following incubation, templates were washed with transcription buffer (see Materials and Methods). MyoD was added either to the first or the second incubation. Following the second 15 min incubation, nucleoside triphosphate mixture was added for 1 h at 30°C. RNA transcripts were extracted and analyzed over a denaturing polyacrylamide gel (see Materials and Methods).

MyoD did not affect transcription if it was added at stages that followed the assembly of transcription complexes (Fig. 1, lane 6). The results presented in Figure 1 suggest that MyoD enhances the binding of basal transcription factors to the DNA template during the preinitiation stage, but does not exclude the possibility that MyoD may also affect their activity in transcription after the assembly.

MyoD can activate transcription after the binding of TFIID to the TATA element

It is well documented that TBP (or TFIID) binding is a major rate-limiting stage in the assembly of the complex because of its slow association with the TATA element (9,25,49). Several activators induce transcription by enhancing the rate limiting stage of TFIID binding (4,9–13). To test whether MyoD affect the slow rate of assembly of preinitiation complexes, the kinetics of their formation was measured in the absence or presence of MyoD. Transcription factors were mixed with template DNA for different periods of time before transcription was initiated. After initiation, transcription was allowed to continue for a short period of 5 min. We assume that in this case levels of transcription reflect the levels of fully assembled competent complexes. The data shown in Figure 2A (lanes 1–6, upper panel and graph) suggest that a slow phase of assembly (lag) is followed by a faster phase. Addition of MyoD did not change the kinetics significantly (Fig. 2A, lanes 1–6, lower panel and graph), suggesting that MyoD did not change the overall rate of PICs assembly. However, the faster phase was significantly stimulated by MyoD (Fig. 2A, lanes 1–6, lower panel and graph). To find out if the lag period was due to the slow interaction of TFIID with DNA, templates were first pre-incubated for 1 h with TFIID. This length of time was sufficient to allow maximal binding of TFIID to the TATA element (not shown). After this time the remaining basal transcription factors were added with or without MyoD for different assembly periods (Fig. 2B). The lag period observed in the previous experiment disappeared (Fig. 2B, upper panel) and the second faster phase was significantly stimulated by MyoD (Fig. 2B, lower panel). These results suggest that MyoD does not influence the slow assembly of TFIID, but affects later stages of assembly.

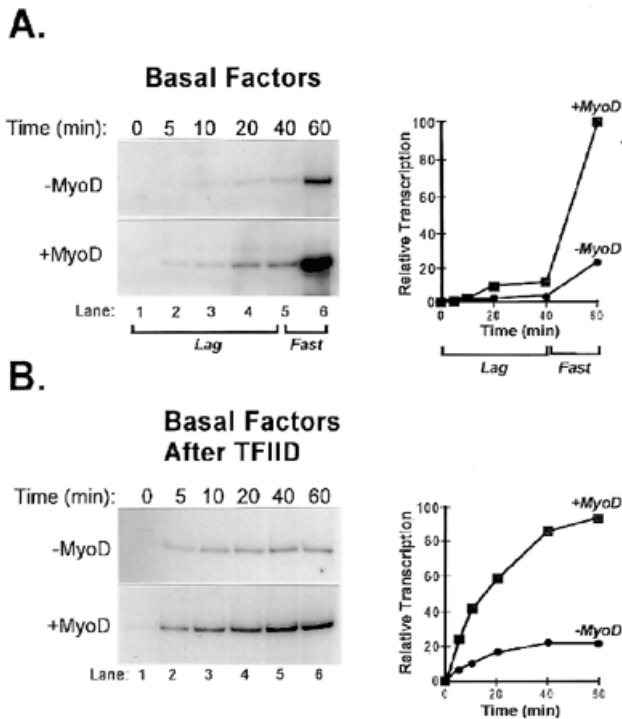


Figure 2. MyoD does not affect the rate limiting stage of assembly of preinitiation complexes. (A) Soluble transcription assay: basal transcription factors with or without MyoD were incubated for different time intervals with a soluble template that contained 6x MBS, as indicated, before ribonucleoside triphosphate mixture was added to initiate transcription. Transcription proceeded for 5 min. Transcripts were separated over denaturing gel. The intensity of bands was quantified using a phosphor imager and plotted against time. The circle plot represents transcription by basal transcription factors only. The bar plot represents transcription by basal transcription factors in the presence of MyoD. (B) TFIID (DEAE-52, 6 µg) was preincubated with the same template for 1 h. The other basal transcription factors with or without MyoD were added and incubated for different periods as indicated. Transcription proceeded for 5 min. The intensity of bands was quantified using a phosphor imager and plotted against time. The filled circle plot represents transcription by basal transcription factors only. The bar plot represents transcription by basal transcription factors in the presence of MyoD.

