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Positive control mutations in the MyoD basic region fail to show cooperative DNA binding and transcriptional activation *in vitro*

EYAL BENGAL*, OSVALDO FLORES*, PUNDI N. RANGARAJAN*, AMY CHEN†, HAROLD WEINTRAUB†, AND INDER M. VERMA*

*The Salk Institute, P.O. Box 85800, San Diego, CA 92186-5800; and †Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104

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ABSTRACT **An *in vitro* transcription system from HeLa cells has been established in which MyoD and E47 proteins activate transcription both as homodimers and heterodimers. However, heterodimers activate transcription more efficiently than homodimers, and function synergistically from multiple binding sites. Positive control mutants in the basic region of MyoD that have previously been shown to be defective in initiating the myogenic program, can bind DNA but have lost their ability to function as transcriptional activators *in vitro*. Additionally, positive control mutants, unlike wild-type MyoD, fail to bind cooperatively to DNA. We propose that binding of MyoD complexes to high affinity MyoD binding sites induces conformational changes that facilitate cooperative binding to multiple sites and promote transcriptional activation.**

The MyoD family of myogenic regulators is necessary and often sufficient for muscle differentiation in mammals (1). Members of this family are transcription factors that share a high conservation across a stretch of 60 amino acids known as the basic helix–loop–helix (bHLH) domain (2–5). The basic region constitutes the DNA-binding motif of these proteins, whereas the helix–loop–helix (HLH) region is a dimerization motif that allows multimerization with other HLH proteins (6–10). Members of the MyoD family do not efficiently homodimerize but readily form heterodimers with ubiquitous HLH proteins which include E47 and E12, the products of the E2A gene (6–8). The basic region is necessary for recognition of the consensus MyoD binding site. Additionally, it is required for muscle-specific transcriptional activation and for the conversion of fibroblasts to muscle (9, 11, 12). Domain swap experiments, in which the basic regions of MyoD, myogenin, and Myf5 were replaced with those of non-muscle-specific bHLH factors like E12 and the achaetescute of *Drosophila*, revealed that the resulting chimeric proteins (positive control mutations) retained DNA binding activity but lost their ability to activate transcription of muscle-specific genes (9, 13, 14). Back mutations of only two residues of chimeric protein corresponding to amino acids Ala-114 and Thr-115 in the basic region of MyoD restores myogenic potential (11). These two residues are conserved in all myogenic regulatory proteins and, together with one lysine residue from helix I, can confer myogenic specificity to E12 (11, 15).

To decipher the role of the basic region, we undertook an analysis of the transcriptional activity of MyoD in a defined biochemical system. We report here that (*i*) bacterially expressed and purified MyoD protein specifically activates transcription from promoters carrying its cognate DNA binding sites, and this activation occurs both in crude extracts (HeLa nuclear extracts) and in a partially purified reconstituted system; (*ii*) *in vitro* transcription by homodimeric complexes is significantly less efficient than that by the heterodimeric complex of MyoD and E47 proteins; (*iii*) several positive control MyoD proteins with mutations in the basic region were able to bind to the DNA recognition site as homodimers and heterodimers with E47 but failed to activate transcription in both oligomeric forms; and (*iv*) MyoD basic mutants that bind well to a single site and fail to activate transcription show decreased cooperativity in DNA binding because they have faster dissociation rates upon binding to multiple DNA-binding sites as heterodimers with E47.

