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A Proximal Promoter Element in the Hamster Desmin Upstream Regulatory Region Is Responsible for Activation by Myogenic Determination Factors*

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The muscle-specific intermediate filament protein desmin is up-regulated during skeletal muscle differentiation. When myoblasts leave the cell cycle and fusion into multinucleated myotubes starts, genes associated with myogenesis become activated. The activation is believed to be mediated by the muscle-specific determination factors. We present evidence that both MyoD and myogenin are able to activate the transcription of the hamster desmin gene. A proximal promoter fragment of 89 base pairs is sufficient for this transactivation process. The single E-box in this region is essential for desmin promoter activity in mouse C2 skeletal muscle cells and upon co-transfection of a myogenin expression vector also in human primary fibroblasts. Mutation of this MyoD binding site abrogates desmin transcription, and transactivation of the promoter no longer occurs. By using gel electrophoretic mobility shift assays, we were able to demonstrate that nuclear proteins from C2 muscle cells and myogenin/E12 glutathione *S*-transferase fusion proteins are able to bind to the functional E-box consensus sequence. A second E-box, situated in a more upstream regulatory region, which also binds to purified Helix-Loop-Helix proteins *in vitro* is only moderately affected by site-directed *in vitro* mutagenesis.

Intermediate filament protein-encoding genes form a large family with a more or less tissue-specific distribution. The proteins can be divided into 6 different subtypes on the basis of their biochemical and immunological properties. One of these, the type III intermediate filament protein desmin is expressed in adult cardiac, skeletal, and smooth muscle. The hamster desmin gene has been cloned and analyzed (Quax *et al.*, 1984, 1985). The promoter regions of the hamster and the human gene have been characterized previously in our laboratory (Pieper *et al.*, 1987, 1988) and by others (Li and Paulin, 1991). The experiments revealed the presence of a proximal region in the hamster promoter directing muscle-specific expression and a more upstream enhancer region in the human promoter.

The transcriptional regulation of several muscle-specific genes has recently been elucidated. In a number of cases, the *trans*-acting factors involved belong to the group of the myogenic determination factors (Lassar *et al.*, 1989; Piette *et al.*, 1990; French *et al.*, 1991; Prody and Merly, 1992; Yutzey and Konieczny, 1992). Each member of the family including MyoD, myogenin, myf-5, and MRF4 (myf-6, herculin) is expressed in skeletal muscle exclusively and seems to play an important role in myogenesis *in vitro* (Davis *et al.*, 1987; Edmondson and Olson, 1989; Wright *et al.*, 1989; Braun *et al.*, 1989; Rhodes and Konieczny, 1989; Miner and Wold, 1990; Braun *et al.*, 1990). These nuclear phosphoproteins are able to induce a skeletal muscle phenotype upon forced expression in fibroblasts and in a variety of nonmesenchymal cells (Choi *et al.*, 1990; Weintraub *et al.*, 1989; for review see Olson (1990), Weintraub *et al.* (1991a), and Wright (1992)). Myf-5 mRNA is the first of the family to be expressed in mouse development at day 8 of gestation, followed about 12 h later by myogenin, just before the appearance of myf-6. MyoD is detected approximately 2 days later, accompanied by the disappearance of myf-6 (Rudnicki *et al.*, 1992; Buckingham, 1992; Ott *et al.*, 1991; Bober *et al.*, 1991; Sassoon *et al.*, 1989). In tissue culture of skeletal muscle model systems like the mouse C2 cell line, the situation is slightly different, since MyoD is expressed in myoblasts and myotubes, whereas myogenin is found primarily in differentiated myotubes (Edmondson and Olson, 1989; Braun *et al.*, 1989). myf-6 (MRF4) is not expressed in most established muscle cell lines (Rhodes and Konieczny, 1989; Braun *et al.*, 1990; Miner and Wold, 1990). It has not been possible to assign a specific role to the individual myogenic factors (Thayer *et al.*, 1989; Braun *et al.*, 1989). Moreover, targeted inactivation of MyoD in transgenic mice has resulted in elevated levels of myf-5, indicating that the absence of expression of one protein may lead to an up-regulation of another one (Rudnicki *et al.*, 1992).