Transcription on the immobilized template allows us to distinguish between stages of assembly of the PIC. Therefore, we used this approach to find out whether MyoD could activate transcription after the binding of TFIID to the template. It was shown by Lin and Green (14) that PICs assembled on immobilized template DNA were not stable. Washing the complexes in the absence of activator released all the basal factors except TFIID that stayed bound to the template (14). We used the same methodology to assemble complexes on immobilized templates (6x MBS) that were incubated with WCE but without MyoD (Fig. 3). The templates were then washed with transcription buffer, and partially purified basal factors with or without MyoD were added before transcription was initiated. No transcription occurred after the wash if basal factors were not added to the second incubation mixture (Fig. 3, lanes 1 and 2). Therefore, some basal factors were not stable and dissociated from the complex. We could identify low levels of basal transcription only if all basal transcription factors with the exception of TFIID and TFIIB were added during the second incubation (Fig. 3, lane 7). Significantly higher levels of transcription occurred if all basal

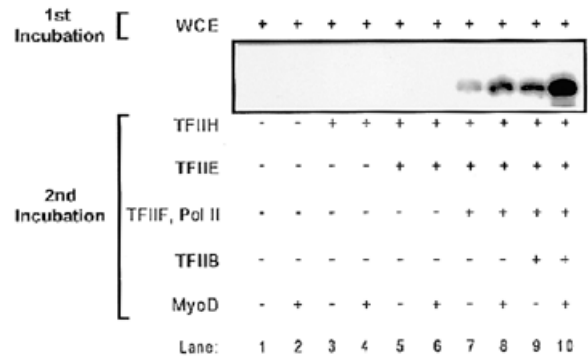


Figure 3. MyoD can activate transcription without recruiting TFIID to the promoter template. Immobilized template (6x MBS) was incubated with WCE (50 µg/reaction) for 1 h. Following incubation, templates were washed in transcription buffer (see Materials and Methods) and partially purified basal factors were added for 20 min incubation with or without MyoD as indicated. Transcription proceeded for 1 h at 30°C. RNA transcripts were extracted and analyzed over a denaturing polyacrylamide gel (see Materials and Methods).

transcription factors, with the exception of TFIID, were added in the second incubation (Fig. 3, lane 9). Therefore, TFIID, and to a limited extent also TFIIB, were the only factors that stayed bound to the DNA template after the wash. MyoD induced higher levels of transcription only if added to the second incubation mixture with most basal factors, with the exception of TFIIB and TFIID or TFIID alone (Fig. 3, lanes 7–8 and 9–10). We conclude that MyoD can induce transcription after the binding of TFIID and/or TFIIB to the DNA template. MyoD may affect the activity of TFIID, TFIIB and/or the activity of subsequent basal transcription factors.

TFIID or TBP stabilize the binding of MyoD to its DNA recognition site at the promoter

Although our study suggests that MyoD does not affect the recruitment of TFIID to the DNA template, the possibility exists that TFIID may affect MyoD. The complex of MyoD with its DNA recognition site (E box) is very labile, as measured by its dissociation rate (38,50). On the other hand, the TFIID/TBP complex with its DNA recognition site (TATA element) is stable (25,51–53). If MyoD interacts with TFIID/TBP, one may expect that the binding of MyoD to its DNA recognition site would be affected by TFIID/TBP. Therefore, we determined the binding of MyoD to DNA while TFIID/TBP was bound to an adjacent TATA element. For that purpose, a DNA fragment that contained the MCK enhancer with binding sites for MyoD and the MCK promoter was immobilized on magnetic beads (see Materials and Methods). Constant amounts of MyoD were added to the immobilized template with different amounts of TFIID or TBP (Fig. 4). The amounts of MyoD and TBP bound to DNA were detected by immunoblotting of the proteins that remained bound to the beads after extensive washes (see Materials and Methods). The amounts of MyoD bound to the DNA fragment were directly proportional to the amounts of either TFIID or TBP that were bound (Fig. 4, lanes 2–4 and 5–7). TFIID or TBP did not augment MyoD binding if the DNA fragment did not contain the TATA element but contained MyoD binding site (data not shown). Therefore, we conclude that the binding of MyoD to its site was augmented by TFIID or TBP. In addition, we noticed that TFIID

