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Biochemistry

A cellular factor stimulates the DNA-binding activity of MyoD and E47

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ABSTRACT **We show that mixing purified MyoD and E47 proteins results in heterodimers that fail to bind DNA, even though MyoD and E47 homodimers can bind DNA efficiently. Addition of cell extracts or a specific fraction from a cell extract enables the heterodimer to bind DNA, but components in this fraction fail to enter the DNA complex. The activity is sensitive to heat and protease and is not ATP-dependent. The activity functions on E47 and MyoD homodimers and can stimulate DNA binding of the basic–helix–loop–helix region of MyoD. The effectiveness of the activity, for MyoD homodimers, depends on the exact DNA sequence of the binding site. Our results suggest that specific factors in the cell might control the DNA-binding properties of helix–loop–helix proteins.**

The helix–loop–helix (HLH) proteins are known to play important roles in development, the regulation of tissue-specific gene expression (MyoD, achaete scute, SCL), and sex determination (daughterless, achaete scute). A family of HLH-containing gene products controls muscle cell identity—MyoD (1), myogenin (2), Myf-5 (3), and Mrf-4 (4–6). MyoD induces the skeletal muscle-specific differentiation program by binding to regulatory sequences present in muscle-specific genes. Only 68 amino acids of MyoD are required for stable conversion of C3H10T½ (10T½) fibroblasts into myoblasts (7). This region contains the DNA-binding domain, which is a putative basic–HLH structure (bHLH). Mutagenesis has revealed that the HLH region is required for dimerization and that the adjacent basic region is required for DNA binding.

Although MyoD can bind DNA as a homodimer, it binds to its target sequence more than 10 times better as a heterodimer with one of the bHLH proteins encoded by the *E2A* gene (8). The heterodimeric species bind DNA with altered sequence preferences relative to their homodimeric counterparts (9). Furthermore, the inhibition of DNA binding of various bHLH proteins *in vitro* by their association with the inhibitory HLH protein Id1, which lacks the basic DNA-binding domain (10), provides the cell with the potential for negative regulation. The related protein EMC plays a similar role during *Drosophila* neurogenesis (11). These observations suggest that the ability of HLH proteins to form hetero- and homodimeric complexes is important for transcriptional regulation. Thus, with the increasing number of HLH proteins being described, the rules for specifying particular interactions and their consequences need to be addressed. Until now, it had been thought that dimerization and DNA binding were inherent properties of these proteins, since previous studies carried out with proteins translated *in vitro* in rabbit reticulocyte lysates established that MyoD and E2A proteins spontaneously form heterodimeric complexes that interact with E-box-containing DNA (8, 12). Here we have characterized the properties of a cellular activity, initially identified in the reticulocyte lysate, that enhances the DNA-binding activity of MyoD and E2A proteins.

MATERIALS AND METHODS

Purified Proteins. All purified proteins were generated by using the bacteriophage T7 expression system developed by Studier and Moffatt (13). The E47 used in this study was the E47N construct described by Sun and Baltimore (14), and the purification protocol was as described. MyoD was expressed and purified as follows. Following creation of an *Nde* I site at the initiator methione codon of the MyoD cDNA, an *Nde* I–*Hin*dIII fragment of 957 base pairs was cloned into *Nde* I/*Hin*dIII-digested T7 expression vector pRK171a (15). The T7 MyoD construct was transformed into *Escherichia coli* BL21(DE3)/pLysS bacteria (16). Cultures were grown to OD_{600} of 0.5 in L broth plus ampicilin (100 μg/ml) and chloramphenicol (10 μg/ml). Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM and the culture was grown for 3 hr. Cells were harvested, washed, and suspended (25 ml/liter of starting volume) in 0.2 M NaCl/20 mM Hepes, pH 7.6/1 mM EDTA/1 mM dithiothreitol (DTT)/10% (vol/vol) glycerol/0.1% (vol/vol) Triton X-100/1 mM phenylmethanesulfonyl fluoride (PMSF) with leupeptin and pepstatin (each at 1 μg/ml). The lysate was frozen once in liquid nitrogen, thawed on ice, and then sonicated on ice 5 times for 2 min each time, with 1-min intervals between each. The sonicated lysate was centrifuged for 30 min at 20,000 × g at 4°C, nucleic acids were removed from the supernatant by addition of polyethylenimine to a final concentration of 0.3%, and the mixture was stirred at 4°C for 30 min. After centrifugation at 20,000 × g at 4°C for 20 min, the supernatant was collected. Ammonium sulfate (2 M in 10% glycerol/20 mM Hepes, pH 7.6/1 mM DTT/1 mM PMSF) was added to the supernatant, to give a final concentration of 0.6 M, with stirring at 4°C and incubation for 30 min. The precipitate was collected by centrifugation at 4000 × g at 4°C for 20 min. The pellet was dissolved by rocking overnight at 4°C in 10% glycerol/20 mM Hepes pH 7.6/1 mM EDTA/1 mM DTT/1 mM PMSF/0.1 M NaCl. The product of this procedure was 90–95% MyoD.