For transactivation, the factors must bind to specific binding sequences defined as E-box sequences consisting of the general consensus CANNTG (Lassar *et al.*, 1989). Detailed mutagenesis studies have revealed that the most essential part of each protein is formed by a so-called basic helix-loop-helix (HLH)[1] structure (Tapscott *et al.*, 1988; Benezra *et al.*, 1990). The basic region is involved in DNA binding, whereas the helix-loop-helix motif is required for a dimerization process (Davis *et al.*, 1990). Most myogenic HLH proteins are able to bind to their target sequence as homodimers. Higher affinity, however, is reached after hetero-oligomerization with other more widely distributed HLH proteins, like the E12 or E47 proteins (Lassar *et al.*, 1991; Finkel *et al.*, 1993). Other E proteins, like ITF2 and HEB, have also been defined (Henthorn *et al.*, 1990; Hu *et al.*, 1992).

There are indications for an involvement of members of the MyoD family in regulating the desmin gene (Weintraub *et al.*, 1989; Choi *et al.*, 1990). In an attempt to understand whether and how transcriptional control is exerted by these proteins, we looked for E-box sequences in the hamster desmin upstream promoter region. At least 8 putative consensus sequences were

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[1] The abbreviations used are: HLH, helix-loop-helix; CAT, chloramphenicol acetyltransferase; bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium.

found, several of which are in previously defined regulatory regions. Here we present the analysis of the sequences involved in muscle-specific regulation of the hamster desmin promoter region and the transactivation of these sequences by MyoD and myogenin.

MATERIALS AND METHODS

Construction of Eukaryotic Expression Plasmids—A 3.4-kilobase *Eco*RI/*Hpa*II fragment containing the hamster promoter region and subfragments, which were inserted in a CAT vector, were described earlier (Pieper *et al.*, 1987). The different promoter lengths used for insertion were obtained by restriction endonuclease digestions, and the length of each fragment was given according to the start site of transcription, −89 bp (6), −450 bp (5), −1600 bp (2), and −3400 bp (1) (see also Fig. 1). Three additional constructs were created by using exonuclease Bal31 and restriction enzyme *Ban*I digestions containing −53, −625, and −926 bp of promoter sequences. Mutant promoter constructs were prepared by oligonucleotide-directed mutagenesis of desmin promoter subfragments. For this purpose, one E-box located at −825 bp from the cap site was changed from CAGCTG into a *Stu*I site (AGGCCT). The E-box at −80 required a similar mutation, ultimately resulting in several combinations of mutated and nonmutated E-boxes which were incorporated in the full 3.4-kb desmin promoter region. All base substitutions were verified by DNA sequencing. M1 contains a mutated −825 box, M2 a mutated −80 box, and M3 contains both mutated E-boxes. M4 is also a double mutant, in which a *Ban*I restriction site was used to destroy a third putative E-box by making a 5′ deletion. Supercoiled plasmid DNA was purified by CsCl gradient centrifugation (Sambrook *et al.*, 1989) and quantitated by means of both ethidium bromide staining and spectrophotometric measurement.

Cell Culture, Immunofluorescence, and CAT Transfections—The C2 skeletal muscle myoblast cells (Yaffe and Saxel, 1977) were grown in Dulbecco's modified Eagle's medium (DMEM) enriched with 20% fetal calf serum. To induce myotube formation, confluent cultures were switched to low mitogen fusion medium (DMEM supplemented with 2% horse serum). C2 cells start to fuse within 24 to 36 h. Human dermal primary fibroblasts from foreskin were cultured for several passages in DMEM supplemented with 10% fetal bovine serum. Cell samples were stored in liquid nitrogen until further use. In order to investigate whether these cells can differentiate along the myogenic lineage, infections with a recombinant retrovirus containing the MyoD coding sequences under the control of a viral long terminal repeat promoter and the geneticin selection marker were performed. Cells were infected by exposure to virus-containing medium overnight in the presence of 4 μg/ml Polybrene (Weintraub *et al.*, 1989). Subsequently, cells expressing virus-encoded proteins were selected by supplementation of the culture medium with geneticin (Life Technologies Inc.) 24 h after infection. For detection of desmin protein by immunofluorescence staining, the medium was changed to DMEM supplemented with 2% horse serum 48 h before harvesting. Staining procedures were performed on cultured cells as described by Krimpenfort *et al.* (1988). We used a polyclonal rabbit antibody (poly-des) to chicken gizzard muscle desmin (Ramaekers *et al.*, 1983).

