Journal of Cellular Biochemistry 50:130–142 (1992)

Monoclonal Antibodies Identify a Possible Regulatory Domain of MyoD1

Francesca Cole, Kimberly S. Timo, and D. Stave Kohtz

Department of Pathology, Mount Sinai School of Medicine, New York, New York 10029

Abstract A panel of monoclonal antibodies (mAbs) to murine MyoD1 was generated. One set of mAbs is shown to react with epitope(s) in the cysteine/histidine-rich (C/H) region while another set is shown to react with epitope(s) in the C-terminal portion of MyoD1. One of the mAbs reactive with a C-terminal epitope sensitively detected MyoD1 in whole cell extracts by Western blotting. Time course studies of total protein accumulation during C2C12 myoblast differentiation revealed only subtle changes in the phosphorylation and quantity of MyoD1 protein present in C2C12 cells from induction to 120 hr after induction. These results suggest that modulation of MyoD1 protein or total phosphorylation levels is not tightly associated with the transition of undifferentiated myoblasts to differentiated myocytes. Monoclonal antibodies to the C-terminal epitope produced supershifted bands in gel retardation assays, indicating that these mAbs had no effect on DNA binding. Although the C/H region of MyoD1 does not participate in DNA binding, mAbs reactive with the C/H region neutralized this activity in gel retardation assays. These data suggest that the conserved C/H domain may serve to modulate MyoD1 DNA-binding activity by interacting with another regulator.

Key words: muscle, myoblasts, C2C12 cells, phosphorylation, transcription factors

Proliferating skeletal myoblasts resemble fibroblasts and display few obvious morphological or biochemical characteristics of skeletal muscle. Several mitogens inhibit the differentiation of skeletal myoblats in culture, including components of serum [Konigsberg, 1963; Konigsberg et al., 1978], components of embryo extracts [Slater, 1976], basic fibroblast growth factor [Gospodarowicz et al., 1976; Linkhart et al., 1980], transforming growth factor β (TGF-β) [Massague et al., 1986; Olson et al., 1986], and insulin-like growth factors [Ewton and Florini, 1980, 1981]. Reducing the ambient concentration of mitogens in their medium induces myoblasts to differentiate [Okazaki and Holtzer, 1966; O'Neill and Stockdale, 1972], a process that is partly mediated by factors secreted by the differentiating cells [Doering and Fischman, 1977; Nadal-Ginard, 1978; Linkhardt et al., 1981]. Differentiation of myoblasts is marked by morphological changes, such as fusion of the mononuclear cells into multinucleated syncitia (myotubes and myofibers), and biochemical changes, including synthesis of the vast array of proteins required for sarcomere assembly and contractile activity.

10T1/2 embryonic fibroblasts are phenotypically closer to somitic mesoderm than are skeletal myoblasts. So far, four distinct cDNA clones have been described that, upon transfection in an appropriate expression vector, confer a myogenic phenotype to 10T1/2 cells: MyoD1 [Davis et al., 1987], myogenin [Wright et al., 1988], myf-5 [Braun et al., 1989a], and MRF4/myf-6/herculin [Rhodes and Kozniecznу, 1989; Braun et al., 1990; Miner and Wold, 1990, respectively]. MyoD1, myogenin, myf-5, and MRF4/myf-6/herculin share a 60-amino acid structural motif, referred to as a helix–loop–helix [Murre et al., 1989a]. This motif is common to a larger family of developmentally relevant genes, including c-myc, N-myc, L-myc, daughterless, achaete–scute, twist, enhancer of split, E12, and E47 (a synopsis is given in Benezra et al., [1990]). Specific binding of a common DNA sequence, demonstrated for the MyoD1 [Lassar et al., 1989], E12, E47, daughterless (da), and achaete–scute (T3) proteins [Murre et al., 1989b], is mediated by a basic region proximal to the helix–loop–helix motif, while dimerization is mediated by

Received May 27, 1992; accepted May 28, 1992.

Address reprint requests to D. Stave Kohtz, Department of Pathology, Mount Sinai School of Medicine, 1 Gustave Levy Place, New York, NY 10029.

the helix–loop–helix domain itself [Murre et al., 1989a].

The pattern of expression for MyoD1, myf-5, myogenin, and MRF4/myf-6/herculin varies between different skeletal myoblast cell lines, and between their differentiated and proliferative states. Myogenin mRNA expression has been observed in all skeletal myoblast cell lines tested so far [Wright et al., 1988; Braun et al., 1989b; Edmondson and Olson, 1989]. Myogenin is expressed at low to undetectable levels in proliferating myoblasts, then induced to high level expression as the cells differentiate. MyoD1 mRNA expression has been observed in C2C12 myoblasts [Davis et al., 1987], primary human myoblasts [Braun et al., 1989b], and 5-azacytidine-induced 10T1/2 myoblasts [Davis et al., 1987], while myf-5 mRNA expression has been observed in BC3H cells and primary human myoblasts [Braun et al., 1989a,b]. MyoD1 and myf-5 are expressed constitutively in skeletal myoblasts, and at slightly higher levels in differentiated cells. MRF4/myf-6/herculin is expressed in adult skeletal muscle, but has not been detected in most cultured muscle cells [Rhodes and Kozniecczny, 1989; Braun et al., 1990; Miner and Wold, 1990].

An important aspect of skeletal myoblast proliferation, first noted by Yaffe [1968] and is the stability of the unexpressed phenotype through long-term continuous passaging. MyoD1 is expressed at high levels in both C2C12 myocytes and myoblasts, and it is possible that one of its functions as a determination protein is to perpetuate the myogenic phenotype in the latter as it replicates [Blau and Baltimore, 1991]. Another study has shown that MyoD1 in vivo is bound to target sequences of myocyte-specific genes only in myocytes and not in myoblasts [Mueller and Wold, 1989], indicating that its DNA-binding activity can in some way be suppressed. Since MyoD1 is a nuclear phosphoprotein [Tapscott et al., 1989], one possible mechanism of regulation could be phosphorylation. As suggested in this report, however, total phosphorylation of MyoD1 does not change dramatically during the course of C2C12 myoblast differentiation. Instead, we show that binding of a monoclonal antibody to an epitope in the conserved cysteine/histidine (C/H)-rich domain neutralizes the DNA-binding activity of MyoD1. Deletion mutation studies [Lassar et al., 1989] have shown that the C/H domain is not involved in binding DNA; therefore, we propose that it constitutes a regulatory element that could control the activity of MyoD1 by interacting with a putative suppressor.

MATERIALS AND METHODS

Cell Culture

C2C12 myoblasts were acquired from the American Type Culture Collection. C2C12 myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (HyClone), 1% bovine embryo extract (Gibco), 2 mM L-glutamine, 1 mM sodium pyruvate, minimal essential medium (MEM) nonessential amino acids (×100 solution, Gibco), MEM vitamins (×100 solution, Gibco), and 100 μg/ml gentamicin. The cells were induced to differentiate in similar medium lacking 20% fetal bovine serum and 1% bovine embryo extract, supplemented with 4% horse serum.