Cell Extracts. 10T½ cells were grown in Dulbecco's modified Eagle's medium plus 15% calf serum (HyClone) and were harvested at about 90% confluence. Cells were washed three times with Tris-buffered saline solution, dislodged by scraping, pelleted by centrifugation for 15 sec at 10,000 × g, were suspended (0.5 ml per culture dish in lysis buffer [20 mM Hepes, pH 7.6/20% glycerol/0.5 M NaCl/1.5 mM $MgCl_2$/0.2 mM EDTA/0.5% Nonidet P-40/1 mM DTT/1 mM PMSF with leupeptin (10 μg/ml), pepstatin (10 μg/ml), and aprotinin (100 μg/ml)]. Lysed cells were vortexed occasionally for

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Abbreviations: HLH, helix–loop-helix; bHLH, basic–HLH; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; MCK, muscle creatine kinase; PMSF, phenylmethanesulfonyl fluoride.

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15 min at 4°C and centrifuged at 10,000 × g for 15 min, and supernatant was dialyzed extensively against lysis buffer containing only 10 mM NaCl instead of 0.5 M NaCl.

Gel filtration chromatography was performed on a Pharmacia FPLC system. 10T½ cell extract (200 mg of protein) was loaded onto a Superose 6 column and eluted with 10 mM HEPES/50 mM NaCl/10% glycerol/0.1% Nonidet P-40/1 mM DTT/0.5 mM PMSF containing leupeptin and pepstatin (each at 1 μg/ml). Protein concentration was monitored by continuous UV absorption at 280 nm.

Oligonucleotides. The following double-stranded oligodeoxyribonucleotides were used: MCK (muscle creatine kinase), 5'-GATCCCCCCAACACCTGCTGCCTGA-3'; WM-1, 5'-CCCCCGTCAGCTGACGCCTGA-3'.

Electrophoretic Mobility-Shift Assay (EMSA). The assays were performed essentially as described (12). Purified proteins were incubated with or without 1–10 μg of 10T½ cell extract for 5 min at room temperature in 20 mM Hepes, pH 7.6/50 mM KCl/3 mM $MgCl_2$/1 mM EDTA/0.5% Nonidet P-40 containing 500 ng of poly(dI-dC) in a final volume of 20 μl. The mixtures were loaded onto a 2% agarose gel (Sea-Plaque low-melting; FMC) containing 50 mM Tris base, 50 mM boric acid, and 1 mM EDTA (same solution in running buffer) and electrophoresed at room temperature or 4°C as indicated.