Approximately 48 h before transfection with desmin-CAT promoter deletion and mutation constructs, C_2 cells were plated in 10-cm Petri dishes. Calcium phosphate co-precipitations were performed as described earlier (van de Klundert *et al.*, 1992). For each construct, equimolar amounts of DNAs were used, and a vector with the β-galactosidase reporter gene linked to the Rous sarcoma virus long terminal repeat (β-Gal) was co-transfected as an internal control. After transfection, cells were kept in DMEM containing 2% horse serum for 48–36 h to obtain myotube formation. Primary human dermal fibroblasts were transfected by electroporation. Briefly, cells were washed twice with phosphate-buffered saline, and $3–5 \times 10^7$ cells were mixed with 10 μg of Rous sarcoma virus β-Gal and 5 pmol of des-CAT construct and 5 pmol of pEMSVscribe-myogenin in 500 μl of phosphate-buffered saline. After a 10-min incubation on ice, cells were electroporated in a 1-ml electroporation cuvette (Eurogentec, Belgium) by a 2000-V pulse from an ISCO type 494 power supply and immediately replated on 10-cm Petri dishes. Cells were refed after 12 h with DMEM containing 2% horse serum and harvested 24 h later. All transfections were performed at least three times with two different DNA preparations. To correct for differences in transfection efficiencies, the amount of lysate used for the CAT assay was corrected for protein content and β-galactosidase activity according to the procedure described by Edlund *et al.* (1985). CAT activities were determined according to Gorman *et al.* (1982).

Preparation of Nuclear Extracts—Nuclear extracts were prepared by a modification of the method described by Dignam *et al.* (1983). Briefly, cells were lysed in 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride. Nuclei were isolated by centrifugation in lysis buffer containing 6.8% sucrose and extracted with 400 mM NaCl, containing 5% glycerol. Nuclear proteins were concentrated by $(NH_4)_2SO_4$ precipitation (33% w/v). Dialysis took place in 20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, with 20% glycerol in the dialysis solution, and 5% glycerol in the dialysis sample. After dialysis, extracts were quickly frozen in liquid nitrogen and stored in small samples at −70 °C.

Bacterial Expression Vectors—The glutathione-MyoD (GST-MyoD) expression plasmid was created as described by Lassar *et al.* (1989). In brief, an *Alu*I fragment of the MyoD cDNA, extending from the third amino acid to 88 nucleotides downstream of the terminal amino acid, was isolated and ligated into a *Sma*I-cleaved glutathione *S*-transferase expression vehicle (Gex 3X; Smith and Johnson (1988)). The vector expressing the glutathione-myogenin (GST-myogenin) fusion protein was a kind gift from Dr. Eric Olson. Amino acids 33 to 224 of myogenin are included in this construct. The purification of both the MyoD and myogenin fusion proteins from bacterial lysates was performed according to Lassar *et al.* (1989). An *Xho*I fragment containing a partial E12 cDNA was isolated, and ends were filled in with T4 polymerase and ligated in-frame downstream of a T7 promoter). The latter fragment contains all the necessary information for heterodimerization (Sun and Baltimore, 1991). Using 20 units of T7 polymerase, an mRNA was synthesized from the linearized E12 plasmid in a 60-μl reaction volume for 1 h at 37 °C (reaction conditions: 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine-HCl, 10 mM NaCl, 5 mM NTP, 10 mM dithiothreitol, 80 μg bovine serum albumin, 50 μM GpppG, 60 units of RNasin). After purification, the RNA was translated in wheat germ extract according to the procedures described by the manufacturer (Promega). The purified GST-MyoD, GST-myogenin, and the *in vitro* translated truncated E12 proteins were analyzed on 10% SDS-polyacrylamide gels. For heterodimerization, equal amounts of fusion proteins and *in vitro* translation products were mixed and incubated at 30 °C for 1 h in the presence of 20 mM EDTA (Murre *et al.*, 1989).