Preparation of trpE Fusion Proteins

The Alu I restriction fragment of murine MyoD1 cDNA (encoding amino acids 3–318) was inserted into the Sma 1 site of the pATH 10 trpE expression vehicle [Tapscott et al., 1988]. The construct (pEXD3) was used to transform *Escherichia coli* (C600). The bacteria were grown in tryptophan-free medium, and expression of the fusion protein was induced with 3-indoleacrylic acid. The fusion protein was isolated from bacterial lysates by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and excision of the band containing the 75-kDa fusion protein. Similar fusion proteins were generated from MyoD1 cDNA fragments encoding amino acids 60–219 (pEXD2) and amino acids 160–307 (pEXD1), and the 37-kDa trpE protein was generated (as a control) with the pATH 10 vehicle. To prepare antigen for enzyme-linked immunosorbent assay (ELISA), induced bacterial pellets were lysed by sonification in a buffer containing 5 mM Tris pH 7.4, 10% β-mercaptoethanol, clarified by centrifugation (100,000*g*, 60 min, 4°C), and equilibrated with 5 mM Tris pH 7.0 by dialysis.

Preparation of MyoD1 and MyoD1 Deletion Mutants

The Alu I fragment (encoding residues 3–318) and deletion mutants of the Alu I fragment (d3–56; d63–99; d102–135; d143–162; d3–56, termination Gln 167 (referred to as C/H–B-

HLH); d3–99, termination Gln 167 (referred to as B-HLH) of the MyoD1 cDNA were generated and used to produce RNA to program a rabbit reticulocyte in vitro translation system as described before [Tapscott et al., 1988]. Glutathione S-transferase fusion proteins (glu–MyoD1, glu–MyoD1 deletion mutants, and glu–myogenin) were generated and purified as described [Lassar et al., 1989]. T7 MyoD1 was generated using the pRK171a vector and isolated from bacterial extracts by ion-exchange column chromatography. These reagents were gifts of A. Lassar and H. Weintraub (Fred Hutchinson Cancer Research Center, Seattle).

Generation of Monoclonal Antibodies

To generate the monoclonal antibodies reactive with an epitope in the C/H region of MyoD1, Balb/c mice (6- to 8-week old) were initially immunized intraperitoneally with 100 μg trpE–MyoD1 (generated as described above) emulsified (1:1) in 200 μl complete Freund's adjuvant. Forty-five days later, the mice were immunized intraperitoneally in 100 μg MyoD1 emulsified (1:1) with 200 μl incomplete Freund's adjuvant; 45 days later, the mice were immunized intravenously with 100 μg trpE-MyoD1 dissolved in 200 μl normal saline. Four days later, splenocytes were harvested from the mice and used to generate hybridomas. To generate the monoclonal antibodies reactive with an epitope in the C-terminal domain, a similar protocol was used, but glu–MyoD1 deletion mutant 63–99 was used as an immunogen.

Sp2/0 Ag14 mouse myeloma cells were cultured in RPMI supplemented with 15% fetal bovine serum (heat inactivated) (Gibco), 2 mM L-glutamine, 150 ng/ml 8-azaguanine, 1 nM β-mercaptoethanol, and 100 μg/ml gentamicin. Two days prior to fusion, the Sp2/0 cells were cultured in the same medium lacking 8-azaguanine. Sp2/0 cells were mixed 1:5 with splenocytes, fused by centrifugation (800*g*, 3 min, 22°C) in polyethylene glycol 1500 (de-ionized) (Sigma). The fused cells were washed with serum-free RPMI and plated at a density of 10^4 cells/well in 96-well plates (100 μl/well). After 24-hr recovery in RPMI supplemented with 15% fetal bovine serum (heat-inactivated), 2 mM L-glutamine, 1 mM sodium pyruvate, MEM nonessential amino acid supplement (Gibco), MEM vitamin supplement (Gibco), 1 nM β-mercaptoethanol, 10 μg/ml insulin, 1 μg/ml transferrin, 1 ng/ml selenium, the cells were selected in the same medium containing 176 ng/ml aminopterin, 1 μg/ml thymidine, and 15 μg/ml hypoxanthine. Approximately 1,000 independent hybridomas (1 colony per well) were generated from a single spleen of an immune animal. Individual clones were expanded to 1-ml wells prior to screening.

Culture medium from each hybridoma was screened for monoclonal antibodies specific for MyoD1 by ELISA. Polyvinyl chloride 96 well plates (Falcon) were incubated with 100 μg/ml trpE–MyoD1 fusion protein or trpE protein (control) diluted in distilled water for at least 24 hr at 4°C. Prior to use, the plates were incubated at 37°C for 1 hr. The plates were then incubated (37°C, 1 hr) with 1 mg/ml bovine serum albumin (BSA) in Tris-buffered saline pH 7.4 (TBS). The plates were washed once with TBS containing 0.1% Tween 20 (TBS/Tween) and incubated with the culture supernatants (2 wells trpE-MyoD1; 1 well trpE) for 2 hr (37°C). The plates were washed twice with TBS/Tween, and incubated with alkaline phosphatase conjugated goat antimouse IgG/IgM (Boehringer/Mannheim) diluted in TBS containing 100 μg/ml BSA for 1 hr (37°C). The plates were washed three times with TBS/Tween, once with distilled water, and developed using 1 mg/ml paranitophenyl phosphate (PNP) (Sigma) in 100 mM glycine, 1 mM $MgCl_2$, 100 nM $ZnCl_2$ pH 10.4. Positive clones were scored as those secreting antibodies reactive with trpE–MyoD1 extracts but not trpE extracts. Positive clones were subcloned by limiting dilution, and Ascites fluid was generated by inoculating pristane-primed syngeneic mice.

Gel Electrophoresis and Immunoblotting

Cells were washed twice with PBS, then immersed directly on the culture plates in sample buffer (100 mM Tris (pH 6.8), 2% SDS, 100 mM dithiothreital (protein quantitation was performed prior to the addition of DTT, which was added last), 10% glycerol, 0.05% phenol red). After removal, the samples were heated to 100°C for 10 min prior to loading; 7–14% gradient polyacrylamide gels were prepared using the discontinuous buffer system described by Laemmli [1970]. For comparisons, equal numbers of cells (cell nuclei were used to quantitate fused cultures) were dissolved in sample buffer, examined by Coomassie stain for anomalies, and loaded.

Proteins were transferred from polyacrylamide gels to nitrocellulose membranes electro-

phoretically using a submersible device sold by Biorad (standard wire electrode mini or maxi model). Transfer buffer contained 50 g glycine, 7.2 g Tris base, 20% methanol (pH was not adjusted). After transfer, proteins were fixed to the membranes by immersion in 10% acetic acid, 25% isopropanol. The membranes were washed three times with TBS/Tween, then incubated (2 hr, 4°C, with shaking with TBS/Tween containing 10% horse serum (EIA grade) (Gibco). Ascites fluid containing monoclonal antibody was then added to a 1:1,000 dilution. Alternatively, hybridoma culture supernatants were made 0.1% in Tween 20 and used at full strength. The membranes were incubated with primary antibody with shaking (18 hr, 4°C). The membranes were then washed twice with TBS/Tween (5 min per wash) and incubated with second antibody. Two second antibodies were used (as indicated in the figure legends): ^{125}I-conjugated rabbit antimouse IgG, which was incubated with the membranes overnight (4°C); and peroxidase-conjugated goat antimouse IgG/IgM (Boehringer/Mannheim) or peroxidase-conjugated goat antirabbit IgG (Boehringer/Mannheim), which were incubated with the membranes for 1 hr (4°C). The membranes were then washed three times with TBS/Tween (30 min per wash for ^{125}I-conjugated antibodies; 10 min per wash for peroxidase-conjugated antibodies), then once (5 min) with 20 mM Tris (pH 7.4), 500 mM NaCl. The blots performed with ^{125}I-conjugated second antibody were autoradiographed using Kodak XAR-5 film and Dupont Lightening plus intensifying screens. The blots performed with peroxidase-conjugated second antibody were developed with 4-chloro-1-naphthol (500 μg/ml) in TBS containing 16% methanol and 0.15% H_2O_2. Rabbit polyclonal anti-MyoD1 was given by A. Lassar and H. Weintraub (Fred Hutchinson Cancer Research Center, Seattle).