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MATERIALS AND METHODS

Construction of Plasmids. MyoD binding sites (MBS) were inserted into the pML-52/260 plasmid. This plasmid carries the adenovirus type 2 (Ad2) major late promoter (MLP) sequences (positions −52 to +10). It also contains a cassette of 260 nt that lacks deoxyguanylate residues ("G-less"). The double-stranded (ds) oligonucleotides of the MBS that were used for cloning are as follows, where boldface letters signify MBS: ds 33-mer containing two wild-type (wt) MBS, 5′-GAT-CCA-GCA-GGT-GTT-GGG-AGG-CAG-CAG-GTG-GAG-3′ and antisense 3′-GT-CGT-CCA-CAA-CCC-TCC-GTC-GTC-CAC-CTC-CTAG-5′; ds 21-mer containing one wt MBS, 5′-GAT-CCA-GCA-GGT-GTG-AAT-TCG-3′ and antisense 3′-GT-CGT-CCA-CAC-TTA-AGC-CTAG-5′; and ds 33-mer containing two mutated MBS, 5′-GAT-CCA-GAC-GGG-TTT-GGG-AGG-CAG-ACG-GGT-GAG-3′ and antisense 3′-GT-CTG-CCC-AAA-CCC-TCC-GTC-TGC-CCA-CTC-CTAG-5′. The ds oligonucleotides were cloned into a *Bgl* II site positioned immediately upstream of the MLP. Each clone was sequenced to verify orientations and the number of inserts. The clones that were used for further studies carried one MBS (one insert of the ds 21-mer), two MBS (one insert of the ds 33-mer), six MBS (three direct repeats of the ds 33-mer), and 4 mut MBS (two direct repeats of the ds 33-mer). The plasmid that was used as a control in each reaction was pML(C_2AT)Δ50. This plasmid contains essentially the same Ad2 MLP sequence as that in the test template (positions −50 to +10), and a G-less sequence of 390 nt.

MyoD was cloned under a phase T7 promoter of the pRK171a vector by creating a *Nde* I site in the initiator methionine by *in vitro* mutagenesis. The basic-region MyoD mutants were generated as described (9). Construction of the expression vector for "E47N," an N-terminally truncated version of the HLH protein E47 (see Fig. 2), has been described (16). All of the constructs of bacterially expressing proteins were transformed into BL21(DE3)pLysS *Escherichia coli*. The MyoD proteins were purified by standard

Abbreviations: MyoD, myogenic differentiation; HLH, helix–loop–helix; bHLH, basic HLH; MBS, MyoD binding site(s); Ad2, adenovirus type 2; MLP, major late promoter of Ad2; ds, double-stranded; wt, wild type; E47N, N-terminally truncated E47; TFIIA–TFIIH, transcription factors IIA–IIH; EMSA, electrophoretic mobility-shift assay; rTFIIB and rTFIIE, recombinant TFIIB and TFIIE.

procedures, and E47N protein was expressed and purified as described (16).

Transcription Extracts and Partial Purification of Basal Transcription Factors. HeLa nuclear extracts were prepared as described (17). Transcription factors IIB and IIE (TFIIB and TFIIE) were purified from recombinant *E. coli* cells as described (18, 19). TFIIA, TFIIB, TFIID, TFIIF, and TFIIH were purified from 500 ml of HeLa nuclear extracts (2.0×10^{11} cells). The phosphocellulose and DEAE-52 chromatographic steps used to generate the TFIIA and TFIID fractions were as described (20). The TFIIB factor was fractionated through two chromatographic steps of phosphocellulose and DEAE-Sephacel as described (20). TFIIF and TFIIH were purified as described (21) up to the DEAE-Sephacel step. The DEAE 5PW and Mono S steps were replaced by a gel filtration step (Superdex 200 HR 16/60, Pharmacia). The Superdex 200 column fractions that contained TFIIF/TFIIH activities in an *in vitro* transcription assay were pooled and further fractionated on a phenyl-Superose column (HR5/5, Pharmacia) as described (21). TFIIF was eluted between 0.7 and 0.5 M ammonium sulfate and TFIIH was eluted between 0.2 and 0.1 M ammonium sulfate. Fractions containing TFIIF and TFIIH were separately pooled, dialyzed against buffer C (20) containing 10 mM KCl, and used to reconstitute *in vitro* transcription reactions. RNA polymerase II was purified essentially as described (22).

Transcription reaction mixtures (40 μl) for crude nuclear extracts contained 10 mM Tris (pH 7.9), 7% (vol/vol) glycerol, 50 mM KCl, 0.1 mM EDTA, 6 mM $MgCl_2$, 1% PEG, and 5 mM creatine phosphate. Fifty to seventy micrograms of nuclear extracts was added together with the designated amounts in each figure of bacterially expressed proteins. Heterodimers were allowed to form at 37°C for 10 min, and then two DNA templates (control and test) were added to each reaction at 50–250 fmol each. Reaction mixtures were preincubated for 45–60 min at room temperature before nucleotides were added to initiate the reactions. The tubes were transferred to 30°C, and 5 min later the NTPs were added to each (0.5 mM ATP, 0.5 mM CTP, and 15 μM UTP plus 1 μl per reaction (10 μCi) of [α-^{32}P]UTP (800 Ci/mmol; 1 Ci = 37 GBq). RNase T1 was added at 20 units per reaction (Boehringer Mannheim). Reactions were carried out at 30°C for 60 min and terminated by the addition of 7 mM Tris (pH 7.9), 3 mM EDTA, 0.2% SDS, proteinase K at 660 μg/ml, and 30 μg of tRNA. After 10 min at 37°C, reaction mixtures were extracted with phenol/chloroform, 1:1 (vol/vol), and transcripts were precipitated with ethanol.

