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Regulation of the mouse desmin gene: transactivation by MyoD, myogenin, MRF4 and Myf5

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ABSTRACT

Desmin, the muscle specific intermediate filament (IF) protein, is expressed at low levels in myoblasts and at the onset of differentiation its expression increases several fold. In an effort to explore the mechanism involved in the tissue-specific and developmentally regulated expression of desmin, we have isolated the mouse desmin gene. Sequence analysis of 976 bp 5′ flanking region revealed several potential cis-acting elements: 1) Three E boxes (MyoD binding sites), namely, E_1, E_2 and E_3, located at −79, −832 and −936, respectively; 2) one MEF2 binding site at −864; 3) a region with homology to M-CAT motif at −587; 4) several GC boxes. Transient transfections with various 5′ flank deletion mutants into C_2C_{12} muscle cells have revealed both positive and negative elements that seem to be involved in the expression of desmin. The first 81 bp upstream of the transcription initiation site, including E_1 box, were sufficient to confer muscle specific expression of the desmin gene. The maximal level of expression was achieved by the construct containing up to −897 base pairs. The region between −578 to −976 behaves as a classical enhancer in the absence of which the region between −578 and −81 suppresses CAT activity. Gel electrophoretic mobility shift assays using both C2C12 muscle cell nuclear extracts as well as in vitro translated myoD/E12 and myogenin/E12 heterodimers, showed that both myoD and myogenin bind to the proximal E1 and the distal E2 boxes of the desmin promoter and enhancer respectively. Co-transfection of myoD, myogenin, MRF4 and Myf5, with the desmin-CAT construct into 10T-1/2 cells demonstrated that all these factors could transactivate desmin gene expression.

INTRODUCTION

Desmin, the muscle specific intermediate filament (IF) protein is encoded by a single gene (1) which is expressed in every muscle cell type (for review see 2). It is one of the earliest known myogenic markers (3–5). During mouse development, desmin first appears at 8.25 days post coitus (dpc) in the neuroectoderm where it is transiently expressed with vimentin and keratin. It appears then in the heart rudiment at 8.5 dpc where no other muscle specific gene product has earlier been found, and at 9 dpc appears in the myotome (6). Desmin is expressed at low levels in satellite cells (7) and replicating myoblasts (5) which express high levels of the growth regulated IF protein vimentin (1,4). During in vitro myogenesis, desmin is up and vimentin is down regulated and it has been shown that this pattern of expression is controlled at the mRNA level (1). Characterization of the 5′ upstream sequences of several IF subunit genes have been reported (8–14; for review see 15), including the hamster (16) and human desmin (17). The factors which regulate these genes have not been identified yet. Several cis-acting regulatory regions necessary for muscle specific expression have been identified. These include the MEF1 and MEF2 sequences of mouse muscle creatine kinase (MCK) which are recognized by the myocyte-specific enhancer binding factor 1 (18), and 2 (19), respectively; and the M-CAT motif in cardiac troponin T (20). Muscle specific enhancers have been identified in the distal 5′ upstream of the MCK gene (18,19), in the proximal 5′ regions of the skeletal a-actin (21,22) and acetylcholine receptors (23–25), in the intron of the quail troponin I (26) and the 3′ end of the rat myosin light chain 1/3 gene (27).

Recent studies have identified a family of myogenic regulators which include myoD (28), myogenin (29,30), MRF4 (31–33) and Myf5 (34) that can convert 10 T1/2 fibroblasts to myoblasts through a mechanism that requires a basic region and a domain with homology to the myc family with potential helix-loop-helix (HLH) conformation (35; for review see 36). It was further shown that hetero-oligomeric complexes formed between myoD and the ubiquitous HLH proteins E12 and E47, which bind the immunoglobulin kappa chain enhancers, bind the MCK enhancer (37), previously referred to as MEF1 (18). Myogenin also binds this enhancer with comparable affinity as heterodimer with E12 (38–39).

To investigate the mechanism by which the mouse desmin gene is regulated during myogenesis, we have isolated the gene and characterized 976 bp of 5′ flanking sequences. We show that both positive and negative elements regulate the tissue-specific and developmental myogenic expression of mouse desmin. An upstream (−976 to −578) muscle specific enhancer seems to be responsible for the high level of desmin expression. MyoD, myogenin seem to bind the observed desmin E boxes and all the

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myogenic regulatory factors identified so far (myoD, myogenin, MRF4 and myf5) could transactivate the desmin-CAT fusion genes in cell culture.

MATERIALS AND METHODS

Isolation of genomic clones and DNA sequence analysis

Mouse desmin genomic clones were isolated by screening with a mouse desmin cDNA probe, a charon 4A mouse genomic library, constructed by partial EcoRI digestion of mouse Balb/c spleen DNA.

A 2.0 kb HindIII fragment containing the 5'-upstream region and part of exon 1 of the mouse desmin gene was subcloned into M13 vector and sequenced by the dideoxynucleotide method (40).

Construction of des-CAT expression vectors

A 1.2 kb HindIII-XhoI fragment containing 976 bp 5'-upstream region and 0.2 kb of exon 1 of the mouse desmin gene was subcloned into HindIII-SalI sites of pBasicCAT (Promega), a promoterless expression vector carrying the bacterial chloramphenicol acetyltransferase (CAT) gene. The other des-CAT expression vectors were derivatives of this one named mD976. The structures of all constructs were confirmed by sequencing.

For construction of 5'-deletion des-CAT mutants, appropriate restriction enzyme sites were used in combination with klenow and T4 DNA polymerase to generate 5'-upstream deletion mutants. These include NsiI (−527 bp, −798 bp), StyI (−574 bp, −833 bp), AvaII (−367 bp), NarI (−61 bp), BamHI (−82 bp), RsaI (897 bp) which together with XhoI generated mutants mD897, mD837, mD798, mD578, mD533, mD370, mD81 and mD61, respectively.