Mobility Shift Assays—A double-stranded oligonucleotide fragment containing the −80 E-box sequences (sense strand 5′ AGGGATCCTGCAGCTGTCAGGGGAGG 3′, nonsense 5′ AATTCCTCCCCTGACAGCTGCAGGATCCCT 3′) were subcloned in the *Eco*RI and *Hin*dII sites of pGEM-3Zf(+) (Promega) using a synthesized *Eco*RI site and a blunt site. To generate a probe containing the −825 E-box element, a 95-bp *Rsa*I/*Hin*dII fragment was subcloned in the *Hin*dII site of pGEM-3Zf(+). The resulting plasmids were digested with *Eco*RI and ^{32}P-end-labeled, after which a second restriction enzyme in the vector polylinker was used to release the fragment followed by gel purification. Binding reactions for the nuclear extracts were performed in a final volume of 25 μl containing 20 mM Hepes, pH 7.9, 100 mM KCl, 1.5 mM $MgCl_2$, 100 mM EDTA, 4 μg of poly[d(I-C)], and 8% glycerol for 15 min on ice in the presence of 1–2 ng of labeled DNA fragment. The incubations with purified fusion proteins (0.1–0.2 μg) contained 20 mM Hepes, pH 7.9, 50 mM NaCl, 1 μg of poly[d(I-C)], and 4% glycerol. Unlabeled competitor DNA was added to the binding reactions 10 min before addition of the protein mixtures. Samples were size-fractionated through a 6% acrylamide gel (1:19 methylenebisacrylamide/acrylamide) containing 6.7 mM Tris, 3.3 mM NaAc, 1 mM EDTA, pH 7.9, at 10 V/cm.

RESULTS

Desmin Transcription Regulation—Desmin expression is up-regulated severalfold upon differentiation of the mouse C2 myoblasts to myotubes. Withdrawal of high serum leads to elongation and fusion of myoblasts and formation of multinucleated myotubes. This process is associated with an elevated expression of myogenic determination factors. Both MyoD and myogenin are expressed in this cell line. MyoD is already present in a relatively undifferentiated stage of the cells, while myogenin expression is detectable in myoblasts but highly up-regulated during differentiation. Using a subclone of the C2 cell line (C2C12), the hamster desmin upstream regulatory region was analyzed previously, showing that the most proximal promoter region harbors sequences responsible for muscle-specific expression (Pieper *et al.*, 1987). The members of the MyoD family are obvious candidates for the regulation of muscle-specific expression of desmin. In the present report, we have a closer look at the desmin upstream region by employing