Transcription reaction mixtures (40 μl) for the fractionated system contained 12 mM Hepes (pH 7.9), 6 mM $MgCl_2$, 2% PEG, 50 mM KCl, 7% glycerol, 5 mM creatine phosphate, 2 μl of TFIIA (DEAE-cellulose fraction), 2 μl of TFIID (DEAE-cellulose), 1 μl of recombinant TFIIB (rTFIIB), 2 μl of recombinant TFIIE (rTFIIE), 2 μl of TFIIF (mono Q), 2 μl of TFIIH (mono Q), and 1 μl of polymerase II (mono Q). In some cases less purified fractions were used: 2 μl TFIID (DEAE-cellulose), 2 μl TFIIE, TFIIF, and TFIIH (DEAE-Sephacel), and 1 μl of TFIIB. Bacterially expressed proteins were added as described in each experiment. Heterodimers of MyoD and E47 were allowed to form at 37°C for 10 min. DNA templates were then added at 50–100 fmol. Activation was optimal between 10 and 20 pmol for wt MyoD, between 2–4 pmol for E47N, and between 1 and 2 pmol of MyoD·E47N heterodimers. The reactions were preincubated at room temperature for 40 min, and transcription was initiated and terminated as described for the crude extracts.

Electrophoretic Mobility-Shift Assay (EMSA). Probes were labeled with polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol). The probe was separated from unincorporated [γ-^{32}P]ATP on a Sephadex G-50 spin column. The typical EMSA mixture (20 μl) contained 12.5 mM Tris (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$ 7.5% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 μg of poly(dI-dC)·poly(dI-dC), different concentrations of the bacterial proteins, and 10–20 fmol of ^{32}P-labeled probe. In most cases, 1 μl of whole-cell extract or nuclear extract (10 mg/ml) was added to the reaction. When heterodimeric complexes were formed, the proteins were added before the probe and left to incubate at 37°C for 10 min. The binding reaction took place at 30°C for 20 min. The reaction mixture was then applied to a 4% polyacrylamide gel in 0.25× TBE (1× TBE = 90 mM Tris/64.6 mM borate/2.5 mM EDTA, pH 8.3) and electrophoresed at 20 mA at 4°C.

In the dissociation-rate experiments, the same binding conditions were applied, but the reactions were scaled-up according to the number of time points that were taken. After 30 min of binding at 30°C, 100 ng of nonradioactive competitor oligonucleotide at each time point was added (200–500 excess over probe DNA). Samples (20 μl) were then taken at different times after the addition of nonradioactive competitor and loaded immediately onto a gel that was running at 4°C.

RESULTS

MyoD and E47 Homo- and Heterodimers Activate Transcription *in Vitro*. To study the transcriptional activation properties of MyoD, several reporter templates were generated that contained high affinity MBS from the muscle-creatine kinase enhancer and the core sequences of the Ad2 MLP (positions −50 to +10) linked to a G-less cassette of 260 nt (Fig. 1*A*). A template containing the minimal Ad2 MLP linked to a G-less cassette of 390 nt was used as an internal control (Fig. 1*A*). Transcription reactions were reconstituted either with HeLa nuclear extract (Fig. 1*B*; ref. 17) or with partially purified basal factors (Fig. 1*C*).

The full-length MyoD protein was expressed and purified from bacteria to about 90% homogeneity. The DNA binding activity of the protein was analyzed by EMSA with the same oligonucleotides that were used to construct the test promoters. When transcription reactions containing HeLa nuclear extracts were supplemented with increasing amounts of MyoD, transcription was induced from the test promoter but not from the control promoter (Fig. 1*B*, lanes 1–3). MyoD protein was not able to activate transcription from a test promoter that carried mutated MBS (Fig. 1*B*, lanes 4–6).