Internal deletion mutants were generated from mD976 with KpnI/EagI (−492/−244) digestion. The extruding termini were blunted with T4 DNA polymerase and different length of internal deletions were generated. All the clones obtained were sequenced to confirm the point of deletion.

To test the enhancer activity, a fragment from HindIII-NsiI of mD976 clone was subcloned in both orientations into the polylinker region of pBLCAT2, which is driven by the thymidine kinase gene promoter.

Cells and culture conditions

The mouse muscle cell line C_2C_{12} (41) was derived from the C_2 cell line (42) and was obtained from the American Type Culture Collection. These cells were maintained in growth medium (GM) composed of Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum and 5% chick embryo extract. To induce differentiation, the confluent culture cells were switched to differentiation medium (DM) containing DMEM with 10% horse serum. 10 T1/2 and NIH 3T3 cells were maintained in DMEM with 10% fetal bovine serum.

Cell Transfection and CAT Assays

Transfection of plasmid DNAs into C2 cells was performed by using the calcium phosphate coprecipitation procedure (43). 5×10^5 C_2C_{12} myoblast cells were plated on 10-cm petri dishes in GM. Approximately 24 hours later, fresh GM was added to the cells 1−3 hours before transfection. Calcium phosphate precipitates were prepared by conventional methods (44). 15 mg of testing plasmids were used in each transfection. To correct the possible variations between experiments, 4 mg plasmid of the β-galactosidase reporter gene linked to the Rous sarcoma virus long terminal repeat (RSVβ-gal) were cotransfected with the testing plasmids as an internal control. Cells were kept in the GM medium in the presence of the precipitated DNA for 12 hours before changing to 10 ml fresh GM. Undifferentiated cultures were harvested 48 hours after transfection. For myotube cultures, cells were changed to DM medium 36 hours after transfection and harvested 48 hours later. Cells were harvested in 1 ml of TEN solution (Tris-HCl, pH 7.4, 1 mM EDTA, 0.15 M NaCl). The cell pellet was resuspended in 100 μl of 250 μM Tris-HCl, pH 7.8, and lysed by three cycles of freezing and thawing. The samples were centrifuged for 10 min and the resulting supernatants were assayed for CAT and β-galactosidase activity.

CAT assays were performed as described by Gorman et al. (45) and were normalized on the basis of β-galactosidase activity performed as described (46). Levels of CAT activity were quantitated by a Betascore 603 blot analyzer (Betagen). For quantitative analysis, the promoter activity of each desmin-CAT construct was defined as the pecentage of conversion $\{[^{14}C]AcCm/([^{14}C]AcCm+[^{14}C]Cm)\}$ in the CAT assays.

To test for the trans-activation effect on des-CAT by myogenic factors, 15 μg of the reporter plasmids mD976 were cotransfected with 5 mg expression vector pEMSVscribe (28) where transcription is driven by the Maloney Sarcoma virus long terminal repeat (MSV-LTR) which carried the cDNA insert of myoD (28), myogenin (29,30), MRF4 (33) and myf5 (31). Transfection and CAT assays were performed as described above.

Preparation of nuclear extracts

C_2C_{12} myoblasts were harvested when almost confluent, and myotube were harvested after two days in differentiation media. Nuclear extracts were prepared by the method of Dignam et al. (47). In brief, cells were washed in cold phosphate-buffered saline, and were rapidly resuspended in 3 ml of lysis buffer (10 mM Hepes, pH7.9, 1.5 mM MgC_{12}, 10 mM KCl, 0.5 mM DTT). The cells were then homogenized with 20 strokes of a Dounce homogenizer (type B), spun at 4,000 rpm in cold room for 5 minutes. The volume of the pellet was measured and was resuspended in 3 volume of extraction buffer (20 mM Hepes pH7.9, 25% glycerol, 0.55 M NaCl, 1.5 mM MgC_{12}, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). The solution were then transfered to a Dounce homogenizer and stroke 10 times. Let sit on ice for 30 minutes with periodically shaking. The nuclei were then pelleted at 10.000 rpm for 15 minute and the supernatant was dialysed against 50 volumes of dialysis buffer (15 mM Hepes pH7.9, 40 mM KCl, 1 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 20% glycerol) overnight in the cold room. The next morning, spin the supernatant at 10,000 rpm and store in 70°C immediately.

In vitro transcription and translation

RNA synthesis was performed using 1 mg of linearized bluescript plasmids carrying myoD and myogenin inserts or pEMSV plasmids carrying E12 inserts and 10 units of T3 polymerase in a 25 μl reaction volume for 1 hour at 37°C as suggested by the manufacturer (Stratagene, mRNA Capping Kit). After phenol/chloroform extraction, the RNA was precipitated and resuspended in 25 μl of TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA). 2 μl of the RNA was used for in vitro translation using rabbit reticulocyte lysate supplemented with 10 μCi ^{35}S-met in a 50 μl reaction volume as recommended by the manufacturer (Promega). The reactions were allowed to proceed for 1 hour

at 30°C. The translation products were analyzed on 10% SDS polyacrylamide gel.

Electrophoretic mobility shift assays (EMSA)

For the EMSAs with in vitro translated products, fragment probes that contained E1 and E2 box regions were obtained by digesting the plasmid pmd976 with BamHI-NarI and HindIII-NsiI respectively, and labeled with [α-^{32}P] dATP by Klenow fragment of DNA polymerase I. The DNA binding was conducted in 20 ul final volumes in medium salt [Final concentration: 40 mM KCl, 15 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM DTT, 5% glycerol]. The reaction mix contained 2×10^4cpm probe and 2 μl of unlabeled in vitro translated products, with 1 μg of poly(dI-dC):poly(dI-dC) (Pharmacia Inc.) as a non-specific carrier for 15 minutes at room temperature. Unlabeled competitor DNA or antibody was added to the binding reaction 10 min before the labeled probe. DNA-protein complexes were resolved on a 4% polyacrylamide gel in 1× TBE (50 mM Tris borate pH 8.3, 1 mM EDTA). The gels were dried and autoradiographed with intensifying screens at −70°C.