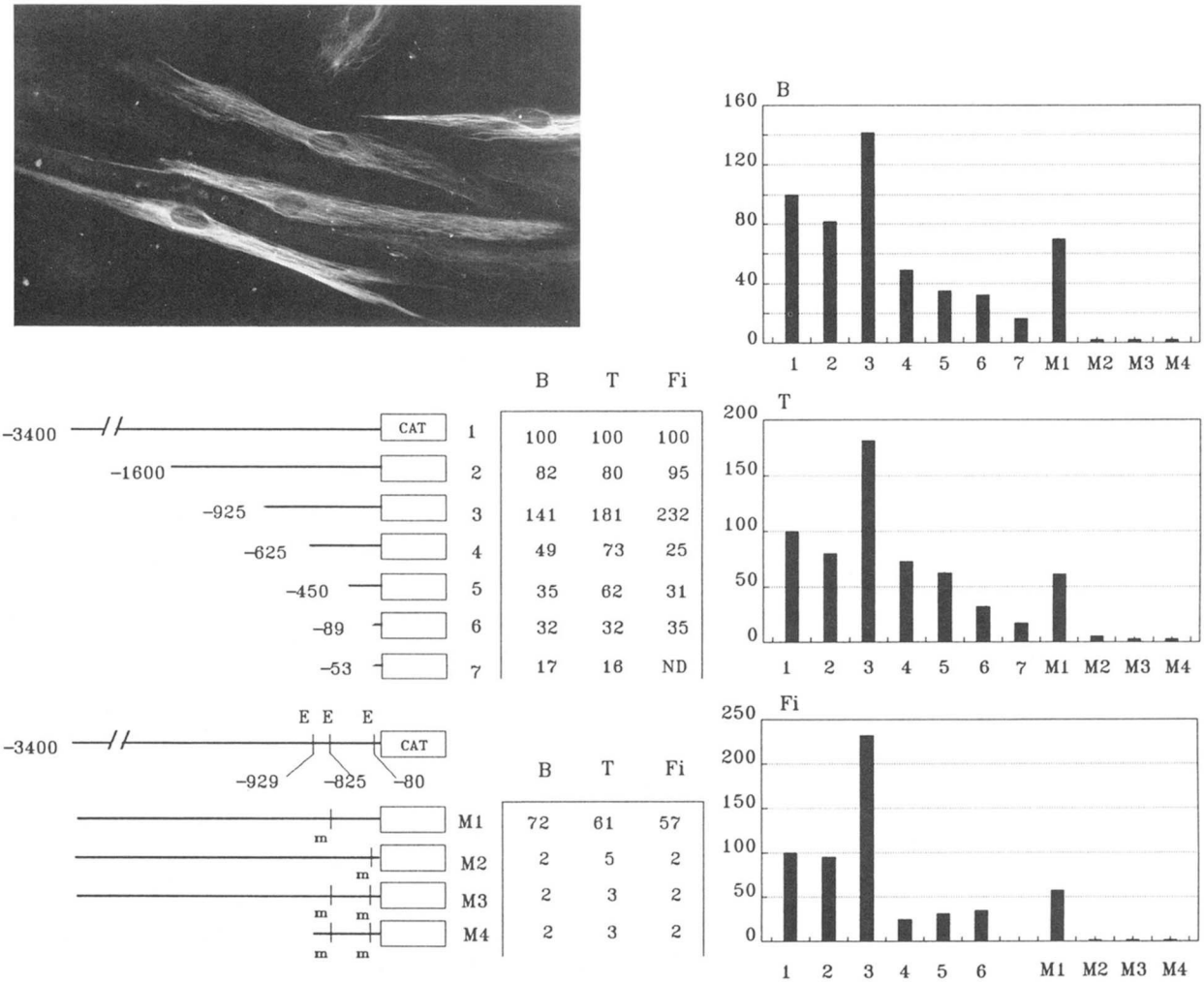

FIG. 1. **Transcriptional activation of desmin in primary fibroblasts and C2 myogenic cells.** Primary fibroblasts were infected with a retrovirus encoding MyoD, leading to expression of endogenous desmin as detected by immunofluorescence. The same cells were used for transient transfections with different desmin CAT deletion constructs upon co-transfection with a myogenin expression vector. Desmin deletion and mutation constructs were also transfected into C2 myoblasts, which were allowed to differentiate into multinucleated myotubes. The 5′ end point of each deletion, created by endonuclease or exonuclease digestion, is shown, and mutation of the E-box sequences (*E*) is as indicated (*m*). All constructs share the same 3′ end point at +25 bp relative to the transcription start site. Myoblasts, myotubes, and fibroblasts were harvested 48 to 72 h after transfection, and CAT values were determined by liquid scintillation counting (*B, T,* and *Fi*). All values are the average of three or four independent transfections and presented in *tables* and *bars*. CAT values are normalized to the activity of the longest promoter fragment, although overall CAT activities are 4–8-fold higher in C2 myotubes compared to myoblasts. This is not reflected in the diagrams because the absolute level of desmin expression varies between the different myotube transfections.

additional deletion constructs and a site-directed mutation analysis. A large part of the hamster upstream region was sequenced earlier (Pieper *et al.*, 1988). We now also detected 8 E-boxes among several putative muscle-specific *cis*-acting sequences. Only 2 of these E-box elements comply to the general sequence rule described by Buskin and Hauschka (1989). These two E-box consensus sequences were chosen for mutation analysis, since they are comprised within hamster and man homology regions (Li and Paulin, 1991). Transfections were performed with mouse C2 cells and nonmyogenic human primary fibroblasts upon co-transfection of a myogenin expression vector. The CAT activities of the different deletion and mutation constructs are shown in Fig. 1. The results indicate that the increased activation of the desmin promoter in C2 myotubes, compared to myoblasts, is also reflected in the higher CAT activity of the different deletion constructs in myotubes. Overall activities are approximately 4–8-fold higher in myotubes compared to myoblasts, with regard to the activity of a construct where the CAT gene is driven by the SV40 promoter/enhancer (results not shown). Comparison of the CAT values of the different deletion constructs in myoblasts and myotubes shows a similar trend (Fig. 1). Highest CAT activity is obtained in myoblasts and myotubes with construct 3 (–926 descat). At –929, an E-box is located which is destroyed by the insertion of this fragment in the CAT vector. In construct 4 (–1600 descat), this E-box is still intact. CAT activity decreases when sequences between –926 and –645 are deleted, indicating the presence of a positive regulatory region. A small decrease in CAT activity can be observed in myotubes, when nucleotides are deleted between –450 bp and –89 bp. Further deletion up to –53 bp, using Bal31 exonuclease, leads to an almost 50% decrease in CAT activity. The mutation analysis demonstrates that the change of the CAGCTG sequence at –80 to AGGCCT, leaving the rest of the full-length promoter intact (M2), leads to almost complete abolishment of the promoter activity. A similar mutation, involving the E-box at –825 (M1), does not exhibit very drastic effects. CAT activity is only 60–70% lower than the activity of the full-length non-mutant. The double mutant with both E-boxes mutated (M3) and the additional deletion of all nucleotides up to –926 (M4) show, as expected, background CAT levels too.