We next reconstituted transcription reaction mixtures with partially purified components. RNA polymerase II and the general transcription factors TFIID, TFIIB, TFIIA/J, TFIIF, and TFIIH were partially purified from HeLa cells. Both factors TFIIB and two subunits of TFIIE were purified to homogeneity from *E. coli*. Reconstitution of the transcription reaction mixtures with purified RNA polymerase II together with TFIID, TFIIA/J, TFIIB, TFIIF, TFIIH, and rTFIIE resulted in basal and activated levels of transcription similar to those observed with crude extracts (Fig. 1*C*). Because basal factors did not show any detectable specific binding to MBS and had no influence on the EMSA patterns of either MyoD or E47 homodimers (data not shown), we suggest that the transcriptional activation is due to homodimeric forms.

In general MyoD and E47N homodimers are weak activators *in vitro*, and MyoD·E47N heterodimers are significantly more active (Fig. 2). Interestingly, MyoD·E47N heterodimers activate transcription significantly only from promoters containing more than one MBS (Fig. 2*A*, lanes 10–12). However, the levels of activated transcription from promoters that contained two or six MBS were similar (Fig. 2*A*, lanes 11 and 12). To understand if enhanced transcription from multiple sites is related to cooperativity in DNA binding, EMSA was used to analyze the stability of the binding of homo- and heterodimers on one and two sites by measuring