Radioimmune Assays

Glu–MyoD1 and glu–MyoD1 mutant proteins were generated as described above; 1 μg of glu–myoD1 and equivalent molar quantities of mutant polypeptides (calculated from their relative molecular masses) were bound to 96-well polyvinyl chloride plates in distilled water (18 hr, 4°C, followed by 1 hr, 37°C). The wells were then incubated with 1 mg/ml BSA in TBS (1 hr, 37°C) to block unbound sites. The wells were washed once with TBS/Tween and incubated with 100 ng (1 μg/ml) of monoclonal antibody diluted in TBS containing 10% horse serum (37°C, 2 hr). The wells were then washed three times with TBS/Tween, and incubated with ^{125}I-conjugated rabbit antimouse Ig in TBS containing 10% horse serum (100 nCi/ml [100 μl per well], 37°C, 1 hr). The wells were washed three times with TBS/Tween, once with distilled water, dried, cut, and counted (γ emissions). All experimental determinations were performed in triplicate. Control values using equivalent dilutions of Sp2/0 (nonsecreting murine myeloma) culture supernatant were subtracted from the values rendered from each polypeptide.

Gel Retardation Assays

C2C12 nuclear extracts were prepared as previously described [Dignam et al., 1983]. MyoD1 (T7) or C2C12 nuclear extract was prepared incubated end-labeled probe, and resolved electrophoretically as described by Buskin and Hauschka [1989] and Lassar et al. [1989]. The right MyoD1 binding site (MEF 1 site), described by Buskin and Hauschka [1989] of the muscle creatine kinase enhancer was generated synthetically for use in these assays. Per 10-μl reaction mixture, 200 ng of MyoD1 (or 5 μg of extract), 10^5 cpm of probe, and 500 ng of poly dI-dC were used; 1 μl ascites fluid (2.5 μg mAb) was added of either mAb 1–8, 14–11, 8A3, or 6F1.

RESULTS

Characterization and Epitope Mapping of MyoD1 Monoclonal Antibodies

Hybridomas were produced by the fusion of spleen cells from a Balb/c mouse immunized with trypE–MyoD1 fusion protein (generated in *E. coli*) with Sp2/0 myeloma cells. Hybridoma supernatants were screened for anti-MyoD1 activity by ELISA. Positive clones were scored by their specific reactivity with a bacterial extract containing trpE-MyoD1 and lack of reactivity with an equivalent extract containing trpE protein. 13 independent clones were isolated that secreted monoclonal antibodies specifically reactive with the extracts containing trpE-MyoD1 fusion protein in this ELISA, all of which reacted with an epitope that was eliminated when the cysteine/histidine-rich (C/H) region of MyoD1 was deleted. Some of these also did not bind to MyoD1 deletion mutants missing other regions of the polypeptide (listed in Table I as complex). Subsequently, mice were immunized

TABLE I. Monoclonal Antibodies Generated Against MyoD1

Monoclonal antibody	Class	Residues containing epitope
1–8	IgG3	63–99, 143–162[a]
14–11	IgG3	63–99, 143–162[a]
18–12	IgM	63–99
1–4	IgM	63–99
11–11	IgM	63–99
19–16	IgM	63–99
16–10	IgM	63–99
6–3	IgM	63–99
6–2	IgM	63–99
11–4	IgG2b	complex
20–10	IgG2b	complex
15–6	IgM	NA
19–10	IgM	NA
8A3	ND	167–318
10A3	ND	167–318
4G1	ND	ND
4D3	ND	ND

[a]These residues are not essential but produce higher-affinity binding. Complex, antibodies that did not bind the C/H deletion mutant and at least one other. ND, not determined; NA, hybridomas that stopped secreting antibody before subcloning.

with glu–MyoD1 deletion mutant 63–99, and 4 monoclonal antibodies were generated, two of which react with an epitope in the C-terminal domain of the protein (Table I).

A panel of MyoD1 deletion mutants generated by the glutathione S-transferase expression system [Smith and Johnson, 1988; Lassar et al., 1989] was given by A. Lassar and H. Weintraub (Fred Hutchison Cancer Research Center). Glu-MyoD1 and mutant glu-MyoD1 polypeptides lacking residues 63–99, 102–135, or 143–162 were immunoblotted with mAb 1–8. Monoclonal antibody 1–8 reacted with native MyoD1 and deletion mutants lacking residues 102–135 or 143–162 but did not react with the deletion mutant lacking residues 63–99 (Fig. 1). Similar results were obtained with mAb 14–11 (not shown). These data indicate that the reactive epitope for mAbs 1–8 and 14–11 lies between residues 63–99. By contrast, a rabbit polyclonal anti-MyoD1 serum reacted equivalently with deletion mutants lacking residues 3–56 (not shown), 63–99, 102–135, or 143–162 (Fig. 1), suggesting that at least one epitope for this antiserum lies between residues 162–318. Neither the polyclonal antiserum nor mAbs 1–8 and 14–11 reacted with glu-myogenin.

Glu-MyoD1, glu-myogenin, and mutant glu-MyoD1 deletion mutants lacking residues 3–56, 63–99, 102–135, 143–162, 168–318 (Gln167), 3–101 and 168–318 (basic helix–loop–helix [B-HLH]), and 3–56 and 168–318 (C/H–B-HLH), were immunoblotted with mAb 8A3 (Fig. 2). This antibody reacted only with recombinant MyoD1 proteins bearing residues 168–318, indicating that the epitope for this antibody lies somewhere in this region. Similar results were obtained for mAb 10A3 (not shown). A map of the major structural features of MyoD1 and the two antibody binding segments is shown in Figure 3.

Differential Binding of MyoD1 Monoclonal Antibodies to Deletion Mutant Polypeptides

Glu–MyoD1 polypeptide and mutant glu–MyoD1 polypeptides lacking either residues 3–56, 63–99, 102–135, 143–162, 168–318 (Gln167), 3–56 and 168–318 (C/H–B-HLH [cysteine/histidine-rich, basic, helix–loop–helix domains]), and 3–99 and 168–318 (B-HLH) were bound to polyvinyl chloride (PVC) plates, and their relative binding efficiency to some of the monoclonal antibodies was determined by radioimmunoassay (RIA). As expected, the deletion mutant lacking residues 63–99 bound none of the monoclonal antibodies known to react with an epitope in this region of MyoD1 (Fig. 4A). In addition, mAbs 11–11, 6–2, 1–8, and 14–11 bound the mutant polypeptide terminating at residue 167 (Gln167), and the deletion 3–56 polypeptide terminating at residue 167 (C/H–B-HLH), but not the deletion 3–101 mutant, terminating at residue 167 (B–HLH) Fig. 4B). This is consistent with the epitope for these antibodies residing between residues 63–99. The reduction in binding efficiency observed with the smaller MyoD1 mutant polypeptides is probably due to an increase in the steric hinderance associated with binding to PVC plates.

