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Characterisation of a genomic clone covering the structural mouse MyoD1 gene and its promoter region

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ABSTRACT

We have isolated the mouse MyoD1 gene flanked by its promoter region by screening a genomic library with synthetic oligonucleotides. The structural gene is interrupted by two G+C rich introns. Transfection of the cloned gene inserted into an expression vector converts fibroblasts to myoblasts. Sequence analysis of about 650 bp of the 5′ upstream region revealed the presence of several potential regulatory elements such as a TATA-box, an AP2-box, two SP1-boxes and a CAAT-box. In addition, there are three half palindromic estrogen response elements, a potential cAMP response element and various muscle specific elements such as a muscle-specific CAAT-box (MCAT) and four potential binding sites for MyoD1. Using S1 protection analysis the major start site of transcription in muscle and myoblast cells was mapped 3 bp upstream of the published cDNA 5′ end. Promoter activity of the 650 bp upstream fragment was tested by *in vitro* transcription and by transfection analysis of myoblasts and fibroblasts. In all promoter test systems used, MyoD1 promoter activity was detected in myoblasts as well as in fibroblasts. Furthermore, DNA methylation was found to turn off MyoD1 promoter activity both in myoblasts and in fibroblasts.

INTRODUCTION

Cellular differentiation is believed to be regulated by lineage-specific changes in gene transcription. In the case of muscle differentiation, activation of a family of transcriptional regulators (MyoD1, Myf-5, Myogenin and MRF4) is known to play a key role in both cellular determination and induction of muscle-specific gene expression (for reviews see references 1–6). The ability of individual members of this myogenic family to convert upon transfection a variety of cell types irreversibly to the myogenic lineage suggests a very tight cell-type and tissue specific expression (7,8). The combined action of a mosaic of positive and negative regulatory factors might ultimately lead to a very restricted gene expression only in the muscle cell lineage. In addition, DNA methylation and the formation of inactive chromatin could influence the action of certain regulatory factors either in a specific (9) or in a more general manner (10).

By far the best characterized member of the family of myogenic regulators is MyoD1. It was originally isolated from an embryonic fibroblast cell line, that upon treatment with the demethylating agent 5-azacytidine started to express MyoD1 and therefore converted the fibroblasts into muscle cells (11). MyoD1 is a nuclear phosphoprotein (12) that interacts by means of its helix-loop-helix domain with other regulatory proteins in a functional (proteins E12, E47) (13,14) or non-functional (protein Id) manner (15). It binds either as homo- or as heterodimer to a specific sequence (CANNTG) (16) and is able to transactivate various skeletal-muscle specific genes (17–22) including itself (23) as well as the other members of the myogenic family (24). Furthermore, MyoD1 gene expression is controlled by serum (25), growth factors (26), electrical activity (27) and by oncogenes such as ski (28), fos and jun (29), ras (29) and myc (30)).

In order to study the positive and negative regulatory mechanisms involved in cell-type and tissue specific expression of one member of the myogenic regulatory family we have isolated the mouse MyoD1 structural gene flanked by its promoter region. We have identified the position of two introns and the start site of transcription using S1 mapping. We show by transfection into myoblasts and by *in vitro* transcription that a fragment of about 650 basepairs (bp) of the 5′ upstream region contains a promoter with common regulatory elements. However, promoter activity is not strictly restricted to myoblasts indicating that additional sequences or DNA modification by methylation are needed for the cell-type and tissue specific expression. Indeed, using methylated promoter constructs we show both by transfection and by *in vitro* transcription, that DNA metylation has the potential to reduce or even block MyoD1 promoter activity in both myoblasts and fibroblasts.

MATERIALS AND METHODS

Cloning and sequencing

A mouse genomic EMBL3 library (Clontech) was screened according to Maniatis et al. (31) by using a 70 bp oligonucleotide corresponding to the 5′ end (pos. 150–220) of the published cDNA of MyoD1 (7). Sequence analysis was performed on both strands using the sequenase according to the protocols provided by the manufacturer (USB).

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Plasmid constructions and DNA methylation

For transfection, promoter test vectors using a MyoD1-β-galactosidase fusion protein as indicator was constructed by replacing the SphI/KpnI fragment of pCH110 (Pharmacia) containing the SV40 promoter by a HindIII/StuI (pMO10) or the HindIII/TaqI (pMO22) fragment containing the MyoD1 promoter region (fig.1). Linker oligonucleotides were inserted at the StuI/KpnI and TaqI/KpnI junction in order to provide the correct reading frame.