The activity of the desmin 5′ upstream region is very low in

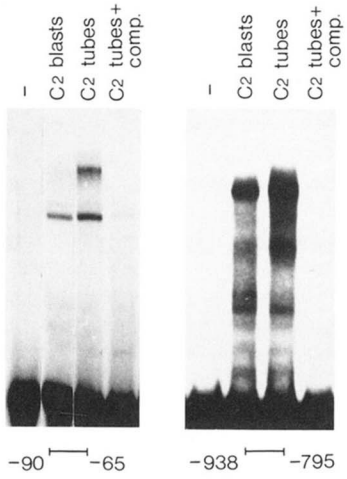

FIG. 2. **Gel mobility shift assays with nuclear extracts from C2 myoblasts and myotubes.** DNA restriction fragments were end-labeled and incubated with myoblast and myotube nuclear extracts. The fragment extending from –90 bp to –65 bp comprises the proximal E-box at –80 bp, and the second fragment (–938 to –795) comprises, for example, the –825 E-box and the putative MEF-2 consensus sequence. The *first lane* of each panel contains DNA without protein extract (–), and, subsequently, incubations with blasts and myotube nuclear extracts are shown. Each *last lane* represents a competition assay with a corresponding unlabeled DNA fragment (100–500-fold molar excess) in combination with a myotube nuclear extract.

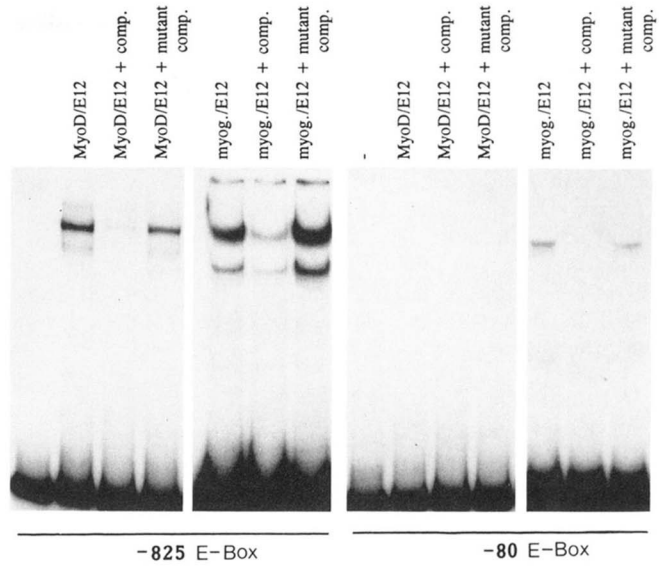

FIG. 3. **Gel retardation assays with purified glutathione *S*-transferase MyoD and myogenin fusion proteins.** Purified fusion proteins were dimerized with *in vitro* translated partial E12 protein before incubation with the same labeled DNA fragments used in combination with the nuclear extracts (see Fig. 5). The *first lane* of each panel contains the probe without protein (–). *Subsequent lanes* represent incubations with either MyoD or myogenin (*myog.*) fusion proteins with or without the appropriate unlabeled competition DNA (500-fold excess). Additionally, in each *last lane*, this competition DNA comprises a mutated E-box.

human primary dermal fibroblasts. Therefore, only the results obtained with the co-transfection of the myogenin expression vector are shown. These results resemble those of the C2 cells very closely. Only the decrease in CAT activity between construct 3 and 4 (–926 descat and –625 descat) is more pronounced.