Two interesting variations were observed in the relative efficiency of binding of the C/H monoclonal to these Glu–MyoD1 deletion mutants. First, while the removal of residues 143–162 (a segment containing the helix–loop–helix motif) had no effect on the binding of mAbs 18–12 (not shown) 11–11 and 6–2, binding of mAbs 1–8 and 14–11 was quantitatively reduced (Fig. 4). Although attenuated reactivity of mAb 1–8 with MyoD1 deletion mutants lacking residues 143–162 was observed by RIA, an obvious loss of signal in immunoblots of the same

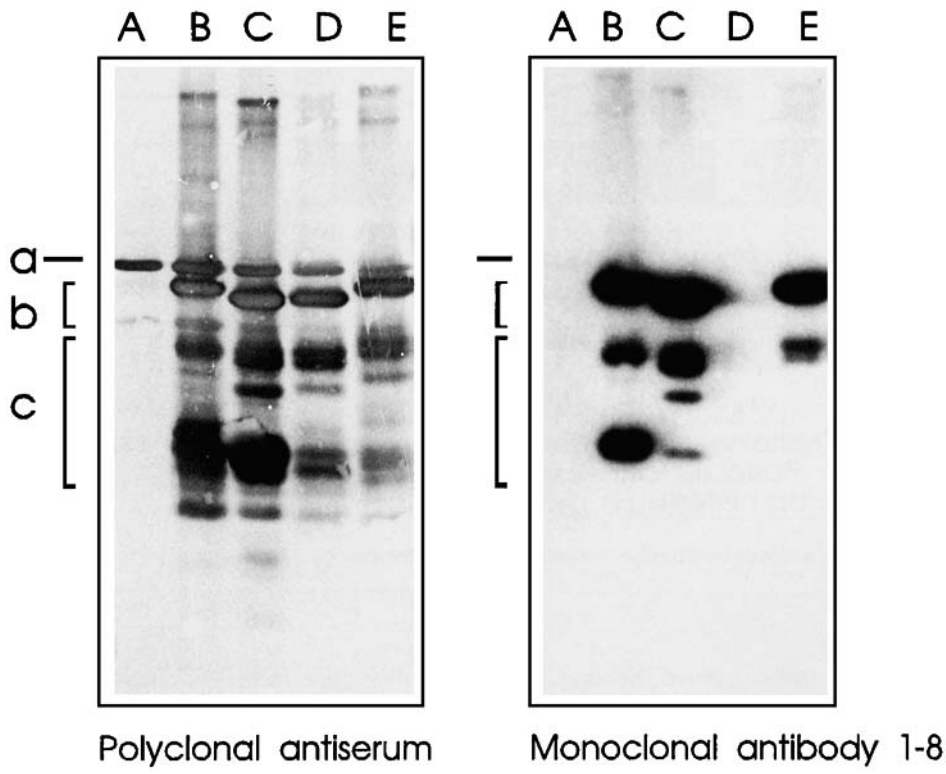

Fig. 1. Deletion mutant analysis of binding site for MyoD1 monoclonal antibody 1–8. A glutathione S-transferase expression system was used to generate and purify glu–MyoD1, glu–MyoD1 deletion mutants, and glu–myogenin (gifts of A. Lassar and H. Weintraub, Fred Hutchinson Cancer Research Center). The recombinant proteins were immunoblotted with either polyclonal rabbit anti-MyoD1 serum (**left panel**) or monoclonal antibody 1–8 (**right panel**). Bands: a, a contaminating protein that reacts with the polyclonal antiserum; b, glu–MyoD1, glu–myogenin, or glu–MyoD1 deletion mutants; c, products of incomplete translation or proteolysis. **Lane A,** glu–myogenin; **lane B,** glu–MyoD1 d102–135; **lane C,** glu–MyoD1 d143–162; **lane D,** glu–MyoD1 d63–99; **lane E,** glu–MyoD1.

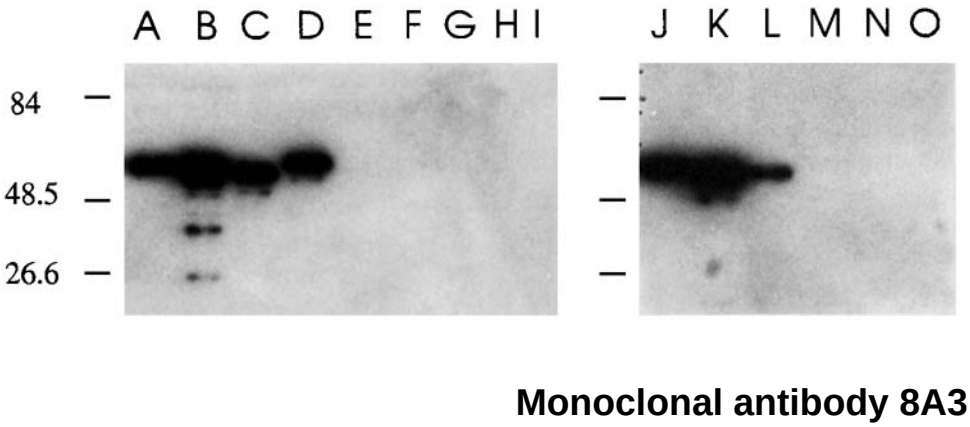

Fig. 2. Deletion mutant analysis of binding site for MyoD1 monoclonal antibody 8A3. Glu–MyoD1 and glu–MyoD1 deletion mutants were immunoblotted with mAb 8A3. **Lane A,** glu–MyoD1 d143–162; **lane B,** glu–MyoD1 d102–132; **lane C,** glu–MyoD1 d3–56; **lane D,** glu–MyoD1; **lane E,** glu–MyoD1 d3–56, d168–318 [C/H–B-HLH]; **lane F,** glu–MyoD1 d168–318 [Gln167]; **lane G,** glu–myogenin; **lane H,** glu–myogenin; **lane I,** glu–MyoD1 d3–101, d168–318 [B-HLH].

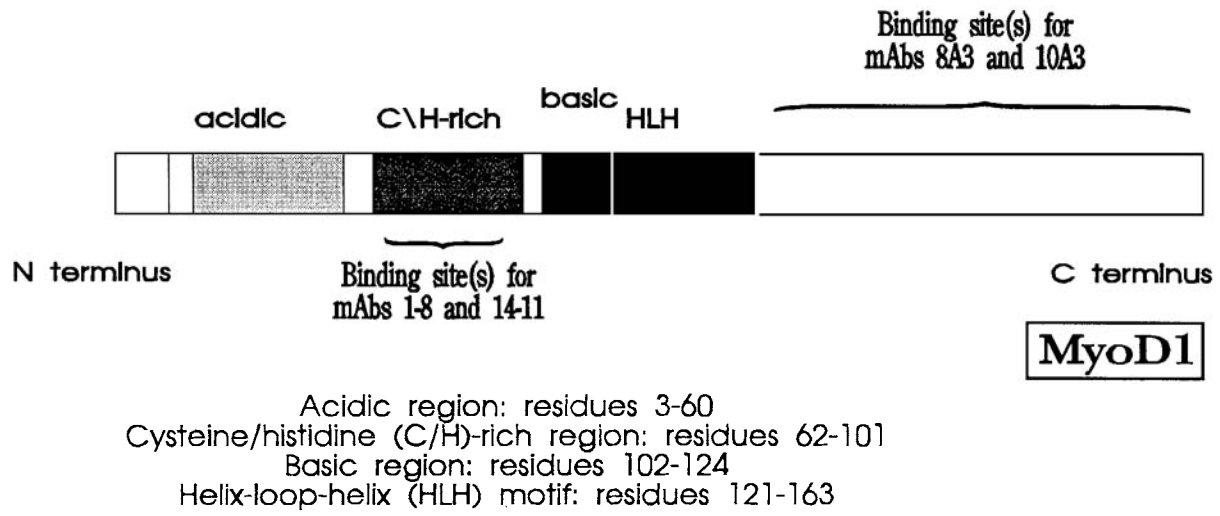

Fig. 3. Diagram of antibody binding sites in relationship to known structural features of MyoD1 (see also Tapscott et al., 1988).

deletion mutant was not observed. Second, removal of residues 102–135 (a region enriched in basic amino acids that directly mediates site-specific binding to DNA) significantly enhanced the binding of mAbs 11–11, 6–2, 1–8, and 14–11 (but not of mAb 11–4) in comparison to wild-type MyoD1 (Fig. 4).