For luciferase assays a promoter test vector was constructed by replacing the SV40 early promoter of pPALU (32) by a Hind III/BspMII fragment containing the MyoD1 promoter (fig.1). Plasmids were methylated with CpG methylase (SssI, Biolabs) according to protocols provided by the manufacturer.

For *in vitro* transcription a vector was constructed (pMO50) by cloning the HindIII/BsmI fragment containing the MyoD1 promoter region into HindIII/AflIII sites of pUC18 (fig.1). An oligonucleotide of 42 bp containing cauliflower mosaic virus gene sequences was used as linker to join the BsmI/AflIII sites and to provide a unique sequence for primer extension.

A MyoD1 expression vector was constructed by replacing a StuI/AvaI fragment of pCH110 by a BspMII/HindIII fragment containing the MyoD1 structural gene interrupted by two introns (fig.1).

S1 mapping

Total RNA was isolated from tissues and cell culture by the guanidinium iso-thiocyanat method (33). Single stranded DNA probe was labelled uniformly by primer extension, purified over an alkaline agarose gel and hybridized overnight at 55°C with 30 μg of total RNA. The hybridisation mixture was then digested with S1 nuclease (Boehringer Mannheim) followed by phenol/chloroform extraction (34). The resulting protected fragments were analysed on a 6% polyacrylamide/8 M urea sequencing gel.

In vitro transcription

Skeletal muscle nuclear extracts from mouse hind limbs were prepared according to Zahradka et al. (35) and nuclear cell extracts from G-8 myoblasts and C3H/T101/2 fibroblasts were prepared according to Shapiro et al. (36). *In vitro* transcription reactions were carried out in a 50 μl volume, containing nuclear extracts (10 μg protein) in 15 mM Hepes (pH 7.6), 30 mM KCl, 10 mM MgCl, 1 mM of each NTP, 12% glycerol, 2.5 mM DTT and 40 units RNAse inhibitor. Optimal transcription was obtained with 200 ng of closed circular template DNA. The reaction mixture was preincubated for 30 min at 30°C before addition of ribonucleotides triphospates, followed by an additional incubation at 30°C for 30 min. The reaction mixture was treated with proteinase K, phenol/chloroform, and RNA was precipitated by ethanol. RNA was analysed by primer extension as described by Andrisiani et al. (37).

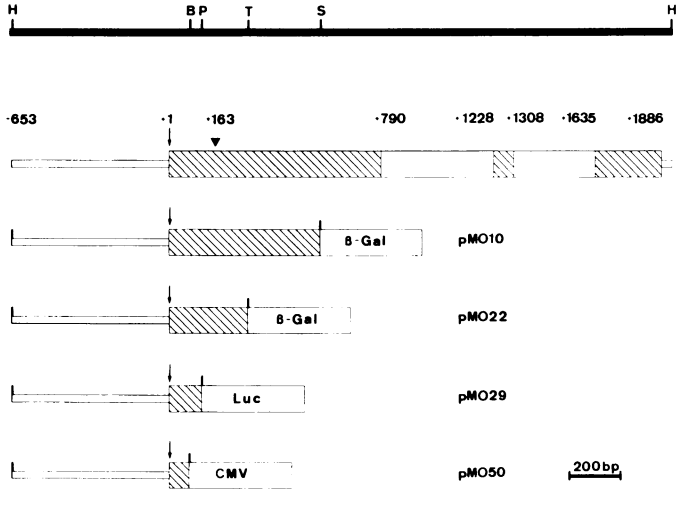

Figure 1: Organisation of the mouse MyoD1 gene. A HindIII (H) fragment contains both the complete MyoD1 gene (striped boxes) interrupted by two introns (open boxes) and the 5' upstream region (open line) of the MyoD1 gene. The arrow indicates the start site of transcription (+1). Positions of exons and introns and the the start site of translation (triangle at +163) are given in bp relative to the start site of transcription. Restriction sites are H (HindIII), B (BsmI), P (BspMII), S (StuI) and T (Taq). Plasmid contructs (for detailed description see materials and methods): MyoD1-β-galactosidase (β-gal) fusions (pMO10 and pMO22); MyoD1-luciferase (luc) promoter test vector (pMO29); MyoD1-cauliflower mosaic virus (CMV) promoter test vector (pMO50). The sequence of the HindIII fragment has been deposited in the EMBL data library (accession number X61655).