RESULTS

DNA-binding Activity of MyoD–E47 Heterodimers Is Stimulated by a Cellular Factor. MyoD and E47 translated in reticulocyte lysates form heterodimeric complexes capable of binding to the MEF-1 site (17) in the MCK enhancer in EMSAs (12, 10). MyoD and E47 generated in the reticulocyte lysates form heterodimeric complexes capable of binding DNA within 5 min when incubated at 37°C. In contrast, proteins purified from bacteria gave rise to homodimer–DNA complexes for both MyoD and E47; these proteins failed to form heterodimeric complexes even after 30 min at 37°C (see below). To determine whether factors present in the reticulocyte lysate contributed to DNA binding of heterodimeric complexes, purified bacterial MyoD and E47 were assayed for MCK probe-binding activity in the presence of the reticulocyte lysate. MyoD and E47 were incubated either alone or together in the presence or absence of reticulocyte lysate for 10 min at 37°C. DNA-binding complexes were analyzed by EMSA (Fig. 1*A*). Homodimeric DNA-binding complexes of MyoD and E47 were not much affected by the addition of the reticulocyte lysate when assayed on the MCK binding site (lanes 1–4). However, the addition of reticulocyte lysate to MyoD plus E47 resulted in a marked increase in the heterodimeric DNA-binding complex (lanes 5 and 6). In addition, eukaryotic cell extracts, such as extracts isolated from 10T½ (mouse fibroblasts), FTO-2B (rat hepatoma), and CV-1 (monkey kidney) cells, were able to stimulate heterodimeric DNA-binding activity of MyoD and E47 (Fig. 1*B*; data not shown for FTO-2B or CV-1 cell extracts). Thus, reticulocyte lysate and a variety of cell extracts contain a factor(s) that increases the heterodimeric DNA-binding activity of MyoD and E47.

The biochemical properties of the stimulatory factor were characterized further by using 10T½ cell extract as a source of activity. Treatment of the cell extract with chymotrypsin caused a dramatic reduction in the stimulatory activity (Fig. 2*A*). This loss of activity was not due to proteolysis of MyoD and E47, since the chymotrypsin was inhibited prior to the addition of MyoD and E47 to the extract. In addition, inhibition of the chymotrypsin prior to addition to the cell extract resulted in little or no affect on the ability of the extract to stimulate heterodimer–DNA binding (Fig. 2*A*, lane 5). The stimulatory factor was heat-sensitive; stimulatory

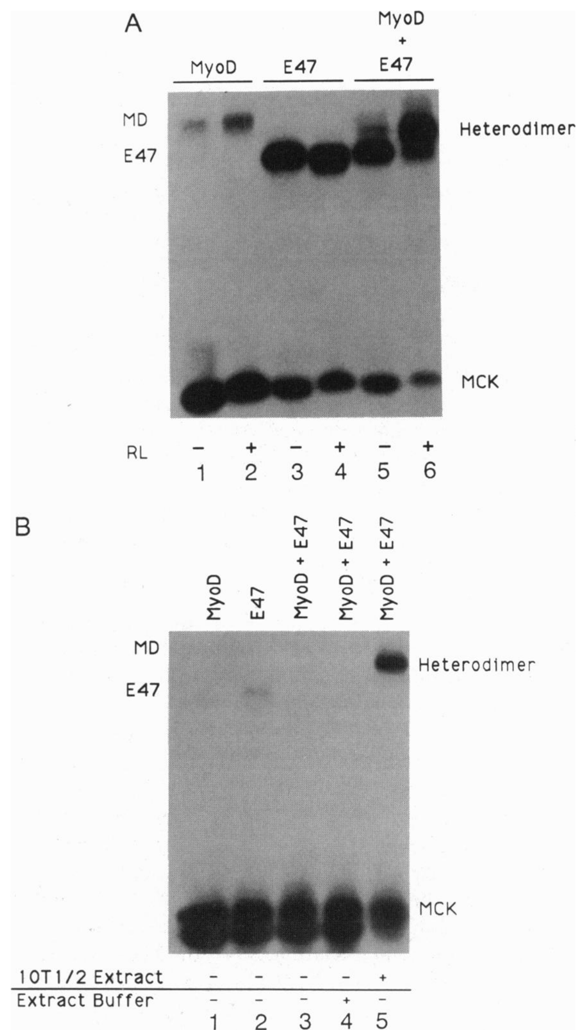

FIG. 1. Enhancement of DNA-binding activity of MyoD–E47 heterodimers by reticulocyte lysate and 10T½ cell extract. EMSAs were performed with purified MyoD and E47 proteins mixed with ^{32}P-labeled MCK enhancer oligonucleotide. Proteins were premixed and incubated either with or without reticulocyte lysate (*A*) or 10T½ cell extract (*B*) for 10 min at 37°C prior to DNA binding. Samples were run in a 2% agarose gel. Positions of protein–DNA complexes for MyoD (MD), E47, and heterodimers are indicated. (*A*) Three micrograms of MyoD protein (lanes 1 and 2), 200 ng of E47 protein (lanes 3 and 4), or 3 μg of MyoD plus 200 ng of E47 (lanes 5 and 6) were incubated with the MCK oligonucleotide probe in the presence (+) or absence (−) of reticulocyte lysate (RL). (*B*) Three micrograms of MyoD (lane 1), 20 ng of E47 (lane 2), or 3 μg of MyoD plus 20 ng of E47 (lanes 3–5) were incubated with probe in the presence (+) or absence (−) of 10T½ cell extract or cell extraction buffer.