Modulation of MyoD1 Total Protein or Phosphorylation Levels Is Not Tightly Associated With the Induction of C2C12 Myoblast Differentiation

C2C12 myoblasts [Blau et al., 1983] were grown in a mitogen-rich medium (20% fetal bovine serum and 1% bovine embryo extract) to reduce the incidence of spontaneous differentiation. The cells were induced to differentiate in mitogen-poor medium (4% horse serum). Whole cell homogenates of myoblasts at various phases of differentiation were resolved by SDS–PAGE and immunoblotted with mAb 8A3. Two bands of 38,000 and 42,000 M_r (using Sigma prestained markers, which may migrate slower than radiolabelled markers) were observed (Fig. 5B). Previous reports [Tapscott et al., 1988] demonstrated that phosphorylation of MyoD1 in vivo significantly reduces its rate of migration during SDS–PAGE, indicating that the slower migrating band represents phosphorylated MyoD1, while the faster migrating band represents hypophosphorylated MyoD1. The previous reports used metabolic labeling with ^{35}S-methionine and immunoprecipitation and were performed in such a way that rates of synthesis were determined rather than total protein accumulation. The total amount of phosphorylated MyoD1 in C2C12 myoblasts exceeded the amount of hypophosphorylated MyoD1, and only at a single point 24 hr post-induction did both forms appear in equivalent quantities (Fig. 5C). Sarcomeric myosin heavy chain (sMHC) (detected by mAb MF-20) [Bader et al., 1982] protein was first observed 48 hours post-induction, and continued to increase up to 72 hr. Sarcomeric MHC levels remained approximately the same at 72, 96, and 120 hr post-induction. By contrast, MyoD1 protein levels dropped after 72 hr, to roughly 50% of the levels observed in myoblasts by 120 hr. These data suggest that regulation of C2C12 myoblast differentiation and variations in MyoD1 total protein and phosphorylation levels are not tightly associated.

Monoclonal Antibodies to the Cysteine/Histidine-Rich Segment of MyoD1 Neutralize DNA-Binding Activity

To determine the effect of mAbs 1–8 (C/H), 14–11 (C/H), and 8A3 (C-terminal) on the site-specific DNA-binding activity of MyoD1, gel retardation assays were performed. MyoD1 (T7) (see Methods) was incubated with an end-labeled synthetic double-stranded 20-mer derived from the muscle creatine kinase gene enhancer and shown previously to contain a specific

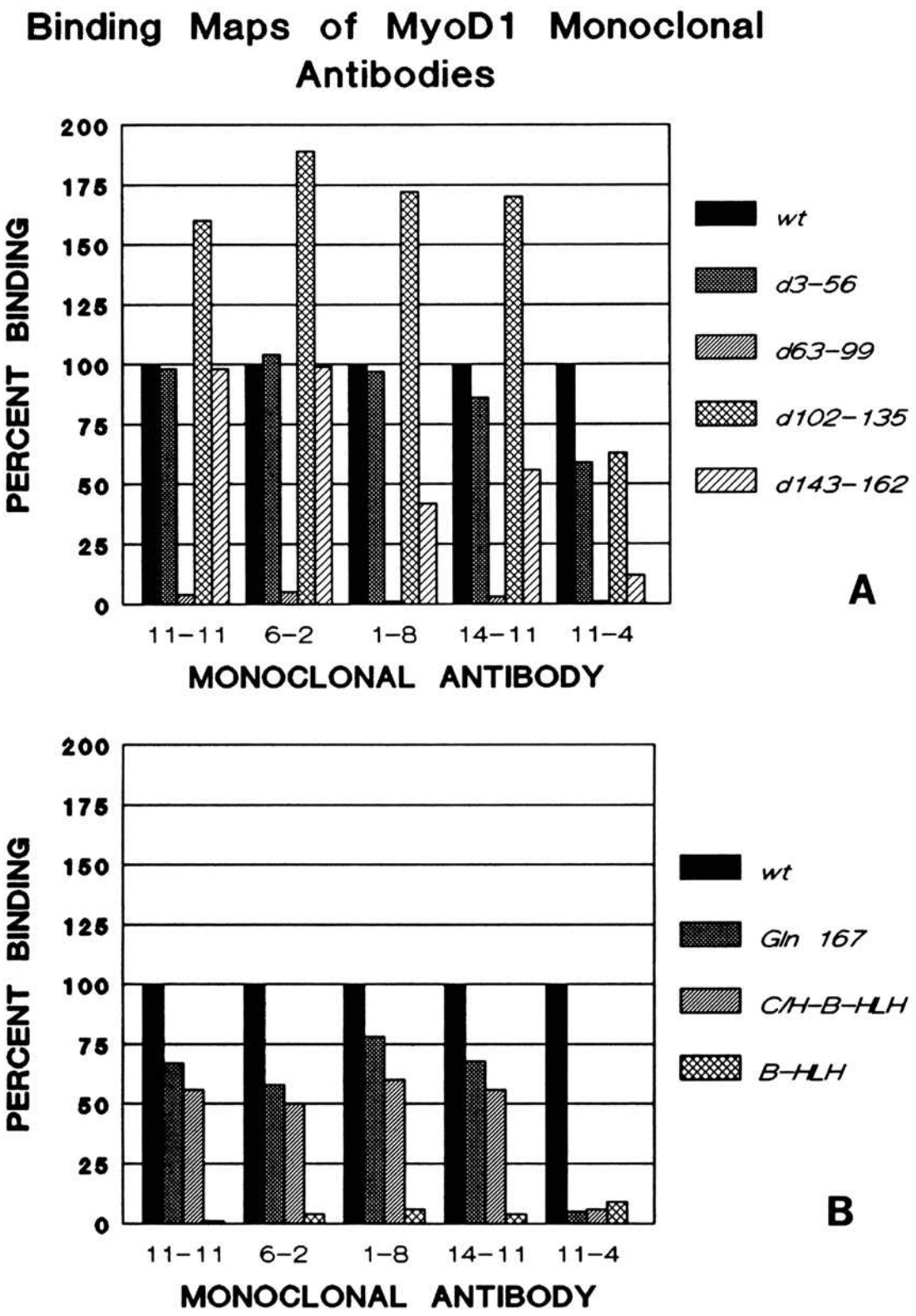

Fig. 4. Radioimmunoassay of relative binding of MyoD1 monoclonal antibodies to mutant polypeptides. The relative binding efficiency of some of the monoclonal antibodies to MyoD1 deletion mutants was evaluated. Molar quantities of Glu–MyoD1 and glu–MyoD1 deletion mutants representing 10-fold excess to antibody were bound to polyvinyl chloride plates and incubated with monoclonal antibodies. Binding was quantified by incubating the wells with ^{125}I-rabbit antimouse Ig second antibody, and counting γ emissions. Values are expressed as percent binding of glu–MyoD1 (wild type). Abbreviated names for mutants are described in the legend for Figure 2.