Figure 2: Sequence of the two introns and the joining exon of the mouse MyoD1 gene. The exon sequence is given in lower case letters. Underlined are nucleotides that are in agreement to the GT-AG rule for splice site selection (40). Positions of the intron bounderies are given in bp relative to the start site of transcription.

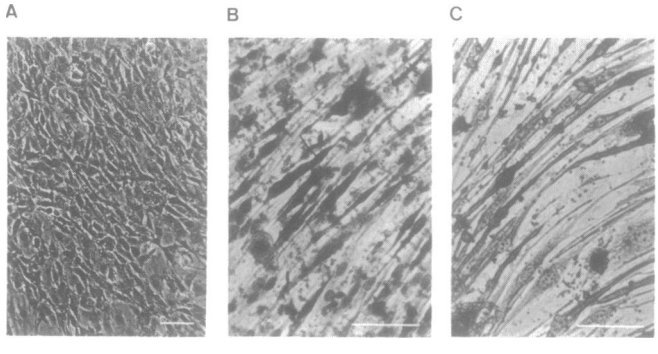

Figure 3: Transfection of C3H/10T1/2 fibroblasts with a vector expressing the MyoD1 gene converts them to myoblasts and myotubes. A) C3H/10T1/2 fibroblasts, B) C3H/10T1/2 fibroblasts transfected with a MyoD1 gene expression vector, C) G-8 myoblasts (Bar = 80 μm).

Cell culture and DNA transfection

Mouse myoblasts (G-8) and fibroblasts (C3H/10T1/2) were both obtained from the American Type Culture Collection. Myoblasts were grown on collagen coated plates in Dulbecco's modified eagle's medium (DMEM) containing 4.5 g/l glucose, 10% heat inactivated fetal calf serum (FCS) and 10% horse serum. Fibroblasts were grown in DMEM with 10% FCS.

Transfection and promoter activity assay was basically performed according to Lucibello and Mueller (38). Briefly, cells were plated in microtiter plates and grown for 20 to 24 hours to a confluency of about 60%. The cells were then transfected using 0 to 0.5 μg plasmid DNA per well. DNA was dissolved in 100 μl serum free DMEM containing 1 μl Lipofectin reagent (Gibco). Cells were cultured for 40 to 48 hours in the appropriate medium. β-galactosidase assays were performed according to Lucibello and Mueller (38). The difference in optical density OD_{405}-OD_{650} was measured by a microtiter plate reader. MyoD1-luciferase constructs were cotransfected with plasmid pCH110 as internal control and assayed according to de Wet et al. (39). For staining, cells were fixed for 10 minutes in 100% methanol and stained with 10% Giemsa.

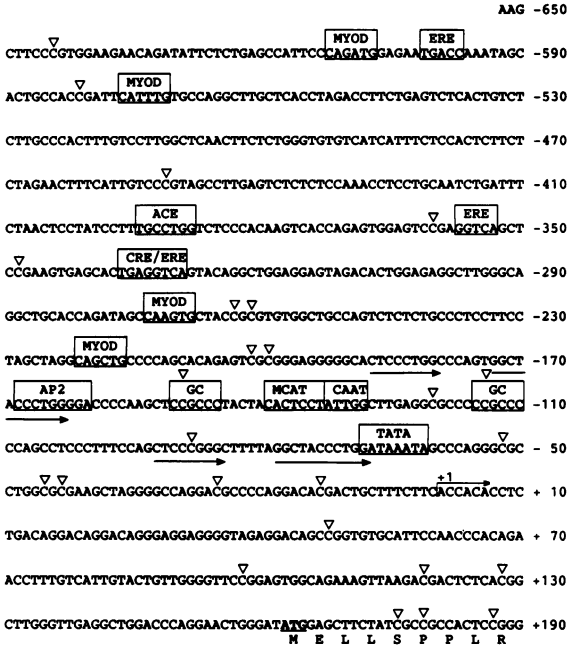

Figure 4: Sequence of the mouse MyoD1 5′ upstream region from the HindIII site to the start of the protein (fig.1). Nucleotide positions are indicated relative to the start site of transcription (+1), the translational start codon is underlined. Potential regulatory elements are boxed (see table 1 for explanation). Potential methylation sites (CpGs) are indicated by triangles. Two pair of nearly fully conserved direct repeats are indicated by arrows below the sequence.