In conclusion, transfection of the above-mentioned deletion and mutation constructs in C2 myoblasts and myotubes demonstrates the presence of a weak negatively regulatory region between –1600 bp and –926 bp, which is not further characterized. The destruction of an E-box located at –929 does not lead to a decrease in CAT activity; instead, an almost 2-fold increase can be observed when the whole region between –1600 and and –926 is deleted. This is also reflected by the results obtained with the primary fibroblasts upon co-transfection of the myogenin expression plasmid. Correspondingly, the positive element located between –926 and –625 can also be clearly recognized in myoblasts, myotubes, and myogenin co-transfected fibroblasts. This element displays an even stronger effect in fibroblasts. We believe that this is due to a higher concentration of transactivating protein upon co-transfection, compared to endogenous levels in C2 cells. A further positive element was found after evaluation of the results upon deletion up to –53 bp (including deletion of the E-box element at –80 bp). The mutation analysis indicates that this E-box is very important, because substitution of 4 nucleotides leads to complete abolishment of the promoter activity.

In Vitro Interaction of Purified HLH Proteins and Proteins in Nuclear Extracts with Elements in the Desmin 5′ Upstream Region—The E-box elements at –80 bp and –825 bp have been chosen for *in vitro* DNA-protein interaction studies. Nuclear extracts have been prepared from myoblasts and differentiated myotubes, and DNA fragments have been end-labeled by Klenow large fragment polymerase. Using an oligonucleotide fragment containing the –80 E-box as a probe, a pattern of retarded bands characterized by only 1 band in myoblasts and 2 bands in myotube nuclear extracts is displayed (Fig. 2). Competition with a 100-fold molar excess of an unlabeled fragment shows that the affinity of the binding proteins is specific. Moreover, the same fragment has also been used for incubation with a HeLa nuclear extract which yields a totally different pattern, suggesting that both protein-DNA complexes are muscle-specific (results not shown). The results obtained with the –825 E-box fragment are much more complex. At least 3 major retarded complexes are visible (Fig. 2). The specificity of all complexes has been confirmed by competition analysis with an unlabeled fragment added in 100-fold molar excess. The latter fragment is an *RsaI/Hin*cII restriction fragment extending from –891 to –796, which not only contains the –825-bp E-box but also comprises an MEF2 site located at –855.

To investigate whether MyoD and myogenin proteins are able to bind to both E-box sequences, we used the pGex bacterial expression system to purify these proteins by affinity chromatography. MyoD and myogenin are able to bind as homodimers *in vitro*, whereas both proteins bind as heterodimers with a third non-muscle-specific protein *in vivo*. *In vitro* heterodimers are known to bind with higher affinity compared to homodimers. Therefore, often a heterodimerization step involving the E12 protein is carried out. The E12 protein we used has been synthesized as a truncated protein by coupled *in vitro* transcription and translation. Despite the fact that it is not a full-length protein, it contains all the necessary information for heterodimerization with MyoD and myogenin, and, as expected, we found higher binding affinities in experiments using heterodimers (results not shown). Fig. 3 shows that prominent retarded complexes are obtained with MyoD and myogenin fusion proteins using the –825 E-box fragment. As a control, we have performed competition experiments with unlabeled DNA fragments containing the wild-type or the mutated E-box consensus sequence. The retarded complexes resulting from incubation of the –80 E-box with heterodimerized myogenin are comparable with the experiment described above. MyoD, however, hardly showed affinity for the labeled oligonucleotide fragment.

The results of the *in vitro* binding studies suggest that the retarded bands obtained with the nuclear extracts from myo-

blasts and myotubes, in combination with the labeled -80 E-box fragment, may be due to the binding of myogenin and MyoD. In the lane with the myotube extract, an additional band is visible; this may be caused by myogenin, which is expressed primarily in myotubes. This is also supported by the observation that bacterially expressed myogenin is capable of binding to this E-box. For the -825 E-box fragment, this is still elusive, since the latter is a much larger DNA fragment and more, yet unidentified, proteins may bind to it. However, it is clear that both myogenin and MyoD are able to bind to the -825 E-box-containing DNA fragment, and that the -80 E-box hardly binds to bacterially produced MyoD, heterodimerized with the E12 protein.