activity was lost after incubation of the extract at 65°C (Fig. 2*B*). These data suggest that the stimulatory activity is a protein.

The amount of E47 homodimer–DNA complex decreased when MyoD and E47 were incubated together in the absence of 10T½ cell extract (Fig. 2, lanes 3). This effect was greater when there was an excess of MyoD protein relative to E47. This indicates that MyoD and E47 are interacting, yet this newly formed complex cannot bind DNA. Direct analysis using analytical ultracentrifugation shows that MyoD and E47 form a heterodimer under these conditions (H.W. and T. Laue, data not shown).

The Stimulatory Activity Can Function at 4°C. Because a MyoD–E47 heterodimer gel shift did not occur at 4°C even in the presence of the cell extract (see below), we attempted to

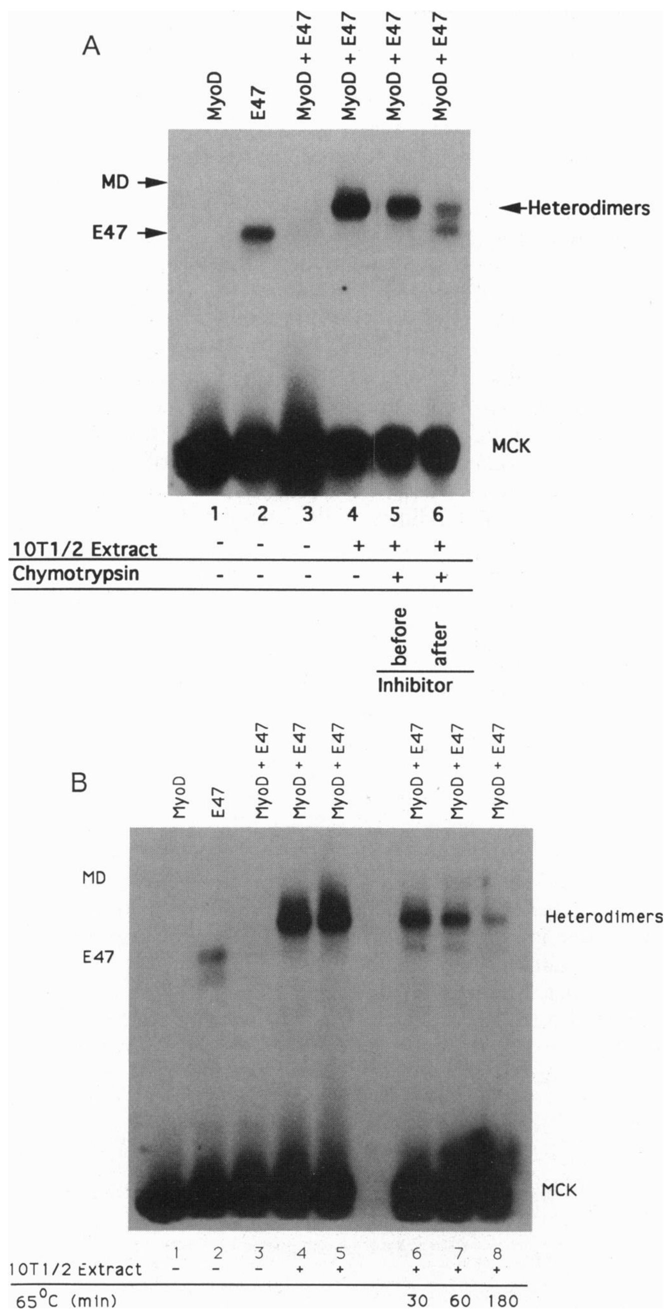

FIG. 2. Characteristics of the stimulatory activity. EMSAs of MyoD–E47 DNA-binding-stimulatory activity were performed following proteolysis or heat denaturation. Positions of protein–DNA complexes for MyoD (MD), E47, and heterodimers are indicated next to the autoradiograms of the 2% agarose gels. (*A*) 10T½ cell extract was incubated with chymotrypsin in the presence (+) or absence (−) of chymotrypsin inhibitor for 15 min at 37°C. Chymotrypsin inhibitor was then added to the proteolyzed sample. Aliquots were subsequently incubated with MyoD (3 μg) and E47 (20 ng) for an additional 15 min at 37°C before addition of the MCK oligonucleotide. (*B*) 10T½ cell extract was incubated at 65°C for 30, 60, or 180 min. Aliquots were subsequently incubated with MyoD (3 μg) and E47 (20 ng) for an additional 15 min at 37°C before addition of the MCK oligonucleotide.