For EMSAs with nuclear extracts, the 20mer synthesized oligonucleotides, shown in the legend of figure 6, were used as probes.

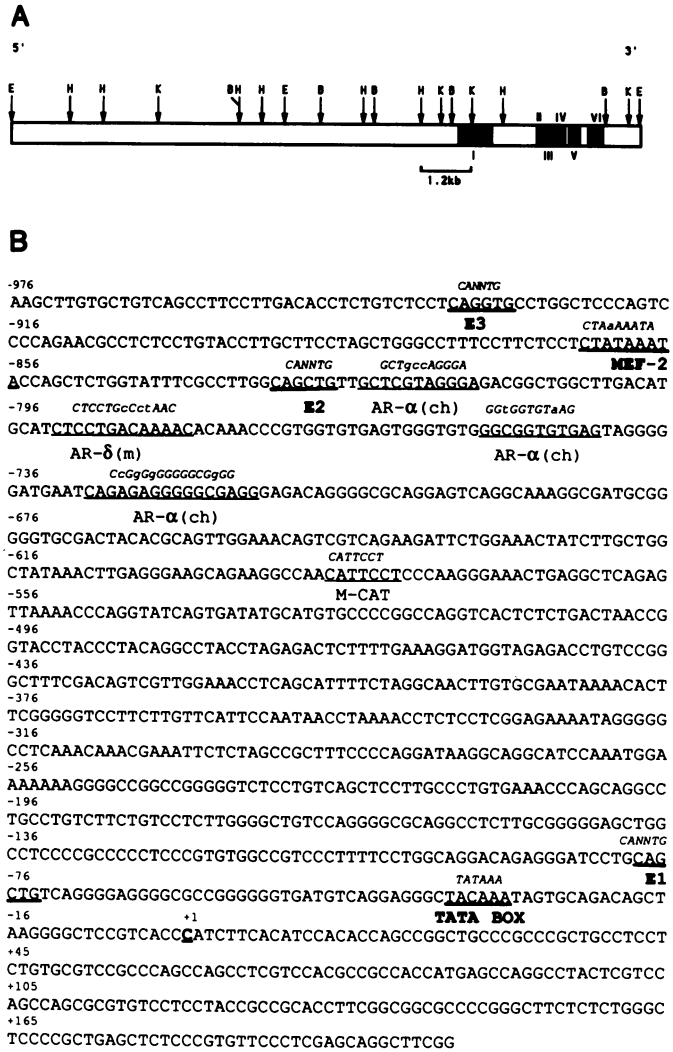

Figure 1. A. Restriction map of the mouse desmin genomic clone. This clone extends from −12 kb upstream of the transcription initiation site to exon 6. The position of exon I was assigned by sequencing; that of the rest exons was deduced from the corresponding position in the hamster desmin gene (57). The sequence in panel B corresponds to the fragment HindIII-XhoI as indicated by the bracket below the map. **B:** BamHI; E: EcoRI; H: HindIII; K: KpnI. B. 5′ flanking sequence of the mouse desmin gene. The nucleotide sequence from −976 to +205 is shown. Putative cis-acting elements are underlined and the corresponding consensus is shown above each sequence. Three E boxes, E_1, E_2, E_3 (37,58), a MEF2 site (19) and M-CAT motif (20) are shown. Sequence similarities with the enhancer of the mouse AchR subunit-δ (AR-δ(1m)) (23) and chicken AchR subunit-α (AR-α(ch)) genes (16) are also underlined and the corresponding sequence is shown above. Small letters show mismatch of the consensus with the mouse desmin sequence.

RESULTS

Isolation of the mouse desmin gene and characterization of its 5′ upstream sequence

Recombinant DNA clones containing the mouse desmin gene were isolated by screening a partial EcoRI mouse Balb/c spleen genomic DNA Charon 4A library using a desmin cDNA probe (1). Restriction map analysis, partial sequencing and comparison with the hamster desmin gene revealed that the obtained genomic clone MD37.1 contains the first 6 exons and 12 kb upstream of the transcription initiation site (Figure 1A). A 2.0 kb HindIII fragment surrounding the initiation of transcription was subcloned into M13 (9) and partially sequenced. The nucleotide sequence of the region extending from 976 base pairs upstream of the transcription start site to the first exon is shown in Figure 1B. Comparison of this region with the hamster desmin gene (16) revealed 87% sequence homology. Further analysis revealed several potential cis-acting regulatory elements. There are three consensus E boxes (CANNTG), potential myoD or myogenin binding sites, designated as E_1, E_2, and E_3 located at −79, −832, and −936, respectively. E_1 and E_2 boxes are the core of putative MEF1 (18) binding sites with only one nucleotide

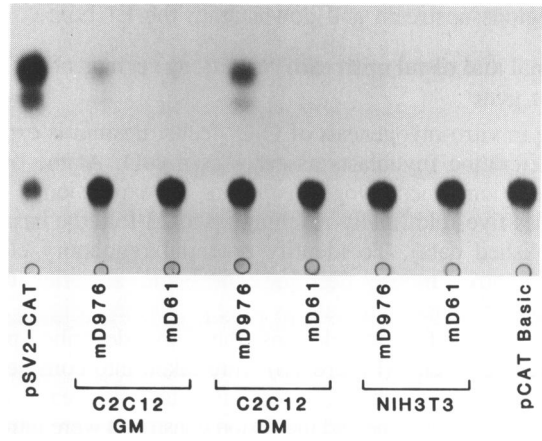

Figure 2. Tissue specific and developmentally regulated expression of the mouse desmin gene. Desmin-CAT fusion gene constructs containing 5′ flanking sequences up to −976 bp (mD976) or −61 bp (mD61) were introduced into the myogenic C_2C_{12} or non-myogenic NIH 3T3 fibroblasts by calcium phosphate precipitation. Transient levels of CAT expression were determined from undifferentiated cultures kept in growth medium (GM) 48 h or after transferring and kept for 36 h in differentiation medium (DM). pSV2-CAT and pCAT Basic are used as positive and negative controls respectively. CAT activity is observed only in myogenic cells and at higher levels in differentiation medium.