DISCUSSION

We have investigated the sequences responsible for the regulation of the hamster desmin muscle-specific expression. A consensus element for the binding of HLH proteins located at -80 bp upstream from the cap site appears to be essential for desmin expression. Substitution of these sequences for a *Stu*I restriction site leads to elimination of promoter activity. Furthermore, an upstream positively acting region has been characterized which functions both in myoblasts and myotubes. The latter region is more prominently displayed when human fibroblasts and co-transfection of a myogenin expression plasmid are used. This is probably due to the excess amount of myogenin created by this procedure. On the other hand, mutation of the HLH binding element, which is situated in this region at -825 bp, has only a moderate effect in both cell lines. Another E-box consensus element located in the same region, which is destroyed by digestion with the *Ban*I restriction enzyme, is probably not important at all, because no negative effects on CAT activity are visible. We also tested the enhancer activity of the upstream positively acting region, but only small effects were detected (results not shown). More detailed experiments in combination with a further mutation analysis of the putative consensus elements situated in the latter region will be performed in the near future.

From our *in vitro* binding assays we may conclude that several nuclear proteins are able to bind to the region surrounding the E-box at -825. Some may well be specific for myotubes because bands of higher intensity appear in the lane with the myotube nuclear extract. Since no other E-boxes are present in this DNA fragment, it is also conceivable that the E-box at -825 bp, which does not contribute to the enhancer activity of the region, can bind to purified glutathione transferase (GST) fusion proteins. *In vitro* synthesized myogenin seems to have a preference for the -80 E-box, which is essential for desmin expression. GST-MyoD binding to this element is only visible after longer exposures. The observation that, for instance, MyoD can bind strongly to the -825-bp E-box, but does not seem to transactivate the same element, has also been reported in other cases (Fujisawa-Sehara *et al.*, 1992). This discrepancy may be caused by a difference between the *in vivo* and *in vitro* situation. Moreover, other factors interacting in the vicinity of the E-box sequence may interfere with binding of MyoD family members *in vivo*.

The analysis of the human desmin promoter region revealed that the upstream positive element is important for high level expression (Li and Paulin, 1991). While our work was in progress, the cloning and characterization of the mouse upstream regulatory region has been published (Li and Capetanaki, 1993). Our results are comparable with the situation in the human and the mouse desmin promoter regions. Additionally, we present a mutation analysis, showing that particularly the E-box at -80 bp is important for the muscle-specific expression of the desmin gene. Another candidate factor, which might be involved in the regulation of expression (also put forward for the human and mouse desmin regulatory regions), is a putative consensus element for the myocyte-specific enhancer factor, MEF-2. This protein complex binds to A + T-rich elements in several other muscle-specific genes (Gossett *et al.*, 1989). Moreover, this complex of binding proteins was shown to be inducible by members of the MyoD family like myogenin and MyoD (Cserjesi and Olson, 1991; Lassar *et al.*, 1991).

A minimum of two MyoD binding sites is often required for enhancer function (Lassar *et al.*, 1989; Piette *et al.*, 1990; Wentworth *et al.*, 1991; Gilmour *et al.*, 1991). In numerous other muscle-specific genes, E-boxes *per se* are not sufficient for high level transcription. Binding sites for other cell type-restricted and ubiquitous factors like MEF-2, CArG, and SpI binding proteins cooperate with the E-box to confer transcriptional activity (Sartorelli *et al.*, 1990; French *et al.*, 1991; Pari *et al.*, 1991). Incidentally, even a single E-box or a single MEF-2 site has been reported to be a target of transactivation (Prody and Merly, 1992; Nakatsuji *et al.*, 1992). The latter situation may be consistent with the situation in the hamster desmin promoter. We cannot rule out, however, the involvement of other factors like the MEF-2 recognition site situated in the upstream enhancer region in contributing to the level of desmin expression. To date, none of the MyoD family members has been detected in heart tissue, but for some other genes expressed in both skeletal muscle and heart, the MEF-1 factor has been reported to play an important role in the latter organ (Nakatsuji *et al.*, 1992; Navankasattusas *et al.*, 1992). Further studies using cardiac muscle cells or transgenic animals can address these questions.

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