determine whether the 37°C requirement applied to the interaction between MyoD and E47 or to the DNA-binding step. Incubation of MyoD and E47 at 4°C did not result in the formation of heterodimeric DNA-binding complexes in either the absence or the presence of 10T½ cell extract (Fig. 3, lanes 3 and 4). In addition, the decrease in E47 homodimer DNA-binding complex seen above in the absence of cell extract did not occur. This indicates that the initial interaction between MyoD and E47 requires incubation at 37°C. However, when MyoD and E47 were preincubated at 37°C prior to addition of 10T½ cell extract at 4°C, there was a decrease in the E47 homodimer DNA-binding complex, indicating an interaction between MyoD and E47. When the extract was subsequently added (at either 4°C or at 37°C) there was a large increase in the heterodimer DNA-binding complex (lanes 5 and 6). Thus the interaction between MyoD and E47 requires incubation at 37°C, resulting in a complex that cannot bind DNA efficiently. Therefore, the stimulatory activity in the 10T½ cell extract does not facilitate the MyoD-E47 interaction but functions on existing MyoD–E47 complexes to enhance their ability to bind DNA, and this enhancement can occur at 4°C.

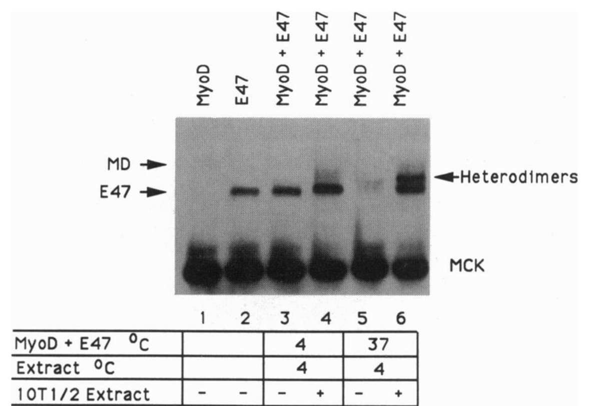

FIG. 3. Stimulation of DNA binding occurs at 4°C. EMSAs of MyoD–E47 DNA-binding-stimulatory activity were performed following incubation at either 4°C or 37°C. One hundred nanograms of MyoD and 10 ng of E47 (lanes 1 and 2, respectively) were incubated at 37°C for 10 min prior to DNA binding. One hundred nanograms of Myo D plus 10 ng of E47 (lanes 3–6) were incubated in the presence (+) or absence (−) of 10T½ cell extract at the indicated temperatures. For lanes 3 and 4, samples were incubated at 4°C during the entire course of the experiment. For lanes 5 and 6, MyoD and E47 were combined and subjected to a preincubation of 10 min at 37°C, incubated at 4°C for 2 min, and incubated with the 10T½ cell extract for an additional 10 min at 4°C. DNA-binding reactions (with the MCK probe) and electrophoresis in 2% agarose gel were performed at 4°C.