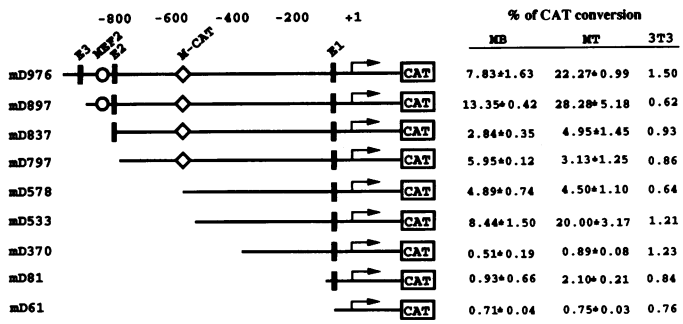

Figure 3. Analysis of the CAT activity of deletion mutants of the mouse 5′ upstream region. Diagrams of the deletion constructs are shown. The names of the constructs are indicated at the left and show the 5′ end point of the deletions used. Dashed lines indicate the internal deletion. The different putative cis acting elements are shown. These constructs were transfected into C_2C_{12} cells together with the β-galactosidase reporter gene (RSV-β-galactosidase). The amount of extract employed for the determination of CAT activity was normalized to β-galactosidase activity. The value of CAT activity was calculated as the percentage of acetylated chloramphenicol derivatives to the total chloramphenicol. The mean values of the percentages of CAT conversion of at least three independent experiments for each construct is shown for comparison, among 48h myoblasts (MB), myotubes (MT) kept 36h in DM and 3T3 fibroblasts.

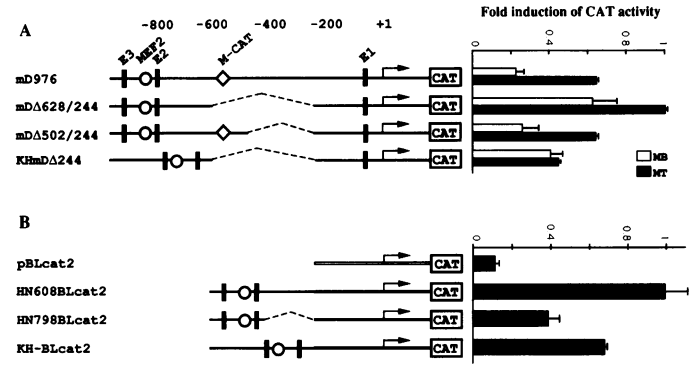

Figure 4. Effect of the desmin enhancer on the homologous (A) and heterologous (B) promoters. Restriction fragments from the upstream enhancer region (−976 to −628 or −976 to −502) were fused to the desmin promoter region at position −244 (in A) of the mD976 CAT-desmin fusion gene or were fused to the heterologous promoter of HSV TK gene (in B) of the pBLcat2 vector as described in Methods. In panel, B HN608 extends from −976 to −608; HN798 from −976 to −798 and KH, in reverse orientation, from −976 to −500. H, N, K derive from the corresponding restriction sites HindIII, NsiI and KpnI used to obtain these fragments. The above constructs were transfected into C_2C_{12} myoblasts and CAT activity was measured in case of panel A in both growing myoblasts (MB □) and differentiating myotubes (MT ■) as in Figure 3 and blotted as fold induction of CAT activity. In panel B the CAT activity of only myotubes (■) is shown because the TK promoter used is very strong in these myoblasts and inclusion of these results would obscure the interpretation of the data. The CAT activity shown is the mean value of at least three experiments. Reverse orientation of the enhancer is indicated by an arrow.

divergence in the established consensus. These boxes contain the symmetrical core consensus sequences (CAGCTG) which in in vitro studies have been shown to be the prefered binding sites for myoD homodimers (48). The E3 box, on the other hand, contains asymmetric consensus more closely resembling the consensus for E47 homodimer according to the same study (48). The surrounding region of the E_3 box deviates significantly from the MEF1 consensus. Between boxes E_2 and E_3, there is a putative MEF2 binding site (19). In addition, between nucleotides −825 and −713 there are three sequences with high homology to the chicken AchR subunit-α (46) and one with the mouse AchR subunit- (23). Furthermore, a region with homology to M-CAT motif (20) CATTCCT exists at position −587 and several GC rich regions upstream and downstream the E1 box.

Proximal and distal upstream regulatory regions of the mouse desmin gene

During in vitro myogenesis of C_2C_{12} cells, desmin is expressed in proliferating myoblasts as early as myoD. At the onset of differentiation, when myogenin appears, the expression of desmin increases five-fold finally reaching up to 23-fold the basal level (unpublished data). To identify potential regulatory elements which control mouse desmin expression, a series of uni-directional 5′ deletion mutations were performed (Figure 3). For the generation of these deletions, the above described putative regulatory elements (Figure 1B) were taken into consideration. The generated fragments were inserted upstream of the promoterless CAT gene and the fusion constructs were introduced into myogenic (C_2C_{12}) and non-myogenic (NIH 3T3) cells. The CAT activity of each construct in transcient transfection is shown in Figures 2 and 3. As shown in Figure 2, the 976 bp long upstream 5′ flanking sequence of the mouse desmin gene contains all the sequences necessary to elicit both muscle-specific and developmentally regulated myogenic expression. CAT activity was observed only in C_2C_{12} muscle cells and no activity was obvious in NIH 3T3 fibroblasts. On the other hand, the CAT transcriptional activity that this construct and a similar one missing the most distal E3 box showed in myotubes (DM) was about 3-fold higher than that of myoblasts (GM). This difference reflects similar values obtained for the steady state desmin mRNA levels (unpublished data). As shown in Figure 3, mD897, the entire fragment missing only E_3 box, showed the highest activity. Initial deletions to nucleotide −578 (mD578) which removes both the E_2 and E_3 boxes and the MEF2 site, as well as the M-CAT box considerably decreases CAT expression. Additional deletion to nucleotide −370 (mD370) diminished the remaining activity. Deletion to nucleotide −81 (mD81), however, increases the CAT activity 2-fold both in myoblasts and myotubes. Further deletion to nucleotide −61 which removes the E_1 box and half of an adjacent downstream GC-rich box decreases the CAT activity to the background level again. The above initial results suggested that upstream sequences up to −81 which include the E_1 box are sufficient to confer low levels of expression in myotubes. Sequences up to −61 are not sufficient to confer muscle specific expression. The sequence between at least −370 and −81 seems to contain negative elements. Finally, the region between −578 to −976 is required for the high level of desmin expression.