RESULTS

Cloning of the mouse MyoD1 gene

Several positive clones were isolated by screening a mouse genomic EMBL3 library with a synthetic oligonucleotide corresponding to the 5′ end of the MyoD1 cDNA. A 2.6 kb HindIII fragment was identified by Southern blot hybridisation with the above labelled oligonucleotide. This HindIII restriction fragment contained the whole coding region of the mouse MyoD1 gene and 653 bp of its 5′ end (fig.1). The coding region of MyoD1 is interrupted by two introns localized at nucleotide positions 786 and 865 of the cDNA (7) corresponding to the positions +790 to +1228 and +1308 to +1635 of our cloned gene (fig.1).

The cloned MyoD1 gene is functional

Sequence analysis of the MyoD1 structural gene did not reveal any sequence difference to the published cDNA sequence (7) except for the presence of two introns. The sequence of the two introns and the joining exon is given in figure 2. The 5′ and 3′ splice sites of both introns are in agreement with the so-called GT-AG rule for splice site selection (40). The introns are 438 and 327 nucleotides long and contain 56 and 55% G+C respectively. Interestingly, both introns are rather poor in CpGs and therefore interrupt the CpG rich exons that have previously been defined as CpG island (41).

Table 1. DNA sequence motifs with possible regulatory function in MyoD1 expression

Element[a]	Consensus[b]	Sequence[c]	position[d]	reference
TATA	TATAAAA	*GATAAAT*	61–68	(42)
GC	GGCGGG	GGCGGG (rev)	110–115	(42)
		GGCGGG (rev)	145–149	
CAAT	CCAAT	CCAAT (rev)	128–132	(42)
MCAT	CATTCCT	CACTCCT	133–139	(45)
AP2	CCCCAGGC	CCCCAGG*G*	160–168	(46)
MYOD1	CANNTG	CAGCTG	216–221	(16)
		CAAGTG	270–275	
		CATTTG	571–576	
		CAGATG	607–612	
ERE	GGTCANNNTGACC	TGACC	598–602	(48)
		GGTCA	354–358	
		GGTCA	329–333	
cAMP	TGACGTCA	TGA*G*GTCA	329–336	(49)
ACE	CCAGGCA	CCAGGCA (rev)	388–394	(47)

[a] The significance of the various elements is discussed in the section result. ACE: myotube specific element found in the promoter of the acetylcholine receptor delta subunit (47).
[b] N stands for any nucleotide.
[c] Deviations from consensus sequence are underlined. (rev) sequence shown in the reverse orientation. Source of sequence, this paper (fig. 2).
[d] Negative position number according to figure 2.

In order to show that we have isolated a fully functional gene, a MyoD1 expression vector that expresses the MyoD1 structural gene under the control of the SV40 early promoter was constructed. The MyoD1 expression vector was cotransfected with plasmid pRSV-neo into C3H/10T1/2 fibroblasts and after two days of incubation stable colonies were selected by addition of G418 (Gibco, 40 μg/ml) to the culture medium. As indicated in figure 3, growing the transfected cells for several days led to the formation of multinucleated myotubes indicating that fibroblasts had been converted into muscle cells.

Sequence analysis of the promoter region

The nucleotide sequence of the 5′ upstream region and part of the first exon is shown in figure 4. As compared to the MyoD1 structural gene which is defined as a CpG island (41) its promoter region is rather poor in CpGs. Analysis of the 5′ upstream sequence revealed several highly conserved elements typical for eukaryotic promoters transcribed by polymerase II (see fig. 4 and table 1) (42−44). A potential TATA-box is located at the unusual distance of 60 bp relative to the major start site of transcription. Various conserved upstream promoter elements for ubiquitous and skeletal-muscle specific factors are located upstream of the TATA-box. A consensus CAAT-box (42) is flanked by two GC-boxes (42) at an equal distance of 14 bp. Interestingly, the CAAT-box and both GC-boxes are in the inverse orientation. Just beside the CAAT-box there is a nearly conserved muscle specific CAAT-box (MCAT) (45). Furthermore, an AP-2 binding site (46), a myotube specific element found also in the promoter of the acetylcholine receptor delta subunit (ACE) (47), three half palindromic estrogen response elements (ERE) (48) and four conserved binding sites for MyoD1 and its relatives (16) can be identified. Not fully conserved, but known to be functional in other promoters is a potential cAMP response element (CRE) (49), that could also represent a potential target for the negative regulation of MyoD1 expression by the oncogene jun and fos (29).