The bHLH Region of MyoD Is a Target of the Stimulatory Activity. Since the above assays were carried out on a DNA sequence that E47 homodimers and MyoD–E47 heterodimers bind to strongly and MyoD homodimers bind to rather weakly (9), MyoD homodimer complexes were not observed even with this relatively high amount of protein (3 μg). Therefore, to determine whether MyoD homodimer–DNA complexes were affected by E47 in the presence or absence of 10T½ cell extract, we utilized a sequence (WM-1) that MyoD homodimers bind to strongly and E47 homodimers bind to weakly (T. K. Blackwell and H.W., data not shown). In the absence of extract, MyoD homodimer–DNA complexes were easily detected with the WM-1 sequence, whereas E47 homodimer–DNA complexes were not detected (Fig. 4*A*). MyoD homodimer–DNA complexes decreased in the presence of E47 without the concomitant appearance of heterodimeric DNA-binding complexes. This is similar to the behavior of E47 homodimer complexes with the MCK sequence when MyoD was added in the absence of cell extract. MyoD–E47 heterodimeric DNA-binding complexes were observed with the WM-1 sequence when 10T½ cell extract was added (Fig. 4*A*, lane 4). These data suggest that MyoD and E47 homodimers, capable of binding DNA as homodimers, interact to form a non-DNA-binding complex. This non-

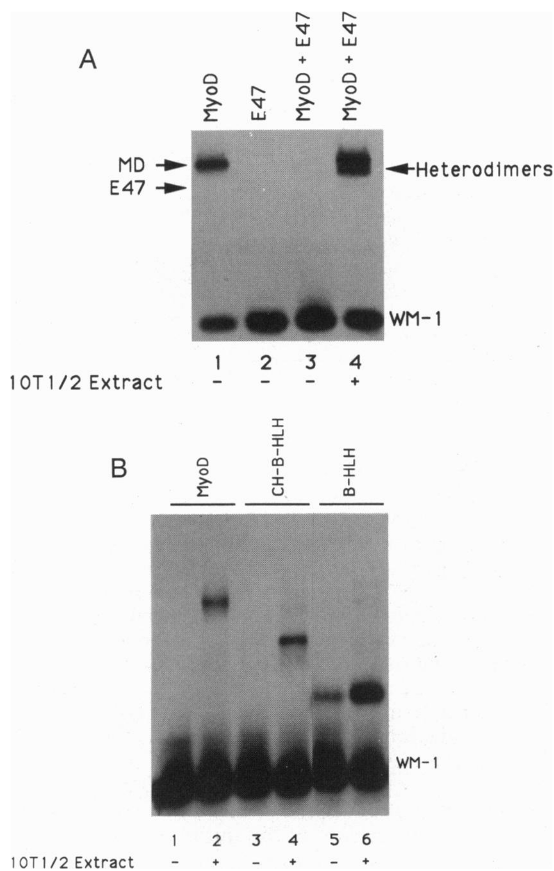

FIG. 4. MyoD is a target for the stimulatory activity. EMSAs of MyoD–E47 DNA-binding-stimulatory activity were performed with a MyoD-preferred DNA sequence, the WM-1 oligonucleotide. (*A*) One hundred nanograms of MyoD (lane 1), 100 ng of E47 (lane 2), or 100 ng of MyoD plus 100 ng of E47 (lanes 3 and 4) were incubated in the presence (+) or absence (−) of 10T½ cell extract. (*B*) One hundred nanograms of full-length MyoD (lanes 1 and 2), 50 ng of MyoD deletion mutant CH-B-HLH (amino acids 57–165) (lanes 3 and 4) or 10 ng of MyoD deletion mutant B-HLH (amino acids 102–165) (lanes 5 or 6) were incubated with WM-1 oligonucleotide at 37°C for 10 min in the presence (+) or absence (−) of 10T½ cell extract. DNA-binding and gel electrophoresis in 2% agarose gel were performed at room temperature.

DNA-binding MyoD–E47 complex is converted to a DNA-binding competent complex by incubation with cell extract.