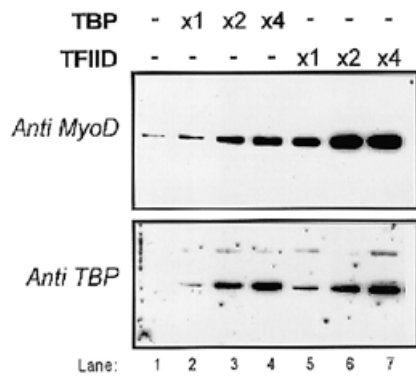


Figure 4. TFIID or TBP that are bound to the TATA element augment the binding of MyoD to its DNA recognition site. A constant amount of MyoD (100 ng) and different amounts of bacterial TBP or partially purified TFIID were incubated with immobilized MCK enhancer-promoter fragment as described in Materials and Methods. After incubation for 60 min at 30°C, the beads were washed extensively in buffer as detailed in Materials and Methods. Each reaction sample was divided into two equal parts that were analyzed over two separate 12.5% SDS-PAGE. Proteins were analyzed by western blotting with anti-TBP antibodies, and with anti-MyoD antibodies. Lanes 2–4: 50, 100 and 200 ng of purified TBP were added to the template DNA, respectively. Lanes 5–7: 1.5, 3 and 6 μ g of partially purified TFIID were added to the template DNA. MyoD was kept at a constant amount of 100 ng.

was more efficient than TBP in recruiting MyoD to its binding site (Fig. 4, compare lanes 2–4 to 5–7). More MyoD was bound to the DNA template with equivalent amounts of TBP as part of TFIID complex than with isolated TBP (Fig. 4). Other basal transcription factors like TFIIA, TFIIB, TFIIF, TFIIE and TFIIH did not play any role in the binding of MyoD to the DNA (data not shown). Therefore, enhancement in MyoD binding to DNA was induced specifically by TBP or TFIID. To find out if MyoD and TBP did bind their cognate DNA binding sites on this template, we further analyzed their binding in a DNase I footprinting assay (Fig. 5A). TBP and MyoD protected specific regions of this DNA fragment from DNase I digestion. TBP protected the TATA element, and MyoD protected mainly the high affinity binding site of MCK enhancer (Fig. 5A, lanes 2–4). To further test the possibility that TBP stabilized DNA binding of MyoD, we used the DNase I footprinting analysis to determine the dissociation rates of MyoD from the MCK high affinity binding site. By adding to the binding reaction excess amounts of unlabeled oligonucleotide that contained the same high affinity MyoD binding site, we could measure the rate of MyoD release from its DNA binding site on the labeled probe. MyoD that was bound to the high affinity site of MCK in the absence of TBP dissociated from its binding site in less than a minute after addition of the unlabeled competitor oligonucleotide (Fig. 5B, lanes 2 and 3). However, simultaneous binding of TBP and MyoD to their corresponding binding sites dramatically increased the stability of MyoD, so much so that MyoD stayed bound to its site on the DNA probe even 40 min after addition of the competitor oligonucleotide (Fig. 5B, lanes 4–8). Similar results were obtained when MyoD–E47 heterodimer complexes bound to the MyoD site rather than homodimers of MyoD (data not shown). This experiment confirms that binding of TBP to its site at the promoter stabilizes the binding of MyoD to its own site.