More detailed deletion analysis was then performed to further characterize the positive and negative elements of this region. As shown in Figure 3 deletion up to nucleotide −897 (mD897) which removes the E_3 box causes increase in the CAT activity in both myoblasts (~1.7 fold) and myotubes (~1.2fold), suggesting that an element in this region has a negative effect on CAT activity in both cases. Further deletion which eliminates also the MEF2 site and left arm of E2 box cause the most dramatic decrease in CAT activity, about 4-fold in myoblasts and 7-fold in myotubes, respectively. Deletion to nucleotide −797 (mD797) which removes the E_2 box completely has no particular effect in myotubes though a 2-fold increase is observed

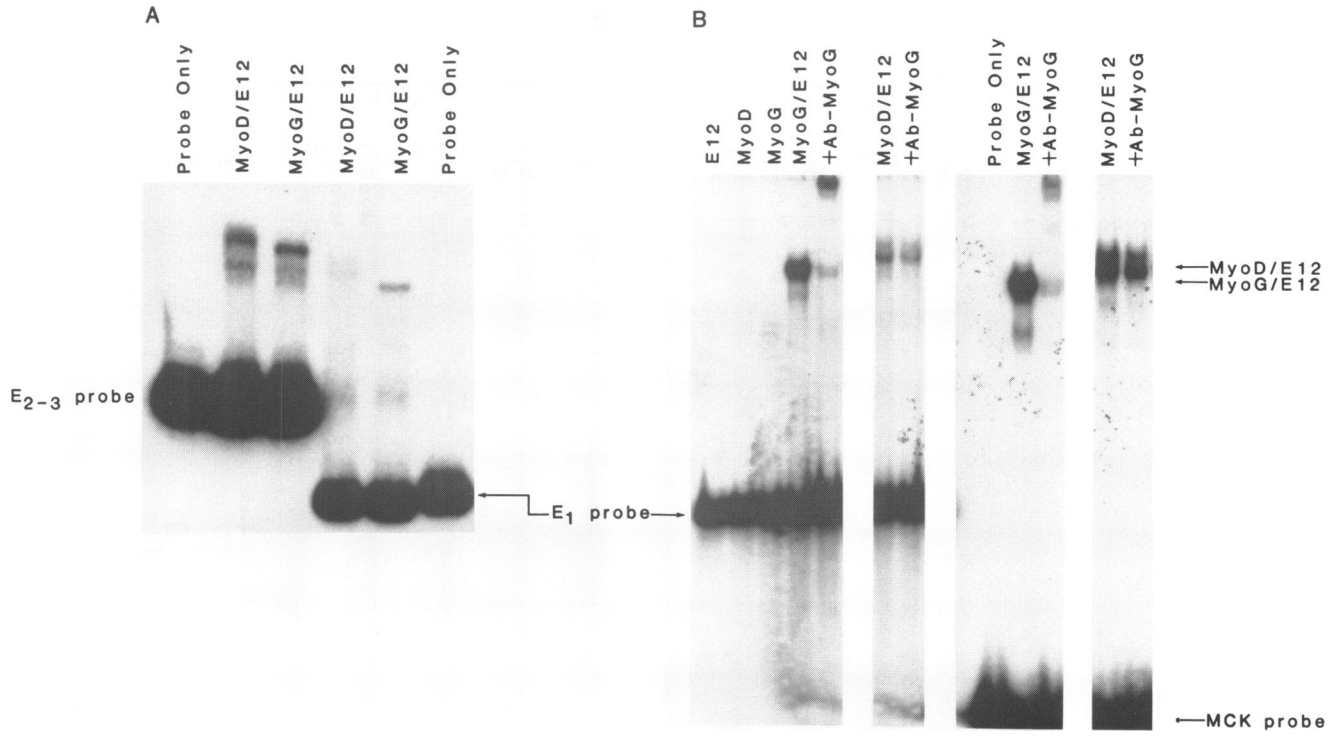

Figure 5. Binding of in vitro translated MyoD and Myogenin to different E box containing regions of the desmin gene. Electrophoretic mobility shift assays (EMSA) of the desmin gene upstream regions were performed with in vitro translated myoD/E12 and myogenin/E12 (myoG/E12) using ^{32}P-labeled fragment E_1 (−84 to +24) containing the E_1 box, fragment E_{2-3} (−976 to −798) containing E_2 and E_3 boxes and MCK, a 30 bp oligomer from the right hand E box of the MCK enhancer core (51), as described in Methods. The specificity of the binding was verified in panel B by the use of 5-fold cold competitor (+5× comp) indicated at the lane on the right of the corresponding assays. For the case of myogenin/E12 as additional control, the complex was incubated with antibodies to myogenin, indicated +Ab-myoG. Note in panel B that homodimers of E12, myoD and myoG do not band shift and in both panels A and B, E_1 probe binds myoD/E12 heterodimer with much higher affinity than myoG/E12. The MCK enhancer right hand E box (56) which bind equally well to both myoD/E12 and myoG/E12 is used here as a control.