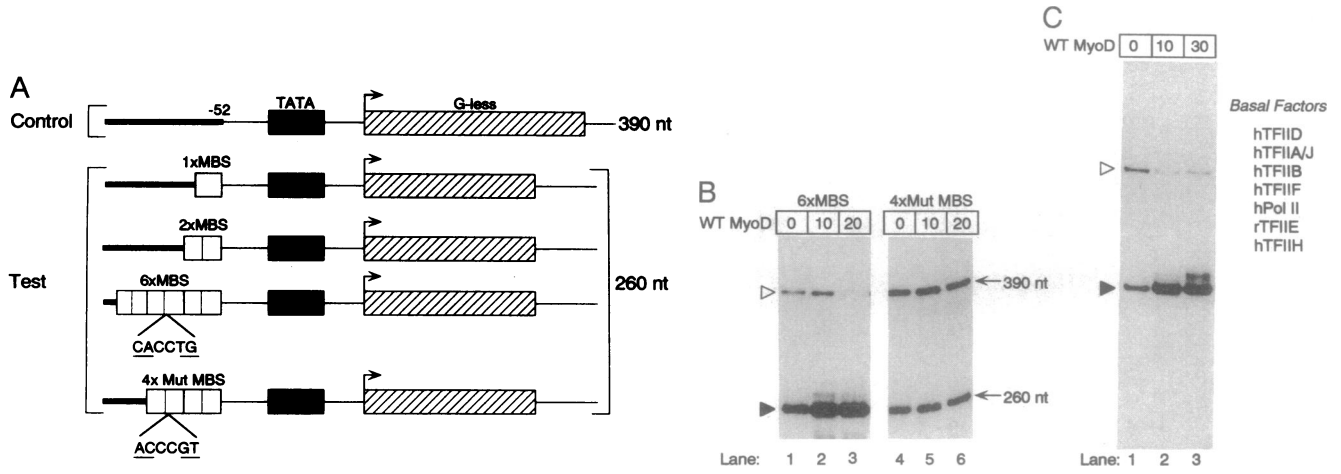

FIG. 1. *In vitro* transcriptional activation by bacterial MyoD. (*A*) Templates for *in vitro* transcription. Reporter templates carry a different number of the MBS from the muscle-creatine kinase enhancer immediately upstream of sequences of the Ad2 MLP (positions −52 to +10 relative to the transcription start site). Promoters are linked to a G-less cassette of 260 nt (23). A template that serves as an internal control contains the same Ad2 promoter sequences with no MBS and is linked to a "G-less" cassette of 390 nt. (*B*) Transcription from circular templates that carry either six MBS or four mutated MBS. Transcripts of 260 nt are designated by the filled arrowhead. In each transcription reaction, a circular control template is included, encoding a 390-nt transcript (transcripts designated by an open arrowhead). The numbers in the boxes indicate the amounts of bacterial proteins in pmol. Transcription was reconstituted with nuclear extracts (17). (*C*) Reconstitution of transcription with the fractionated basal transcription factors, which were purified from HeLa nuclear extracts as detailed in text. TFIID (DEAE-cellulose, 2.8 μg/μl), TFIIA/J (DEAE-cellulose, 3 μg/μl), rTFIIB (Q Sepharose, 0.3 μg/μl), TFIIF (Mono Q, 0.05 μg/μl), RNA polymerase II (Mono Q, 0.4 μg/μl), rTFIIE (0.25 μg/μl), and TFIIH (Mono Q, 0.2 μg/μl) were added with the indicated amounts of bacterially expressed MyoD (in pmol) to reconstitute transcription from control and test (six MBS) templates. The open arrowhead indicates transcripts of the control templates, and the filled arrowhead indicates transcripts of the test template. Numbers in boxes indicate the amounts of bacterial proteins in pmol.

dissociation rates from an oligonucleotide that carries two MBS. Fig. 2*B* shows that MyoD and E47N homodimers dissociated very rapidly from both one and two binding sites (lanes 1–12). In contrast, the rate of dissociation of MyoD·E47N heterodimers from two sites was much slower (lanes 13–18). Therefore, we conclude that MyoD·E47N heterodimers cooperate in binding to two MBS and suggest that cooperativity may contribute to the ability of heterodimers to activate transcription from promoters that contain multiple MBS.

Mutations Within the Basic Region of MyoD Impair Activation. The role of the basic region of the muscle-specific bHLH proteins in DNA binding and activation of the myogenic program has been well-documented (9, 11–14). Some of the mutations within the basic region of MyoD that were crucial for the activity of the protein *in vivo* were expressed in bacteria and analyzed for DNA binding and transcriptional activation *in vitro*. Two mutations—one in which the basic region of MyoD was replaced with the basic region of the *Drosophila* neurogenic HLH protein achaete-scute (T4Basic; ref. 9), and the other in which Ala-114 in the basic region was replaced by aspartic acid (Ala-114 → Asp)—were demonstrated to be unable to either activate transcription *in vivo* or to trigger the differentiation of fibroblasts to myoblasts (9, 11). Another MyoD mutant, in which Thr-115 was replaced with asparagine, did retain much of its normal activity *in vivo*. The DNA binding activity of the different mutant MyoD homodimers was analyzed. The overall affinity of MyoD T4Basic and Ala-114 → Asp mutants for the MBS was lower by a factor of 4 than that of wt MyoD, while the affinity of the