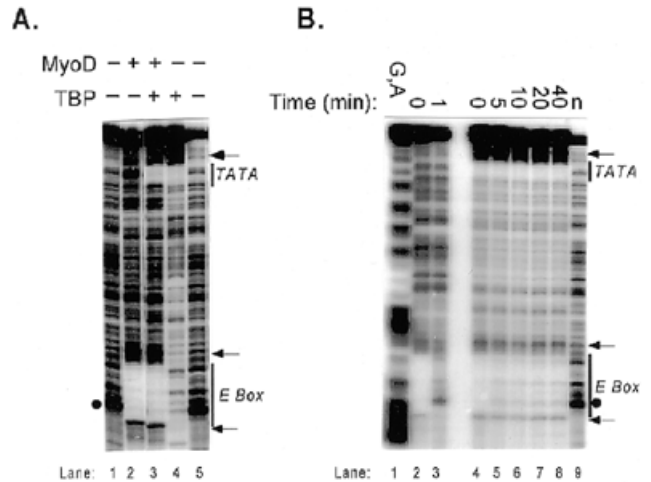


Figure 5. TBP stabilizes the binding of MyoD to the high affinity binding site of MyoD at the MCK enhancer. (A) DNase footprinting analysis of MyoD and TBP binding to MCK fragment. Purified MyoD (400 ng) and TBP (200 ng) were incubated separately or together with an end-labeled MCK enhancer-promoter fragment (see Materials and Methods). Following 1 h of incubation, reaction mixtures were treated briefly with DNase I and DNA fragments were extracted and separated over a sequencing gel. (B) Dissociation rate of MyoD from its high affinity binding site in the absence or presence of TBP. Binding of MyoD to the same MCK end-labeled fragment occurred as described in (A) in the absence or presence of TBP. Following incubation for 1 h, a non-radioactive high affinity binding site of MyoD was added to each reaction at a 50-fold molar excess over the radioactive probe. Samples were removed at different times, as indicated, before or after the addition of the competitor, and treated briefly with DNase I. DNA fragments were extracted and analyzed over a sequencing gel. Protected regions from DNase I digestion are indicated by lines, and the hypersensitive sites-by arrows. The first lane represents the same end-labeled fragment treated with dimethyl sulfate (DMS) to analyze the G,A nucleotide sequence.

MyoD interacts with TBP in solution

To find out if protein–protein interactions between MyoD and TBP are involved in the stabilization of MyoD at the promoter, we analyzed the interactions of several deletion mutants of MyoD with TBP in solution. Recombinant TBP protein that contained several histidine residues in its N-terminal domain (His-TBP) was incubated with several recombinant MyoD proteins and protein complexes were isolated on nickel-NTA resin. Similar amounts of three distinct MyoD proteins were incubated with the His-TBP protein as seen in Figure 6A. The MyoD proteins included the wild-type protein (wt MyoD), a mutant that lacked N-terminal residues 3–56 (Δ 3–56) and a mutant that lacked the N-terminal residues and terminated at amino acid 167 (Δ 3–56tm167) (Fig. 6A). Both the wild-type protein and Δ 3–56 mutant bound efficiently to TBP (Fig. 6A, lanes 4 and 5). The second mutant of MyoD, Δ 3–56tm167, did not interact with TBP (Fig. 6A, lane 6). This mutant contains an intact bHLH domain and therefore is capable of binding to the DNA recognition site (not shown). We analyzed the activity of the three proteins in the reconstituted transcription system. Interestingly, the wild-type protein, as well as Δ 3–56, activated transcription efficiently while the other mutant of MyoD, Δ 3–56tm167, failed to activate transcription (Fig. 6B). The correlation between the binding of MyoD protein to TBP and its ability to activate transcription suggests that MyoD–TBP interactions play a role in the activation of transcription. Also of