in myoblasts. Further deletion to −578 (mD578) which eliminates the M-CAT box also did not cause further change in the CAT activity. However, removal of 44 extra nucleotides caused a considerable increase of the CAT activity in both myoblasts (3-fold) and myotubes (4-fold) suggesting the presence of another putative negative element in their region. To further analyze this region, two internal deletions were made: mDΔ628/244 and mDΔ502/244 (Figure 4A). The first deletion removes this putative negative element together with part of the other more downstream negative region, between −370 to −81 bp, suggested from the initial deletion analysis in Figure 3. The second deletion removes only the downstream element. The results from these internal deletions support the presence of potential negative elements in the region between nt −244 to −628. The most upstream seems to be the strongest one.When 161 more nucleotides were deleted from mD533 creating mD370 the CAT activity was entirely lost, suggesting the presence of sequences which in the absence of the upstream (−628 to −502) putative negative element increase considerably the CAT transcriptional activity. In conclusion, the above serial and internal deletions suggest that throughout the 976 bp upstream flanking region of mouse desmin there are at least 3 potential positive elements and possibly 3 negative ones. In the most distal positive region, the area including and surrounding the MEF2 site and E2 site seems to be the most important element for high expression of desmin. Surprisingly, removal of E_2 as well as the M-CAT elements does not seem to influence strongly the activity of the presently used constructs. Furthermore, E3 box not only does not contribute to the high level of desmin expression, but on the contrary, these 79 nt most upstream region which includes E3 seems to influence the rest of the construct negatively. From the three potential negative elements the middle one (−578 to −533) seems to be the strongest.

The deletion studies so far have suggested that the region between −897 to −578, which is responsible for the high transcription activity of the mouse desmin gene and contains elements characteristic of other muscle specific genes studied to date, is probably a classical enhancer. To test this hypothesis several constructs were prepared to study the properties of this fragment. As shown in Figure 4A, when this putative enhancer fragment (−976 to −628) is placed next to its own promoter at position −244 in normal (mDΔ628/244 or mDΔ502/244) or inverted orientation (KHmDΔ244), the CAT-activity is similar or higher than the entire upstream region (Figure 4A). Similarly, when this fragment was placed in front of the thymidine kinase promoter in plasmid pBLCAT2 in both orientations (HN608/BLcat2 and KHBLcat2) very high induction (10-fold) of this promoter was observed in myotubes (Figure 4B). The region that contained only E_2, MEF2 and E_3 (HN798, from −976 to −798) could function as an enhancer as well but it showed 60% lower enhancement than the fragment extending to position −608.

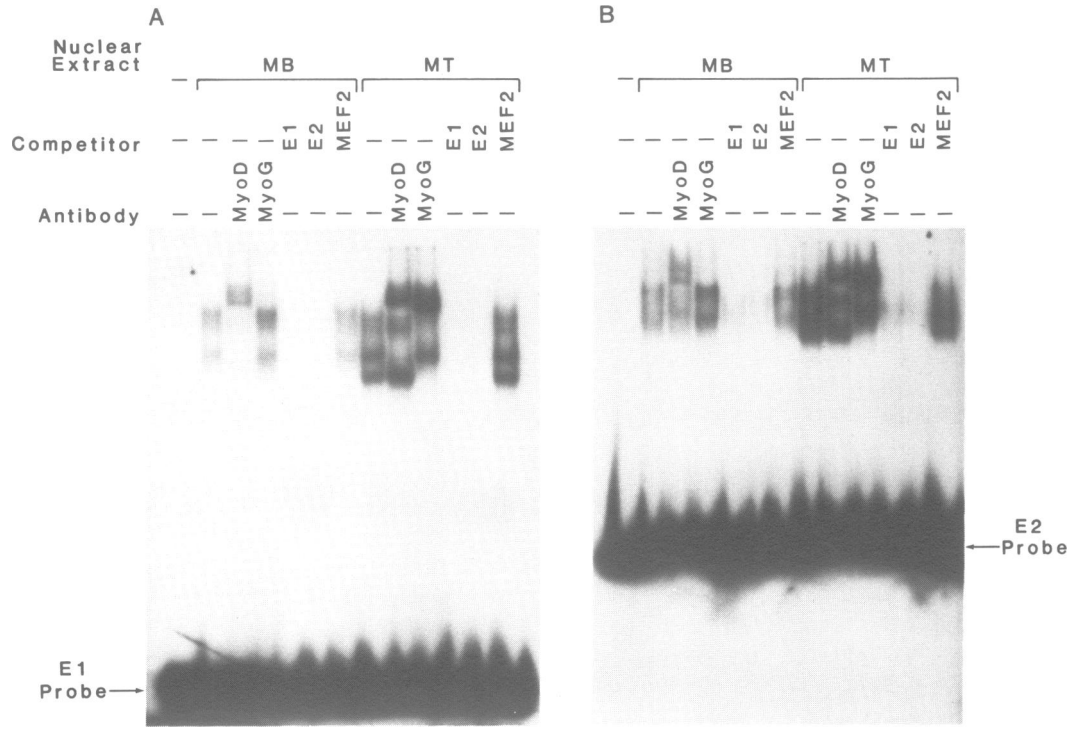

Figure 6. The desmin E boxes (E1, E2) bind specifically to MyoD and Myogenin in C_2C_{12} nuclear extracts. Oligonucleotode probes (20-mer) containing the sequences for E1 and E2 box of the mouse desmin gene were used in EMSA. E1 (A) or E2 (B) double-stranded oligomer was end labeled and incubated with C_2C_{12} nuclear extracts from myoblasts and myotubes as indicated. The binding co-specificity was determined by competition with 100-fold unlabeled E1, E2 or MEF2 oligomer, and the identity of the shifted bands was confirmed by specific antibodies against myoD and myogenin. E1: GGATCCTGCAGCTGTCAGGG 3′; E2: 5′ AGCTTGGCAGCTGTTGCTCG 3′