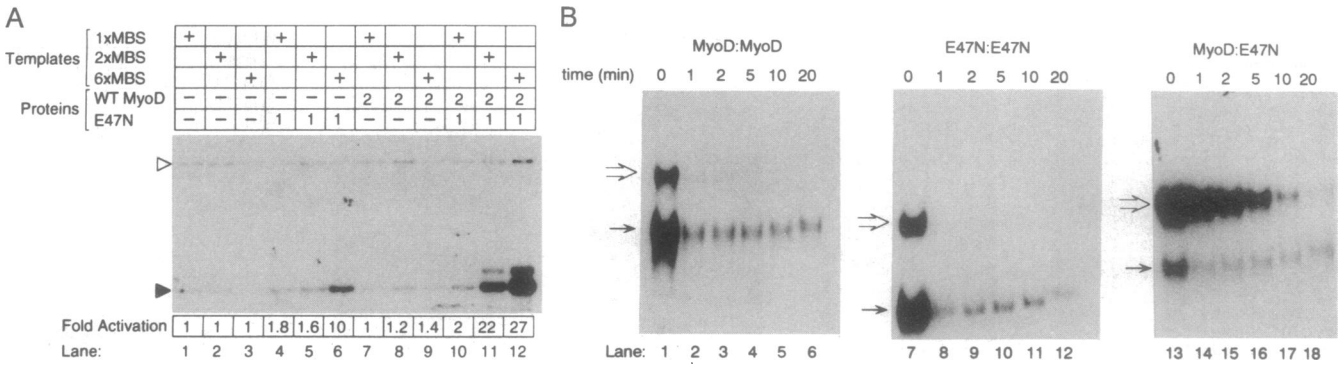

FIG. 2. Differential activation of transcription by homo- and heterodimeric complexes. (*A*) Cooperativity in transactivation. The transcription reaction mixture was reconstituted with the following partially purified fractions: TFIID (DEAE-cellulose, 2.8 μg/μl); TFIIE, TFIIF, TFIIH, and polymerase II (DEAE-Sephacel, 4 μg/μl); and rTFIIB (Q Sepharose, 0.3 μg/μl). The bacterially expressed E47N is an N-terminally truncated protein of 157 amino acids (residues 494–651 of E47). The bacterially purified MyoD and E47N were added together with the basal factors at the concentrations indicated (pmol). Three different templates that carry one, two, and six MBS upstream of the Ad2 MLP were used to drive transcription. Transcripts are indicated by arrowheads as in Fig. 1C. The relative intensities of bands were measured by densitometry reading of the autoradiograms, and the fold activation was calculated in each case relative to transcription from the control template. (*B*) Dissociation rates of the different dimeric complexes from oligonucleotides containing two binding sites as analyzed by a gel mobility-shift assay. Thin solid arrows indicate dimeric complexes binding to a single DNA binding site; thick open arrows indicate complexes associating with two binding sites.

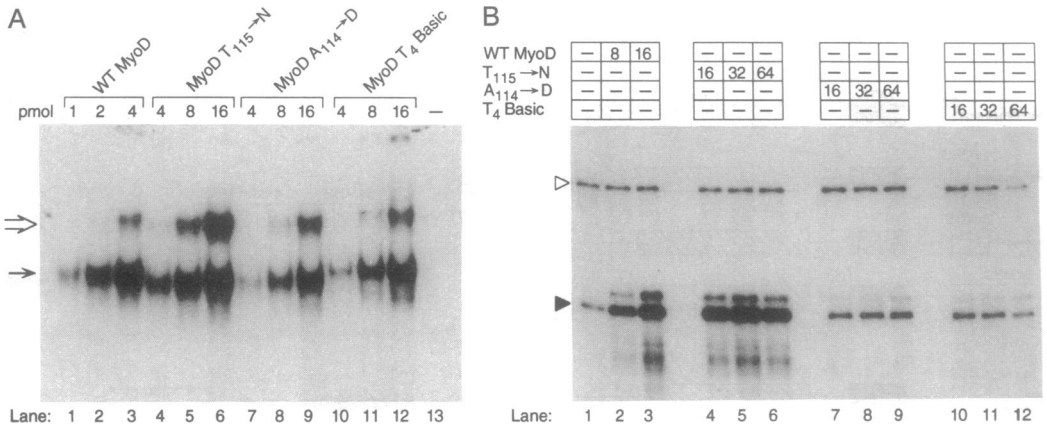

FIG. 3. Homodimeric MyoD complexes of basic-region mutants that bind to the DNA recognition site but do not activate transcription. (*A*) Quantification of DNA binding of several MyoD basic-region mutants. Several concentrations of each MyoD mutant were tested for DNA binding to a ^{32}P-labeled oligonucleotide that contained two MBS in a gel mobility-shift assay. Complexes are indicated by arrows as in Fig. 2*B*. (*B*) Transcriptional effects of the MyoD basic mutants. Transcription reactions were reconstituted as described in Fig. 2*A*. The test template carries six MBS within its promoter sequences. Transcripts are indicated by arrowheads as in Fig. 1*C*. The numbers in the boxes represent the amounts of bacterial proteins in pmol. T_{115}-N, Thr-115 → Asn; A_{114}-D, Ala-114 → Asp; T_4Basic, MyoD mutant in which the basic region of MyoD was replaced with that of *Drosophila* T_4 achaete-scute (AS-C).

Thr-115 → Asn mutant was lower by only a factor of 2 (Fig. 3*A*). The dissociation constants of the proteins were calculated by using EMSA in which various amounts of proteins were added and a constant excess amount of the high-affinity MCK site was maintained. The differences in affinities were reflected in the dissociation constants (K_d), which were in the range of 10^{-11} M, in general agreement with the previously reported values (16). Each of these mutant proteins was then tested for its ability to activate transcription as a homodimer at a maximum concentration that was 8 times higher than the concentration of the wt protein required for detectable transcription activation (Fig. 3*B*). We would expect that under these conditions the steady-state occupancy of two sites would be comparable for mutant and wt proteins. Only the Thr-115 → Asn mutant MyoD could activate transcription, although less efficiently than wt MyoD (Fig. 3*B*). The two positive control mutants, Ala-114 → Asp and T4Basic MyoD, did not activate transcription at the same concentrations (Fig. 3*B*).