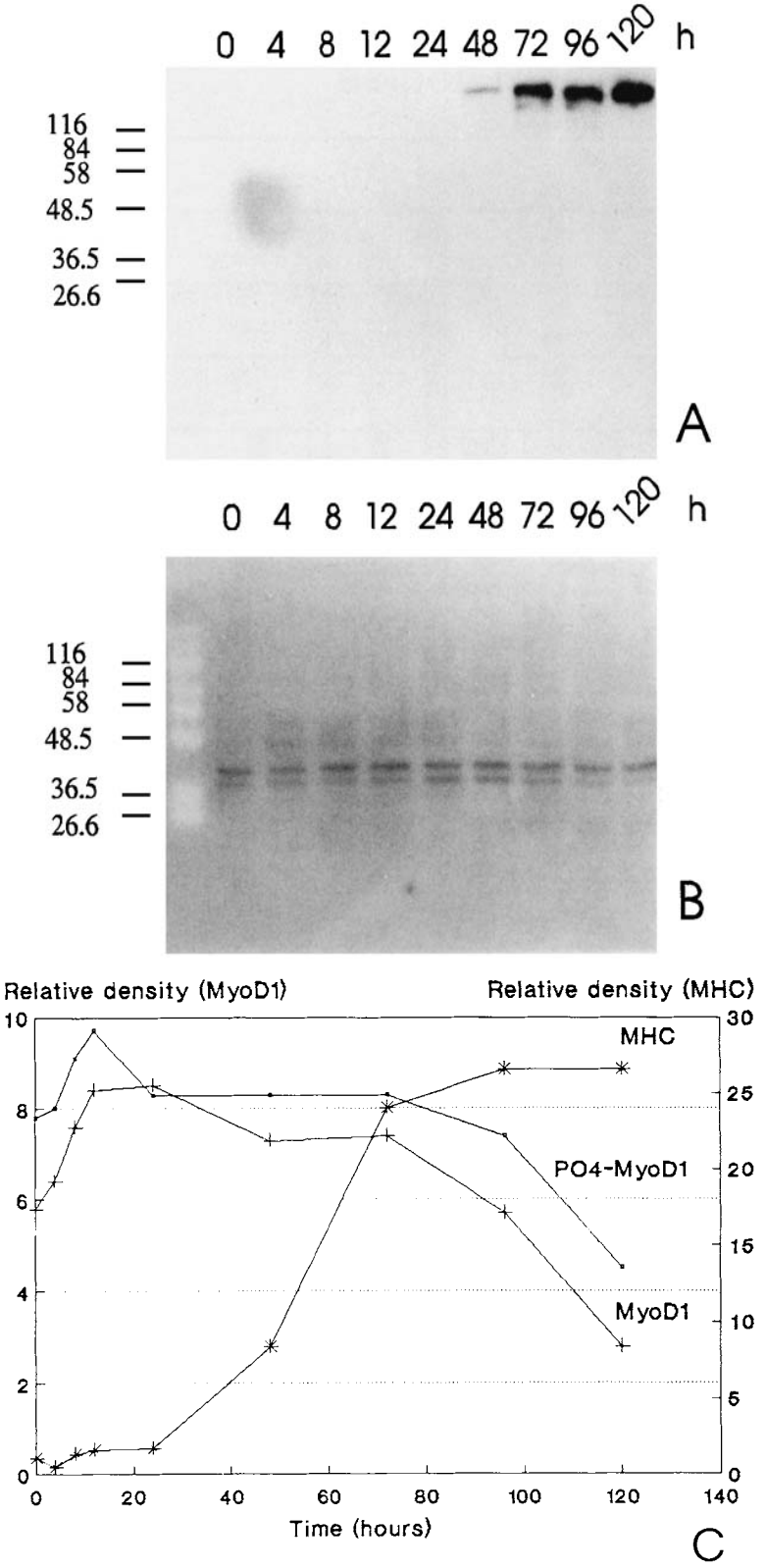

Fig. 5. Evaluation of MyoD1 protein expression during C2C12 cell differentiation. C2C12 myoblasts were induced to differentiate by culturing in mitogen-poor medium for various times (as indicated in hours). Whole cell homogenates were then resolved by SDS–PAGE, and parallel gels were transferred to nitrocellulose and immunoblotted with either mAb MF-20 (antisarcomeric myosin heavy chain **A**) or mAb 8A3 (anti-MyoD1 **B**). **C:** Microdensitometry scans of the data in A and B.