Binding of MyoD and myogenin to the distinct E box regions of the mouse desmin

We have further investigated the putative regulatory elements of the desmin promoter and enhancer region by looking initially at their binding pattern with in vitro translated myogenic factors using gel eletrophoretic mobility shift assays (EMSA). Two fragments were used as probes in this assay, one from −81 to +24 (E_1) containing the proximal E_1 box and the other from −976 to −798 (E_{2-3}) containing part of the enhancer including boxes E_2, MEF2 and E_3. These fragments were end labeled and incubated with in vitro translated myoD, myogenin and E12 homodimers and co-translated myoD/E12 and myogenin/E12 heterodimer. As shown in Figure 5, the heterodimers myoD/E12 and myogenin/E12 specifically interact with both E_1 and E_{2-3} fragments. The specificity of this binding to these DNAs was confirmed by competition using cold MCK enhancer fragment (data not shown), and at least for myogenin, by further band shifting using myogenin antibodies (Figure 5B). The affinity of MyoD/E_{12} for the E_1 probe seems to be lower than the affinity of myogenin/E_{12} shown for the same probe (figure 5A). On the other hand, both myoD/E12 and myogenin/E12 bind with comparable high affinity to E_{2-3} fragment of the desmin enhancer. The low affinity of myoD for the E_1 box containing fragment was confirmed by control comparison of the binding pattern obtained with the MCK enhancer when the same amount of in vitro translated myoD/E12 heterodimer was used (Figure 5B). As stated above, the E1 and E2 box core consensus are

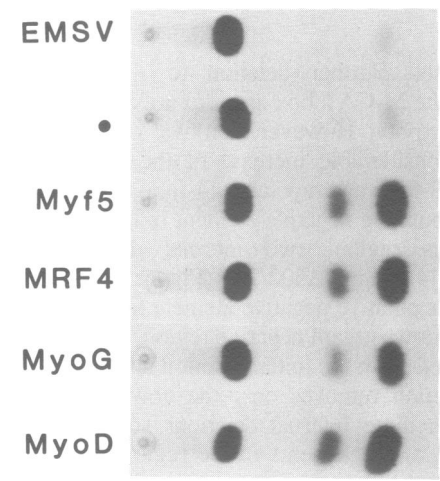

Figure 7. Transactivation of the mouse desmin gene by myoD, myogenin MRF4 and Myf5. 15 mg of pMD976-CAT plasmid were transfected into 10 T1/2 cells together with 5 mg of pEMSV expression constructs (see Methods) carrying MyoD, myogenin, MRF4 or Myf 5 cDNA inserts CAT assays were performed as in the previous figures. This is one out of four independent experiments which have shown very similar results.

symmetrical and according to reported in vitro studies (48) this could predict higher binding affinity to myoD homodimers. The present data, however, shows that this is not the case at least

for the present desmin fragment used (Figure 5A). It seems that heterodimer formation of myoD or myogenin with E12 is required for strong interactions with the E-boxes in these assays. It should be pointed out here that the E_{2-3} fragment shows multiple band shifts with the in vitro translated myoD/E12 complex, but only a single band shift with the myogenin/E12 complexes. This observation could suggest that either this complex binds to more than one site, as expected, or that higher order complexes (eg tetramers) except of the heterodimer, bind to each site.

Similar gel electrophoretic mobility shift assays were performed using nuclear extracts from C2C12 cells. To be more specific in these assays, we used synthetic oligonucleotide (20-mer) probes containing the corresponding E1 and E2 boxes, shown in the legend of figure 6. As shown in this figure, the patterns obtained with both probes were similar. When nuclear extracts from myoblasts (MB) were used, two distinct bands of retarded mobility were seen with both probes (panel A and B, figure 6). The identity of at least one of the proteins responsible for these band shifts was confirmed using myoD and myogenin antibodies. Both bands were further retarded by myoD antibody and, as expected, none with myogenin antibody, indicating that at least one of the proteins responsible for these retarded bands was myoD. The binding was specific, since both bands were competed with either E1 or E2 cold probes but not MEF2 cold probe. When nuclear extracts from myotubes were used, three bands were seen with both probes (panel A and B, figure 6). The binding specificity was confirmed by competition with cold oligonucleotide probes as in the case of myoblast extracts. By using antibodies to myoD and myogenin, we further demonstrated that the lower and higher bands of this triplet were due to myogenin binding whereas the middle band was due to myoD binding to the probe. The myogenin-specific upper band seems to be a doublet not always obvious in these assays.

Transactivation of the mouse desmin gene by MyoD and myogenin MRF4 and Myf5 in 10 T-1/2 cells

The functional significance of the binding of myoD and myogenin to the desmin regulatory sequences (promoter and enhancer) was studied by co-transfection experiments. Vectors expressing myoD and myogenin were co-transfected into 10T1/2 fibroblast cells with the desmin-CAT fusion constructs, mD976. As shown in Figure 7, both myoD and myogenin seem to transactivate the desmin-CAT fusion gene with full length regulatory sequences (mD976) at comparable levels.

Similar to myoD and myogenin, MRF4 (33) and Myf5 (31) can convert fibroblasts to myoblasts by probably binding to muscle specific enhancers and transactivating muscle gene expression. Contrary to Myf5, however, to date, MRF4 has been found, by co-transfection in 10 T-1/2 cells, to transactivate only the mouse acetylcholine receptor a subunit (59) and the human α-actin promoter (57). Co-transfection experiments of the desmin-CAT fusion construct mD976 with either of these factors demonstrate that both of them can equally well transactivate the desmin gene (Figure 7).