We next compared the relative DNA binding and the transcription activity of the different heterodimeric complexes of MyoD mutants with E47N. MyoD mutants that are altered in their basic region heterodimerize efficiently with E47N and bind with nearly identical affinities to one DNA recognition site (data not shown), but at several protein concentrations they differ in binding to two MBS. Whereas heterodimers of wt and Thr-115 → Asn mutant MyoD with E47 occupy mostly two binding sites, heterodimers of the Ala-114 → Asp and T_4Basic MyoD mutants with E47 preferentially occupy largely one binding site (data not shown). This may suggest that the cooperativity in binding to two sites is impaired in the case of some of the heterodimers where the MyoD partner is mutated in its basic region. To further explore this possibility, dissociation rates from doubly occupied DNA binding sites were compared between the different heterodimeric complexes (Fig. 4*A*). Dissociation of heterodimers that contain either wt MyoD or Thr-115 → Asn mutant MyoD with E47N occurs at a similar relatively slow

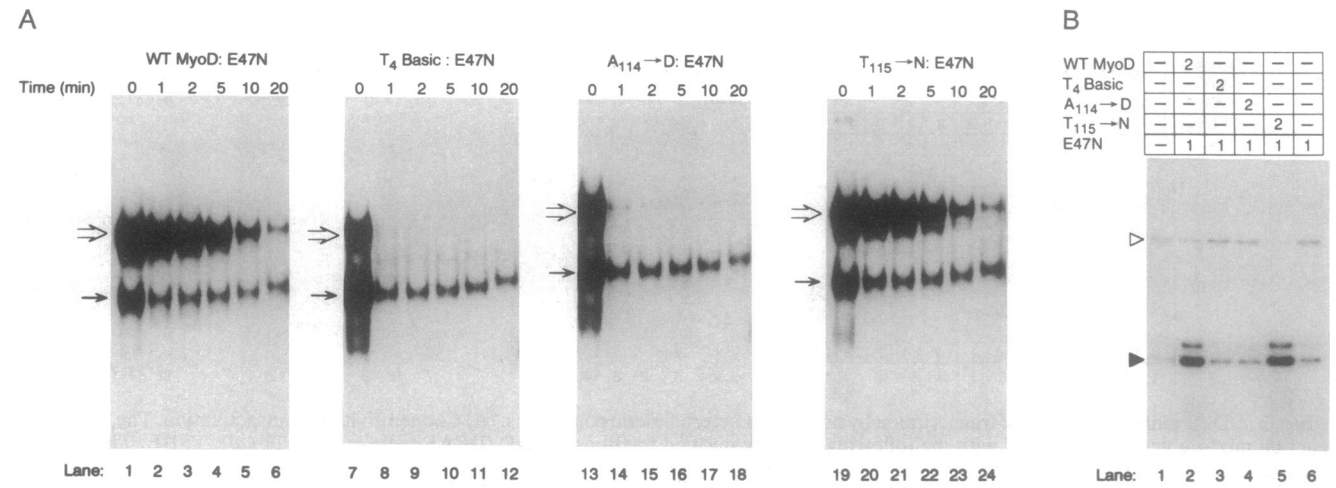

FIG. 4. Heterodimers of E47N with mutants of the MyoD basic region that fail to cooperate in DNA binding and do not activate transcription *in vitro*. (*A*) Dissociation-rates of the different heterodimeric complexes from oligonucleotides containing two binding sites. The details of the experiment are described in text. The heterodimers in all cases were reconstituted from 2 pmol of MyoD protein and 1 pmol of E47N protein. Complexes are indicated by arrows as in Fig. 2*B*. (*B*) Transcription reactions were reconstituted as described in Fig. 2A. The test template carries six MBS in its promoter sequences. Transcripts are indicated by arrowheads as in Fig. 1*C*. The numbers in the boxes represent the amounts of bacterial proteins in pmol per reaction. Abbreviations are as in Fig. 3.

rate ($t_{1/2} \approx 2$ min) under these conditions, whereas dissociation of the positive-control basic mutants, Ala-114 → Asp and T_4Basic, is nearly instantaneous (Fig. 4*A*). These results suggest that the positive-control mutations in the basic region diminish cooperativity in binding to two sites.