Surprisingly, MyoD homodimer-DNA-binding complexes were stimulated by the 10T½ extract when assayed on the WM-1 sequence (see below and Fig. 4*A*, lane 4, and Fig. 4*B*), but were not significantly stimulated when assayed on the MCK binding site (Fig. 1*A*, lanes 1 and 2). Studies with deletion mutants have demonstrated that the 68-amino acid bHLH domain of MyoD is sufficient for DNA binding and conversion of 10T½ cells to muscle cells (7). To determine whether the stimulatory activity could function on a specific region of MyoD, two deletion mutants were tested for stimulation of DNA binding. The CH-B-HLH mutant retains amino acids 57–167, containing the cysteine and histidine (CH)-rich, the basic, and the HLH region. The B-HLH mutant retains amino acids 101–167 and contains only the bHLH domain. The DNA-binding activity of both of these mutant polypeptides was stimulated by addition of 10T½ cell extract (Fig. 4*B*). In addition, heterodimer–DNA binding was also stimulated when these mutant MyoD proteins were mixed with E47, as well as with full-length MyoD (data not shown). Therefore, the bHLH domain of MyoD is a target of the stimulatory activity.

10T½ Cell Extract Contains Multiple Stimulatory Activities. To determine the chromatographic properties and estimate the molecular weight of the stimulatory activity, 10T½ cell extract was fractionated on several columns, and the fractions were tested for stimulatory activity. When 10T½ extract was fractionated on an anion-exchange column (Mono Q), the majority of proteins were present in the flowthrough. The stimulatory activity was retained and was eluted with the 0.4 M NaCl fraction (data not shown). In contrast, when the 10T½ cell extract was analyzed by gel filtration (Superose 6), two different activities were observed (Fig. 5). As expected, an activity that stimulated MyoD–E47 heterodimer DNA-binding was present, eluted in a broad peak with the highest level of activity at ≈70 kDa. Surprisingly, a second activity that appeared to stimulate E47 homodimers was present in a sharp peak of activity centered at ≈40 kDa. Although this homodimer-stimulating activity appeared to be specific for E47 homodimers when the MCK site was used, MyoD homodimer DNA-binding was also stimulated when the assay was carried out on the WM-1 sequence (data not shown). Therefore, the 10T½ cell extract contains at least two activities capable of stimulating the DNA-binding activity of either heterodimeric or homodimeric DNA-binding complexes of MyoD and E47. Whether these two activities represent different proteins or are different activities of the same protein awaits further purification of the two activities. During the preparation of this manuscript it was reported that the 90-kDa heat shock protein (HSP90) can stimulate MyoD–DNA binding 2- to 3-fold (18). At present, it is difficult to know how this activity of HSP90 relates to the activity described here, since HSP90 activity was not tested on MyoD–E47 heterodimers and our 10T½ activity is more complex (see Fig. 5). Most important, the 10T½ activity gives a greater increase in DNA binding, sometimes as high as 50-fold, and is not stimulated by pretreatment of cells with heat shock (data not shown). Most important, our activity is retained on 10-kDa, but not 30-kDa molecular-sieve filters, where the HSP90 dimer is retained (M.J.T., unpublished observations).

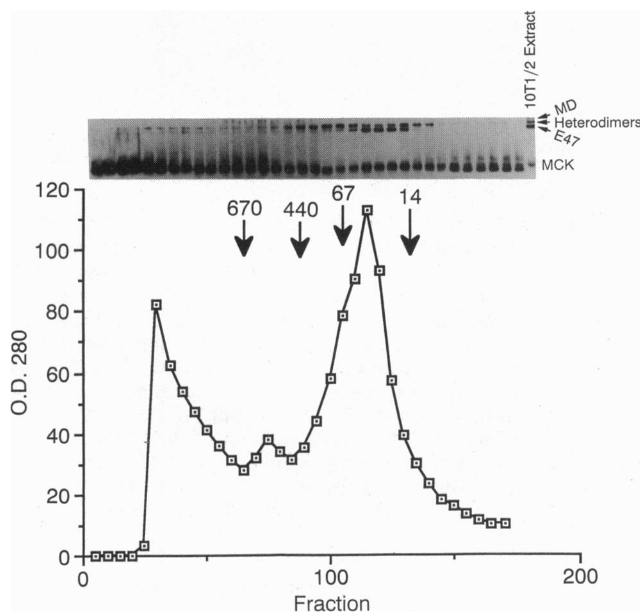

FIG. 5. Protein elution (OD_{280}) and corresponding gel retardation profile (EMSAs) of the 10T½ extract on a Superose 6 column. Molecular size standards included thyroglobulin (669 kDa), ferritin (440 kDa), bovine serum albumin (67 kDa), and RNase A (14 kDa).