DISCUSSION

The present studies identified initially three major regulatory elements in the 5′ upstream region of the mouse desmin gene. A proximal positive element between −81 and −1, containing a TATA box at −36 and an E box, E_1, at −79 and a GC-rich region between them, is considered as the desmin promoter and can confer low levels of muscle specific expression. Similar observations have been reported for the hamster (16) and human (17) desmin gene. Upstream of this promoter, there is a negative region between −81 and −578. Further upstream, between −976 and −578, an enhancer-like element is responsible for high level expression of the desmin gene. The presence of such positive and negative regulatory elements has also been reported recently for the human desmin gene (17). The enhancer region contains several elements previously identified important for muscle specific gene expression. These include two more E boxes one of which (E_2) with its surrounding sequences shows the highest overall similarity to the MEF1 site, previously identified in the mouse MCK enhancer (18). The surrounding region of the most distal E_3 box, at −936, does not resemble MEF1 sequences despite the fact that by itself is a perfect MEF1 core consensus. Furthermore, its assymetric consensus CAGGTG resembles the one which favors E47 homodimer binding according to in vitro studies (48). Since there is no binding studies with this box at the present time, it is not easy to suggest how these sequence characteristics correlate with the negative effect this element has on the desmin enhancer. The present studies demonstrated that myoD and myogenin from both nuclear extracts and in vitro translated proteins bind both E1 and E2 boxes. The symmetric core consensus of both these elements, according to the same studies (48), should favor the binding of myoD homodimers. However, our band shift assays with in vitro translated products do not favor this hypothesis (Figure 5B). Although we do not know what is the situation in vivo, the very low aboundance of such homodimers in the cell does not support such mechanism. The distance between E_2 and E_3 boxes is ~100 bp, that is longer than the longest, ~44 bp, identified till now. In all these cases both sites are required. This observation could suggest a different behavior for the present two E boxes. Indeed these elements do not seem to be funtioning as a pair, at least under the present deletion studies. Between boxes E_2 and E_3, there is a putative MEF2 element initially identified in the MCK enhancer (19). The MEF2 site seems to be a key element of this enhancer. It is not clear from the present studies what the role of the E_2 box is. It might be required for the proper function of the MEF2 element. By itself, in the absence of MEF2, it does not seem to play any significant role. On the other hand, the band shift assays showed that both myoD and myogenin can bind the E2 box in the absence of the MEF2 site. We do not know however, if the presence of the MEF2 site enhances the binding of myoD and myogenin to the E_2 box. There are actually two pieces of data that could support such possibility. The first is based on the differences of the binding affinity of myoD and myogenin for the two E boxes, depending on the presence or absence of neighboring sequences. When long probes are used with both in vitro translated products of the myogenic factors (Figure 5) or nuclear extract (data not shown), we observed differential binding affinity of these factors to the distinct E1 and E2 boxes. However, when short oligonucleotides probes were used with nuclear extracts (Figure 6), both myoD and myogenin showed similar binding affinity to both E boxes. The second piece of data is more direct and derives from the work of W. Funk and co-workers (58) who showed by using a CASTing (Cyclic Amplification and Selection of Targets) method, similar to SAAB's (selected and amplified binding studies) method (48) that

myogenin interacts cooperatively with MEF_2 and other proteins in multicomponent complexes.

When further analysis of the negative region (−578 to −81) was performed, it was shown that it contains a strong 44 bp long negative element between nucleotides −578 and −533 and a weaker negative element between −370 and −81 where actually the putative 'SRE'-like element (49,50) is located. Furthermore, it was shown that between these two negative elements there is a positive one between −533 and −370 which in the absence of the strong upstream negative element could replace the enhancer. Sequence analysis of these elements revealed that the strong negative one (−578/−533) does not show any specific known sequence characteristic except of a region (−551 to −556) which shows six consecutive nucleotides identical to the MEF2 site (60% identity). The weaker negative element (−370/−81) contains a 'SRE'-like site with four instead of 6 (A/T)s and a region (−329/−320) with 80% identity to MEF2 consensus (Figure 1B). It is of interest to notice that inside both of the negative elements there is a pseudo MEF2 site which somehow might be responsible for the negative control of the region. Finally the positive elements between these two negative elements (−533/−370) include a MEF2-like site (51) in opposite orientation with 80% identity to the consensus. There is also an interesting repeat [GG/CC TAC]4 in this region. The significance of all these sequences in these elements is completely unknown. Site directed mutagenesis, binding and transfection studies will further elucidate these issues.

Co-transfection experiments in 10 T1/2 cells demonstrated that all the members of the myoD family known to date can transactivate the desmin promoter. Of interest is the transactivation of the desmin promoter by MRF4 since this factor has not been found to transactivate any other gene (57) except of the human cardiac actin (52,57) and the mouse acetylcholine receptor a subunit (59). Recently it has been shown that the ability of myogenin and MRF4 to discriminate between muscle specific enhancers is determined by domains surrounding the bHLH region (51). Similar co-transfection experiments with all these myogenic transcription factors demonstrated that the negative regulation conferred by the fragment located between −578 and −81 to the desmin promoter takes place directly by inhibiting at least in part the functional activity of these factors (data not shown).

During embryonic muscle development, desmin first appears at 8.25 dpc in the neuroectoderm where it is transiently expressed with vimentin and keratin. None of the known myogenic factors have been identified there so early. The question then arises as to what factor(s) induce desmin expression at that stage. Desmin then disappears from neuroectoderm and appears again exclusively in the heart rudiment where none of the above transcription factors have yet been identified. What then regulates desmin in cardiac muscle? Desmin is a single gene expressed in all muscle cell types, but how its regulation takes place is yet unknown. Desmin first appears in the myotome at 9 dpc. Since transcription starts earlier, the only known factor that may be involved in the induction of desmin expression in the myotome is Myf5 (31). The transcripts of this factor can be identified in the myotome as early as 8 dpc. However, we do not know when the Myf5 protein first appears. The next myogenic factor is myogenin, detected at 8.5 (or 9) dpc, followed by myoD, at 10 dpc and finally MRF4 (31,53). We do not know yet if our data in vitro reflect the situation in vivo. Possibly all these factors transactivate desmin at different stages during development, alone or in combination.

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