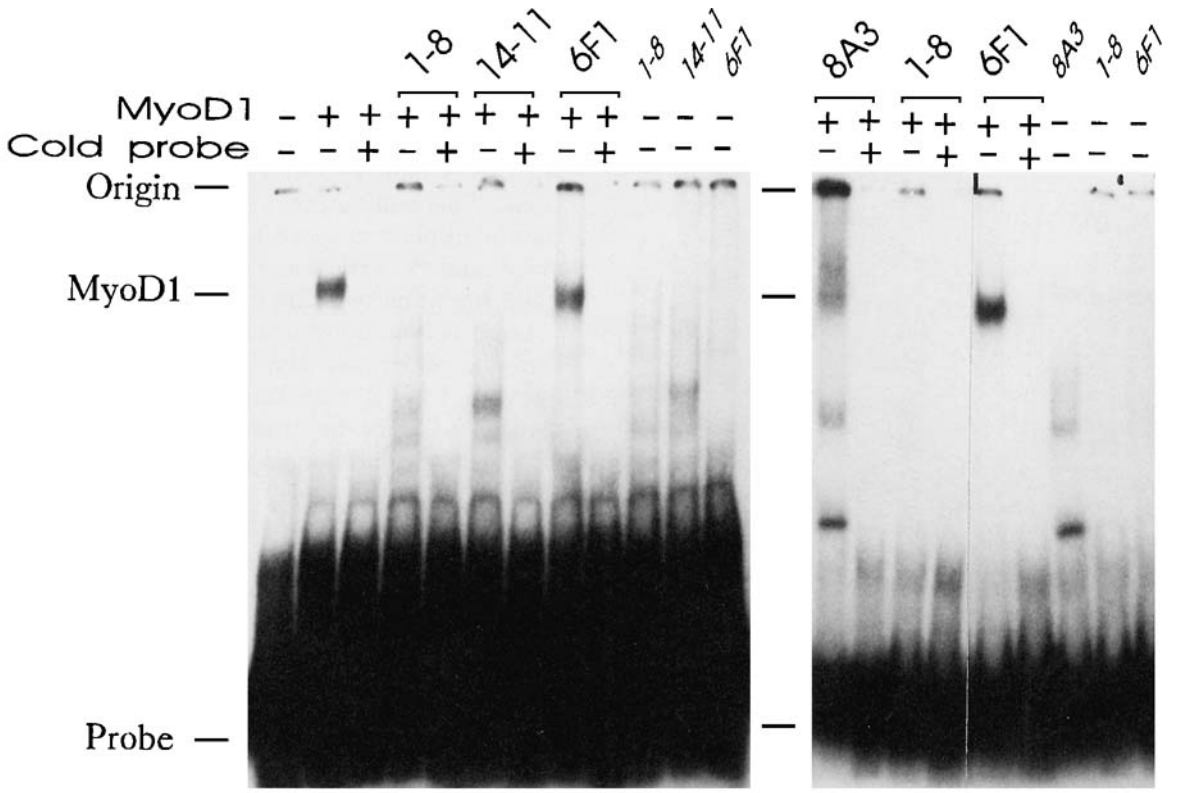

Fig. 6. Neutralization of DNA-binding activity of MyoD1 synthesized in *Escherichia coli* (homodimers) by monoclonal antibody 1–8 MyoD1 (generated in bacteria from a T7 promotor construct and given as purified protein by H. Weintraub, Fred Hutchinson Cancer Research Center) was assayed for binding to the MyoD1 binding sequence (MEF1 site) of the muscle creatine kinase (MCK) enhancer in the presence mAbs 1–8, 14–11, 8A3, or 6F1 (a control monoclonal IgG). Controls are also shown using antibody preparations incubated with probe in the absence of MyoD1, since some additional background bands are generated (note 8A3). Site-specific DNA binding of MyoD1 was inhibited by mAbs 1–8 and 14–11, whereas 8A3 produced a supershifted band at the gel origin and a minor one migrating slightly slower than the MyoD1 band. Control antibody 6F1 produced no change in MyoD1 binding to DNA.

binding site for MyoD1 [Buskin and Hauschka, 1989; Lassar et al., 1989]. Reactions were performed alone and in the presence of mAbs 1–8, 14–11, 8A3, and 6F1 (a control monoclonal IgG reactive with histone H1). The control antibody (6F1), added in equivalent mass and volume, had no effect on MyoD1 binding to probe (Fig. 6). Monoclonal antibodies 1–8 and 14–11 inhibited the formation of a complex between probe and MyoD1, while mAb 8A3 produced a supershift. Two supershifted bands were observed in the presence of mAb 8A3: one migrated at the position of an antibody linked pair of MyoD1 dimers, the second (much darker) migrated only to the origin, and probably represents a polymer of alternating antibody and MyoD1 dimer. Both supershifted bands were competed by unlabeled probe. As only the monoclonal antibodies reactive an the epitope in the C/H region of MyoD1 inhibit DNA binding (and similar inhibitory effects were observed with mAbs 18–12 and 16–10), their effect can be considered site specific. This effect also has not been observed with polyclonal anti-MyoD1, which also produces a supershift [Lassar et al., 1989]. The effect is not restricted to MyoD1 dimers, as DNA binding by MyoD1 complexed with E12/E47 in cell extracts (Lassar et al., 1991) is also inhibited (Fig. 7). This is also shown in a report from another laboratory [Sartorelli et al., 1990]; the antibody referred to as anti-MyoD1 is mAb 1–8.

DISCUSSION

In the penultimate paragraph of his 1963 review, Konigsberg [1963] identified two features of cultured skeletal myoblasts that renders them particularly useful for studies of growth and differentiation: clones of proliferating skeletal myoblasts, although free of any obvious features that distinguish them from

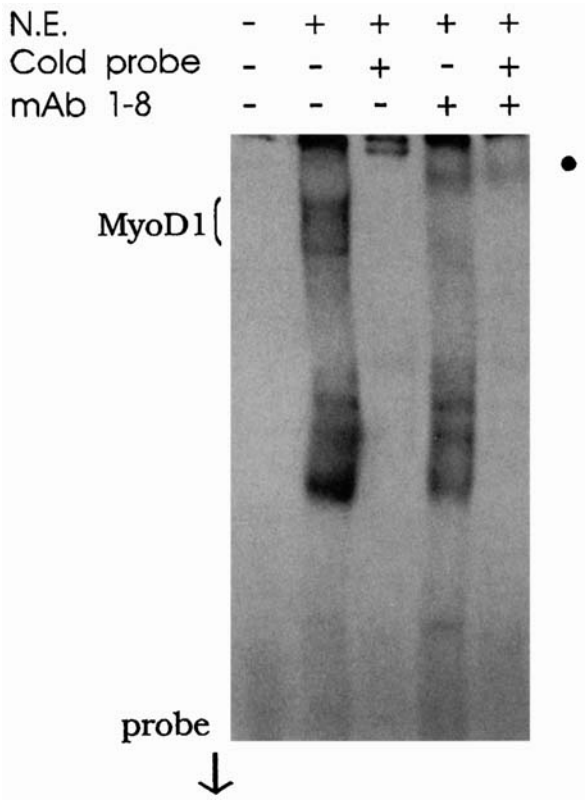

Fig. 7. Neutralization of DNA-binding activity of MyoD1 synthesized in vivo (heterodimers) by monoclonal antibody 1–8. Nuclear extracts from C2C12 cells were assayed by electrophoretic mobility shift assay for binding to complementary oligomers derived from a MyoD1-binding site in the muscle creatine kinase enhancer (as above). Monoclonal antibody 1–8 inhibited formation of the heterodimeric MyoD1-DNA complexes formed in vivo. The extra background band (indicated with a solid dot) is contributed by the Ascites and is not eliminated by competition with cold probe.

fibroblasts, maintain their determined myogenic state in culture; furthermore, the ability of skeletal myoblasts to differentiate biochemically and morphologically into myotubes makes them stand out, even in living cultures, as myogenic cells. Rendering the problem similar in form to the Heisenberg uncertainty principal, Konigsberg emphasized that the identification of myogenic clones was possible without fixation, and therefore, the growth and recovery of proliferating clones of cells determined to differentiate into muscle was possible. This characteristic eventually proved useful, 20 years later, in identifying through serial transfection the myogenic determination locus myd [Pinney et al., 1988]. Although the cloning of four other myogenic determination genes (MyoD1, myogenin, myf-5, and MRF4/myf-6/herculin) did not require serial transfections, these genes were selected nonetheless for their ability to confer myogenic determination to proliferating cells rather than for their ability to confer the terminally differentiated skeletal muscle phenotype. Studies of these genes are therefore concerned with their ability to execute two distinct functions of determination: (1) maintenance of the imminent phenotype while the myoblasts proliferate, and (2) expression of the myogenic program when the cells differentiate.

Immunoblot analysis with mAb 8A3 revealed that phosphorylated MyoD1 is the predominant form in C2C12 myoblasts and differentiated myocytes. At 24 hr post-induction, the levels of phosphorylated and hypophosphorylated MyoD1 were transiently equal, whereas before and after this time point the levels of phosphorylated MyoD1 exceeded those of hypophosphorylated MyoD1. The functional significance of this variation is not obvious. Active MyoD1 exists in vivo as a dimer with the product of the E2A gene, and phosphorylation of MyoD1 is enhanced by this binding [Lassar et al., 1991]. Consequently, hypophosphorylated MyoD1 may represent MyoD1 homodimers, whose presence could have a specific role in inducing the myoblasts to differentiate. Further experiments will be necessary to confirm this interpretation.