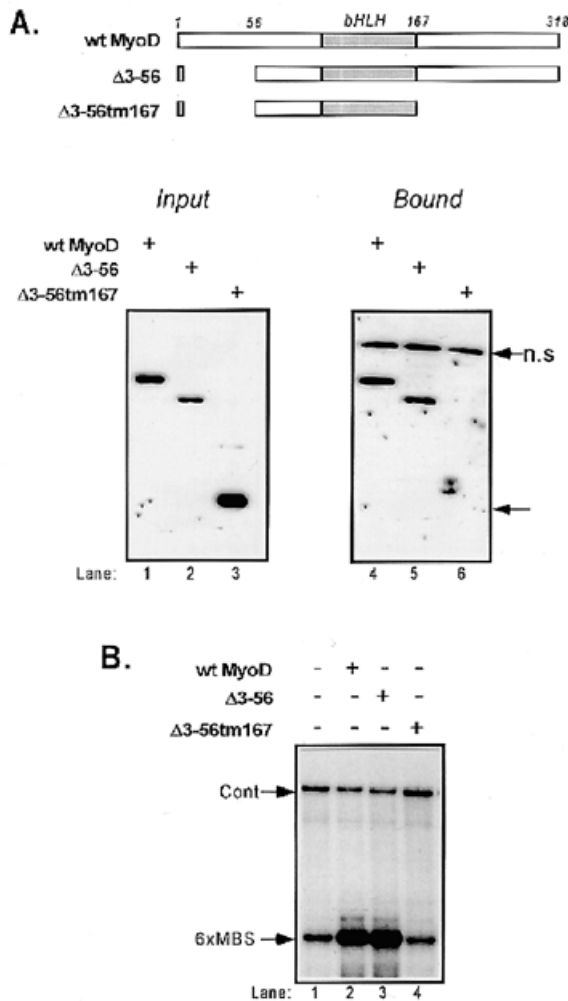


Figure 6. MyoD interacts with TBP in solution. (A) Protein-protein interaction assay. Equal amounts of recombinant MyoD proteins purified from bacteria were incubated with His-TBP protein as described in Materials and Methods. The input amount of MyoD proteins was analyzed by western blotting with anti-MyoD and is presented in the left panel. Protein complexes that were bound to a nickel-NTA resin were then analyzed by western blotting with anti-MyoD and are presented in the right panel. (B) Reconstituted transcription assay. Transcription was reconstituted with basal transcription factors and the same MyoD proteins as used in (A). Two DNA templates were used in the assay; a control template that contained the Ad2 major late promoter that produced a transcript of 390 nt and the test template that contained 6x MBS upstream to the Ad2 MLP and produced a transcript of 260 nt (see Materials and Methods). Abbreviations: n.s., non specific; $\Delta 3-56$, a mutant of MyoD protein that does not contain amino acids 3-56; $\Delta 3-56tm167$, a mutant of MyoD protein that does not contain amino acids 3-56 and terminates at amino acid 167; Cont., control template.

interest is the finding that the $\Delta 3-56$ MyoD mutant which lacks the known activation domain can activate transcription in the reconstituted system to full extent (see Discussion).

MyoD stabilizes the binding of TFIIB to the preinitiation complex

The stabilization of MyoD binding by the core factor of the preinitiation complex, TFIID/TBP, suggests that the effect of MyoD on the preinitiation complex occurs in later stages. Several

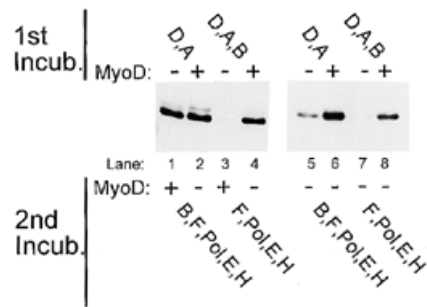


Figure 7. MyoD recruits TFIIB to the preinitiation complex. Transcription reaction with immobilized templates. Partially purified transcription factors were added to immobilized templates as indicated, for a 1 h incubation at room temperature. Following the first incubation, templates were washed several times in transcription buffer as indicated in Materials and Methods. Basal transcription factors that were missing in the first incubation were added for another 30 min at room temperature. Transcription proceeded for 30 min at room temperature. RNA transcripts were extracted and analyzed over a denaturing gel. Basal factors added to the transcription reaction: 5 μ g TFIID, 6 μ g TFIIA, 200 ng rTFIIB, 250 ng rTFIIE, 100 ng TFIIF, 800 ng Pol II and 400 ng TFIIF.

acidic activators were demonstrated to affect the binding and activity of TFIIB (26,54). To test the effect of MyoD on TFIIB, transcription was reconstituted with basal factors on immobilized templates.