To determine how these mutants may function as heterodimers with E47N in transcription, various mutant and wt MyoD proteins were added with the basal factors to a template that carries six MBS. The amount of proteins used was identical to that used in the DNA binding assay shown in Fig. 4*A*. Under these conditions, low transactivation levels were observed with E47N homodimers (Fig. 4*B*, lane 6), while the different MyoD homodimers did not stimulate transcription (data not shown). The heterodimers formed with the positive-control mutant MyoD proteins (Ala-114 → Asp and T_4Basic) had little or no effect on transcription (Fig. 4*B*, lanes 3 and 4). However, E47 heterodimer with wt MyoD or Thr-115 → Asn MyoD activated transcription significantly (Fig. 4*B*, lanes 2 and 5). Identical results were obtained when transcription initiated from promoters that contained two MBS or when the amount of positive control MyoD was increased 2- or 4-fold (data not shown). Therefore, we conclude that the heterodimers, like the homodimers of these mutated proteins, are severely affected in their transcription activation potency even when their steady-state level of occupancy of the template is rendered comparable to wt MyoD. Moreover, these positive control mutants have an increased rate of dissociation from two MyoD binding sites relative to the wt complexes.

DISCUSSION

The role of the basic region in transcriptional activation may be explained in at least two ways. (*i*) An additional factor that specifically interacts with the basic region of the myogenic proteins is required for muscle-specific transcription. Presumably such a factor would bind to the myogenic basic region but not to the basic region of nonmyogenic bHLH proteins. Our data do not exclude this possibility. (*ii*) Positive-control mutations in the basic region fail to induce a conformational change in MyoD that affects its ability to activate transcription. This hypothesis is supported by the present data, which demonstrate that the "mutant heterodimers" do not show cooperative binding (Fig. 4) and, consequently, may fail to undergo a conformational change upon DNA binding.

It has been suggested (12) that in its unbound form, the MyoD activation domain is masked by other parts of MyoD and that binding to DNA may induce a conformational change that exposes the activation domain for interactions necessary for its function. This was based, in part, on the observation (12) that MyoD fusions with the yeast GAL4 DNA-binding domain gave a much lower level of activation than did fusions with just the 53-residue N-terminal activation domain alone or fusions with a variety of internal deletions of MyoD. We propose that this conformational change does not occur in the positive-control mutants and, consequently, that the activation domain may not be exposed. Homodimers of MyoD bind cooperatively to DNA but fail to do so after deletion of the N-terminal activation domain (24). Similarly, heterodimers of the N-terminal deletion mutant of MyoD with E47N also fail to bind cooperatively (data not shown). Together, these observations suggest that a correct conformation of the N-terminal domain of MyoD is important not only for protein–protein interactions leading to cooperativity in DNA binding but also for contacts with the basal transcription machinery.

The crucial residues Ala-114 and Thr-115 are in intimate contact with DNA in the MyoD–DNA cocrystal (25). Perhaps a "conformational" change is initiated if a second domain on MyoD is released upon interaction of these residues with DNA. Alternatively, the "communication" with the second MyoD binding site might be via the DNA and not through the protein, in which case, our assumption that MyoD undergoes a conformational change would be incorrect. However, there is very little distortion of double-helix structure in the cocrystal (25). Whatever the change that occurs as a consequence of MyoD binding to the first site (be it through the protein or through the DNA), we anticipate that it could influence interactions with other regulatory proteins present in much more complex myogenic enhancers, some of which respond dramatically to MyoD yet contain only a single binding site (e.g., the cardiac actin enhancer; ref. 26). This raises a related problem. Many cellular oncogenes and growth factors control MyoD activity (5, 27). A standard test for evaluating this inhibition is whether or not a MyoD gel mobility shift is observed when an extract is challenged with a single site. Clearly, much of the control of MyoD might be focused on the cooperative transition.

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