Although mAb 1–8 does not react with the DNA-binding region of the polypeptide, it does inhibit DNA-binding by MyoD1 in gel retardation assays. This contrasts results obtained with polyclonal anti-MyoD1 and mAbs which react C-terminal to the HLH domain (e.g., 8A3), which produce tertiary bands in retardation assays, a result of simultaneous binding by antibody and DNA. An alternate hydropathy analysis [Hopp and Woods, 1981] of residues 63–99 of murine MyoD1 revealed a single antigenic sequence of 6 amino acids (from residues 76–81). This predicted antibody-binding site would lie 30 amino acids away from the DNA binding domain of MyoD1, indicating that local steric hinderance could not account for the neutralizing effect of the antibodies. These data suggest that the C/H region may serve as a regulatory domain of MyoD1, that operates either through an intramolecular mechanism and/or by mediating the binding of another regulatory molecule. In support of the former possibility, enhanced binding of mAb 1–8 and others reactive with the C/H region to mutant MyoD1 lacking the basic, DNA-binding region of the polypeptide suggests that the basic region may fold into contact with their

epitope. Further experiments should elucidate the structural significance of the C/H region, and identify any cellular components that bind to it and regulate MyoD1 activity in vivo.

ACKNOWLEDGMENTS

The authors thank A. Lassar, R. Davis, and H. Weintraub for biological materials, suggestions, and reading an early version of this manuscript. This work was supported by grants HL40659 and HL43583 from the NIH/NHLBI.

REFERENCES

Bader D, Masaki T, Fischman D (1982): Biochemical analysis of myosin heavy chain during avian myogenesis in vivo. J Cell Biol 95:763–770.

Benezra R, Davis R, Lockshon D, Turner D, Weintraub H (1990): The protein id: A negative regulator of helix–loop–helix DNA binding proteins. Cell 61:49–59.

Blau H, Baltimore D (1991): Differentiation requires continuous regulation. J Cell Biol 112:781–783.

Blau H, Chiu C-P, Webster C (1983): Cytoplasmic activation of human nuclear genes in stable heterokaryons. Cell 32:1171–1180.

Braun T, Buschhausen-Denker G, Bober E, Tannisch E, Arnold HH (1989a): A novel human muscle factor related to but distinct from MyoD1 induced myogenic conversion in 10T1/2 fibroblasts. EMBO J 8:701–709.

Braun T, Bober E, Buschhausen-Denker G, Kohtz DS, Grzeschik K-H, Arnold HH (1989b): Differential expression of myogenic determination genes in muscle cells: Possible autoactivation by Myf gene products. EMBO J 8:3617–3625.

Braun T, Bober E, Winter B, Rosenthal N, Arnold HH (1990): Myf-6, a new member of the human gene family of myogenic determination factors: Evidence for a gene cluster on chromosome 12. EMBO J 9:821–831.

Buskin JN, Hauschka SD (1989): Identification of a myocyte nuclear factor which binds to the muscle-specific enhancer of mouse creatine kinase gene. Mol Cell Biol 9:2627–2640.

Colmenares C, Stavnezer E (1989): The ski oncogene induces muscle differentiation in quail embryo cells. Cell 59:293–303.

Davis RL, Weintraub H, Lassar AB (1987): Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51:987–1000.

Dignam J, Lebovitz R, Roeder R (1983): Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl Acids Res 11:1475–1489.

Doering JL, Fischman DA (1977): A fusion promoting macromolecular factor in muscle conditioned medium. Exp Cell Res 105:437–443.

Edmondson DG, Olson EN (1989): A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev 3:628–640.

Ewton DZ, Florini JR (1980): Relative effects of the somatomedins, multiplication-stimulating activity, and growth hormone on myoblasts and myotubes in culture. Endocrinology 106:577–583.

Ewton DZ, Florini JR (1981): Effects of somatomedins and insulin on myoblast differentiation in vitro. Dev Biol 86: 31–39.

Gospodarowicz D, Weseman J, Moran JS, Lindstrom J (1976): Effect of fibroblast growth factor on the division and fusion of bovine myoblasts. J Cell Biol 70:395–405.

Hopp TP, Woods KR (1981): Prediction of protein antigenic determinants from amino acid sequences. Proc Natl Acad Sci USA 78:3824–3828.

Konigsberg IW (1963): Clonal analysis of myogenesis. Science 140:1273–1284.

Konigsberg IR, Sollman PS, Mixter LO (1978): The duration of the terminal G_1 of fusing myoblasts. Dev Biol 63:11–26.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of bacteriophage T4. Nature 227:680–685.

Lassar AB, Buskin JN, Lockshon D, Davis RL, Apone S, Hauschka, SD, Weintraub H (1989): MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bond to the muscle creatine kinase enhancer. Cell 59:823–831.

Lassar AB, Davis RL, Wright WE, Kadesch T, Murre C, Voronova A, Baltimore D, Weintraub H (1991): Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell 66:305–315.

Linkhart TA, Clegg CH, Hauschka SD (1980): Control of mouse myoblast commitment to terminal differentiation by mitogens. J Supramol Struct 14:384–498.

Linkhart TA, Clegg CH, Hauschka SD (1981): Myogenic differentiation in permanent clonal mouse myoblast cell lines: Regulation by macromolecular growth factors in the culture medium. Dev Biol 86:19–30.

Massague J, Cheipetz S, Endo T, Nadel-Ginard B (1986): Type transforming growth factor is an inhibitor of myogenic differentiation. Proc Natl Acad Sci USA 83:8206–8210.

Miner JH, Wold B (1990): Herculin, a fourth member of the MyoD family of myogenic regulators. Proc Natl Acad Sci USA 87:1089–1093.

Mueller P, Wold B (1989): In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science 246: 780–785.

Murre C, McCaw PS, Baltimore D (1989a): A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell 56:777–783.

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, Weintraub H, Baltimore D (1989b): Interaction between heterologous helix–loop–helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58:537–544.

Nadal-Ginard B (1978): Commitment, fusion, and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. Cell 15:855–864.

Okazaki K, Holtzer H (1966): Myogenesis, fusion, myosin synthesis, and the mitotic cycle. Proc Natl Acad Sci USA 56:1484–1490.

Olson E, Sternberg E, Hu J, Spizz G, Wilcox C (1986): Regulation of myogenic differentiation by type β transforming growth factor. J Cell Biol 103:1799–1805.

O'Neill MC, Stockdale FE (1972): A kinetic analysis of myogenesis in vitro. J Cell Biol 52:52–65.

Pinney DF, Pearson-White SH, Konieczny SF, Latham KE, Emerson CP (1988): Myogenic lineage determination and

differentiation: Evidence for a regulatory gene pathway. Cell 53:781–793.

Rhodes SJ, Kozniecny SF (1989): Identification of MRF4: A new member of the muscle regulatory factor gene family. Genes Dev 3:2050–2061.

Sartorelli V, Webster KA, Kedes L (1990): Muscle specific expression of the cardiac α-actin gene requires MyoD1, CArG-box factor and Sp-1. Genes Dev 4:1811–1822.

Slater CR (1976): Control of myogenesis in vitro by chick embryo extract. Dev Biol 50:264–284.

Smith DB, Johnson KS (1988): Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene 67:31–40.

Tapscott SJ, Davis RL, Thayer MJ, Cheng P-F, Weintraub H, Lassar AB (1988): MyoD1: A nuclear phosphoprotein requiring a myc homology to convert fibroblasts to myoblasts. Science 242:405–411.

Tapscott SJ, Lassar AB, Davis RL, Weintraub H (1989): 5-bromo-2′-deoxyuridine blocks myogenesis by extinguishing expression of MyoD1. Science 245:532–535.

Wright WE, Sassoon DA, Lin VK (1988): Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell 56:607–617.

Yaffe D (1968): Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc Natl Acad Sci USA 61:477–483.