A subset of basal transcription factors were added with or without MyoD to immobilized DNA template (6x MBS). Following incubation, the immobilized templates were washed and the factors absent in the first stage were added for further incubation. Thus, MyoD and all the basal factors were added to each transcription reaction (Fig. 7, lanes 1-4). We assume that if all the factors in the first incubation stably associate with the template, transcription should occur upon addition of the missing factors. However, if any of the factors of the first incubation do not stably associate with the template, it will be washed out; it will therefore be lacking from the final reaction mixture and transcription will not occur. In such a case, we ask if the presence of MyoD can stabilize the binding of loosely bound basal factors. Transcription was similarly activated whether MyoD was added to the promoter concurrently or after the binding of TFIID and TFIIA (Fig. 7, compare lanes 1 and 2 with lane 5). These results are in agreement with the results presented in Figures 2 and 3 that suggest that MyoD functions after the binding of TFIID. When the first reaction mixture contained TFIID, IIA and IIB, the subsequent washing procedure precluded all transcription (lane 3). In contrast, when the first reaction mixture contained only TFIID and IIA, transcription occurred after the washing procedure (lanes 1 and 5). Therefore, in the absence of MyoD, TFIIB was the most likely factor to be washed out from the transcription reaction. When the first reaction mixture contained MyoD in addition to TFIID, IIA and IIB, transcription occurred (lanes 4 and 8). Therefore, we concluded that MyoD recruited and/or stabilized TFIIB to the template. Our conclusions were confirmed by analyzing the protein levels of TFIID and TFIIB that were bound to the immobilized templates. We found that the levels of TFIID bound to the template were not affected by MyoD, while TFIIB binding to the template was significantly enhanced by MyoD (data not shown). In these activities, MyoD resembles the acidic activator VP16 described earlier by Lin and Green (14).

DISCUSSION

In the present work the relationship of the myogenic transcription factor MyoD with basal transcription factors was studied for the first time. Our studies suggest that MyoD interacts functionally and physically with TFIID/TBP in a way that stabilizes the binding of MyoD to its recognition site and promotes its effect at later stages of assembly of PIC.

Interactions of activators with TFIID were reported in many studies (2–9). In several of these, it was suggested that the activator augments and stabilizes the binding of TFIID to the TATA element (4,8–13). As the binding of TFIID to the promoter is a major rate-limiting stage in transcription, its recruitment to the promoter is believed to be an important stage in the activation of transcription (55). We have generated several lines of evidence suggesting that MyoD activates transcription after binding of TFIID to the promoter. Firstly, we observed that the assembly of PIC was a slow process mainly due to the slow rate-limiting binding of TFIID (Fig. 2B). MyoD did not affect the slow stage, but stimulated significantly the subsequent faster stage (Fig. 2). Secondly, MyoD activated transcription from a promoter template that contained a pre-bound TFIID factor (Figs 3 and 7). Thirdly, MyoD did not affect the amount of promoter-bound TFIID when added together to DNA template (not shown). Studies of Lin and Green (14) and White and colleagues (56) suggested that GAL–VP16 activated the steps of PIC assembly occurring after the binding of TFIID. Therefore, although recruitment of TFIID to the promoter may be an essential stage for some activators, others like MyoD, affect transcription at other rate-limiting stages.