DISCUSSION

It has been proposed that the terminal differentiation of myoblasts into myotubes is controlled by the combination of HLH proteins within these cells and the homo- and heterodimeric complexes they form (10). In the present study we have described the activity of a cellular factor that stimulates the ability of MyoD and E47 homo- and heterodimers to interact with DNA. The stimulatory factor is present in a variety of cell lines, is sensitive to protease and heat treatment, and is capable of functioning at 4°C on existing heterodimers to increase DNA-binding activity. MyoD is known to be extensively phosphorylated (7). However, the characteristics of the stimulatory activity suggest that it is not a kinase or a phosphatase. The activity was active after extensive dialysis and column chromatography, procedures that remove the low molecular weight cofactors and energy sources required for kinase activity. Furthermore, addition of ATP to the reaction did not produce an increased stimulation of DNA binding, and the addition of nonhydrolyzable ATP analogs had no effect on the stimulatory activity (data not shown).

The DNA-binding activity of Fos and Jun heterodimers was found to be regulated by a cellular factor called Ref-1 (19). Ref-1 stimulates the DNA-binding activity of Fos–Jun heterodimers by mediating the reduction of a conserved cysteine residue in the DNA-binding domains of Fos and Jun. We have tested Ref-1 in our system and see only marginal stimulation. Furthermore, Ref-1 can be replaced by high levels of DTT. These levels of DTT have no affect in our system. It is unlikely that the 10T½ cell activity described here is involved in catalyzing the formation of MyoD–E47 complexes, since heterodimer complexes form in the absence of extract as assayed by analytical ultracentrifugation (T. Laue & H.W., data not shown); however, as shown here these complexes are inefficient in DNA binding and, in fact, inhibit DNA binding by E47 or MyoD homodimers. MyoD–E47 heterodimers do eventually form a DNA-binding-competent complex in the absence of cell extract; however, this process requires incubation at 37°C for at least 60 min (data not shown; also see ref. 14). In addition, increasing the NaCl concentration to above 150 mM also increases the DNA-binding activity of MyoD–E47 heterodimers. Therefore, given time, temperature, and salt these complexes eventually can bind DNA, but the cell extract greatly accelerates the process.

In *Drosophila*, sex determination is controlled by the X-to-autosome ratio. A main determinant is the ability of achaete-scute T4 to form a heterodimer with daughterless (similar to E47). Surprisingly, a number of other loci also contribute to the monitoring of the X/autosome ratio (20, 21). It is possible that some of these loci may be functioning as the activity described here—to bring achaete-scute and daughterless together to form an effective DNA-binding heterodimer. It will be interesting to know whether activities like the one described here play a more general role in controlling interactions between HLH proteins and DNA.

While the stimulatory activity functions with the bHLH domain of MyoD homodimers (on the WM-1 sequence), the background binding of the bHLH protein is much higher than for full-length MyoD, suggesting that full-length MyoD contains a domain that negatively regulates its own binding to DNA. The 10T½ activity stimulates binding of MyoD to the WM-1 sequence but is much less stimulatory for binding to the MCK sequence. The reason for this sequence specificity is not understood, but factors in the extract are not stable components of the complex, since the stimulatory activity does not alter the mobility of the shifted complexes. Perhaps the activity acts on the DNA sequence so that it is presented to the heterodimer in a more suitable conformation, or the activity could act more directly on the heterodimer so that it presents its DNA-binding surface more favorably. Recent data suggests that the basic region of basic–leucine zipper proteins becomes α-helical with DNA binding (22). Our activity might encourage α-helix formation in the basic region of MyoD. Alternatively, perhaps there is already some α-helical tendency so that the basic region forms a continuous coil with helix 1 in solution under our conditions. Such a continuous coil might then form a coiled-coil structure with its heterodimer partner. Under these circumstances, the activity we describe might function to release the basic region from these coiled-coil interactions and bind the basic α-helix to the DNA. In any case, it is surprising that the DNA sequence seems to be a factor in determining the stimulation of DNA binding by MyoD.

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