An interesting finding was that TFIID or TBP were able to stabilize the binding of MyoD to its DNA site (Figs 4 and 5). We as well as others recognized earlier that MyoD binding either as a homodimer or heterodimer with E12/E47 proteins was not stable (38,50). Dimers of MyoD dissociate rapidly from the DNA binding site. Unlike MyoD, TFIID or TBP bind stably to the TATA element. Two approaches were taken to analyze binding of transcription factors to a DNA fragment that contained MCK regulatory sequences. In one experiment the fragment was immobilized on magnetic beads and the binding of MyoD was measured in the presence of different amounts of TFIID or TBP. The amounts of MyoD bound to the DNA fragment were directly proportional to the amounts of either TFIID or TBP that were bound (Fig. 4). TFIID was more efficient than TBP in recruiting MyoD to its binding site (Fig. 4). This difference suggests that TBP associating factors (TAFs) that are part of the TFIID complex may contribute to the stable binding of MyoD. Indeed, interactions of activators with TAFs play an important role in mediating transcriptional activation (2). A DNase I footprinting assay was used to further analyze the binding (Fig. 5A). The simultaneous binding of TBP and MyoD to their corresponding binding sites dramatically affected the stability of MyoD; in the absence of TBP, the half-life of MyoD–DNA complex was extremely short, while in its presence the half-life of the complex was remarkably longer (Fig. 5B). The continuous presence of the activator at its binding site is necessary to activate reinitiation of transcription (57). TFIID is the only basal factor known to remain bound to the template during elongation of transcription (58). Therefore, it is reasonable for TFIID to anchor the activator to its DNA site, especially if the activator is as unstable as MyoD. In this respect, it should be mentioned that TBP not only stabilized homodimers of MyoD but also heterodimers of MyoD and E47 (not shown). We conclude from this experiment and others

that homodimers of MyoD and heterodimers of MyoD and E47 displayed similar functions.

The concept that a basal transcription factor recruits the activator to the promoter was recently suggested for two activators. In one study it was shown that TFIID or TBP stabilized the binding of p53 to its binding site (59). Chen and colleagues suggested that this mechanism might compensate for the limiting amounts of p53 in cells. MyoD, another scarce protein that is moreover unstably bound to DNA, may also require this mechanism to ensure its function in transcription. In another study, interferon regulatory factors (IRFs) that regulate the transcription of interferon gene were demonstrated to function cooperatively with TFIIB (30). Interactions of TFIIB with IRF-1 and IRF-2 facilitated the binding of these activators to their DNA recognition site.

The functional interaction of MyoD with TBP may occur as a result of the physical interaction between the proteins. We have demonstrated that MyoD physically interacts with TBP in solution (Fig. 6A). Deletion mutants of MyoD that did not contain N- and/or C-terminal residues suggested that the N-terminal residues were not needed for interaction with TBP and for the transcriptional activity of MyoD. These results were surprising in view of our previous knowledge that the N-terminal residues of MyoD were defined as the activation domain (37). The activation domain was defined in transfected cells using GAL–MyoD chimera proteins. Other domains of MyoD did not function as classical activation domains when fused to DNA binding domain of GAL4. However, it is possible that these domains may function only in the natural context of the MyoD protein. Indeed, it was suggested that the bHLH region of the protein affect the activity of other domains of the protein (38). Also, in transfected cells, MyoD protein that did not contain the activation domain was still active in transcription although less potent than the wild-type protein (35,60). In the *in vitro* transcription system, the same mutant (Δ 3–56) was as potent as the full length protein. This raises the possibility that MyoD contains additional domains at its C-terminus that contribute to the interaction with TBP/TFIID. A recent study has shown that other domains of MyoD in addition to the N-terminal domain contribute to MyoD transcriptional activity in cells (60). Gerber and colleagues demonstrated that regions within the C-terminal region of MyoD were needed for MyoD to activate the transcription of endogenous muscle-specific genes (60). However, we should also consider the possibility that the *in vitro* transcription system represents an artificial activity that does not occur in living cells.

Although of potential importance, the interactions of MyoD with TFIID/TBP do not explain how MyoD may affect transcription. Most of the known activators affect two subcomplexes: TFIID and a complex comprising TFIIB, PolII and the rest of the basal factors (29). We studied the second stage of assembly of PICs, the binding of TFIIB. We suggest that MyoD stabilizes the association of TFIIB with the TFIID–TFIIA complex. The effect of MyoD on TFIIB was observed in a reconstituted transcription reaction (Fig. 7) and at the protein level using a western technique to identify TFIIB in PICs (not shown). The results of both assays led us to conclude that MyoD stabilized the association of TFIIB with the complex.

We suggest that MyoD belongs to the family of activators that affect the second rate-limiting stage, i.e., the recruitment of TFIIB and not to those activators that affect the first rate-limiting stage of TFIID binding. Nevertheless, MyoD does interact with TFIID and this interaction is significant for its function because it stabilizes significantly the binding of MyoD to its DNA site.

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