The organisation of the regulatory elements of the MyoD1 promoter is reminiscent of other eukaryotic promoters such as the Herpes simplex thymidine kinase promoter (50), where the TATA box is also preceded by a CAAT-box flanked by two SP-1 boxes. Comparison of the MyoD1 promoter sequence with the promoter sequence of other muscle-specific genes studied to date revealed a number of small regions of sequence homology (not shown). None of these, however, were present in more than one other muscle-specific gene promoter. Two pairs of direct repeats (fig.4) can be found in the MyoD1 promoter, but their potential significance, if any in terms of promoter function, remains to be determined.

Start site of the *in vivo* transcription of MyoD1

In order to determine the transcriptional start site and the cell-type and tissue specific expression of the MyoD1 gene, the S1 nuclease protection assay was used. As shown in figure 5 and as reported by others (7), MyoD1 transcripts can only be detected in RNA isolated from skeletal muscle and from G-8 myoblasts (lanes 3 and 6), but not from adult heart muscle, liver, spleen or C3H/10T1/2 fibroblasts (lanes 1,2,4,5). As seen in figure 5 the main protected band has a size of about 95 bp and therefore extends the previously published 5′ cDNA end of MyoD1 by 3 bp (7). Therefore transcription is initiated at one major start site both in fully differentiated, adult skeletal muscle cells and in G-8 myoblasts.

In vitro transcription of cloned MyoD1 gene

The 5′ upstream region of the MyoD1 gene was tested for promoter activity by an *in vitro* transcription assay using nuclear

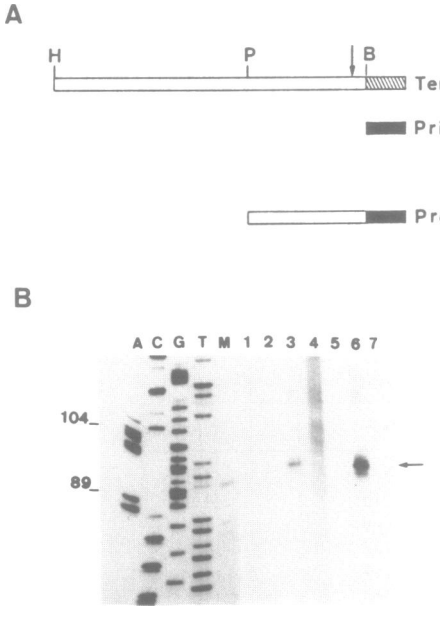

Figure 5: S1 nuclease mapping of the 5′ end of the MyoD1 mRNA synthesized *in vivo*. A) Map of the DNA template used for preparing the single stranded DNA probe. The template containing 271 nucleotides of the 5′ sequence (open line) and 42 nucleotides of the cauliflower mosaic virus (striped line) was used for synthesizing the probe by means of a unique primer (black line) (see materials and methods) and sequenase. The arrow indicates the position of the start site. Restriction sites are H (HindIII), P (PvuII) and B (BsmI). B) Autoradiogram of 6% acrylamide gel used to analyse primer extension products of total RNA. Lane M contains molecular weight markers with sizes in nucleotides; lane 1, heart RNA; lane 2, liver RNA; lane 3, muscle RNA; lane 4, spleen RNA; lane 5, C3H/10T1/2 fibroblast RNA; lane 6, G-8 myoblast RNA and lane 7, yeast tRNA. The arrow indicates the major protected band that corresponds to the transcription start site shown in figure 4.

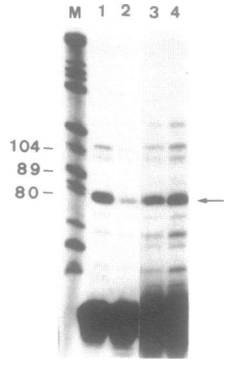

Figure 6: Electrophoretic separation (6% polyacrylamide/ 8 M urea gel) of the *in vitro* transcription product directed from the MyoD1 promoter. *In vitro* transcription was carried out with 10 μg protein and 200 ng of closed circular plasmid DNA (pMO50) as described in materials and methods. Lane M, molecular weight standard with sizes in nucleotides; lane 1, muscle nuclear extract; lane 2, muscle nuclear extract plus 3 μg/ml α-amanitin; lane 3, C3H/10T1/2 fibroblast nuclear extract and lane 4, G-8 myoblast nuclear extract. The arrow indicates the position of the correct initiation site of transcription.

extracts isolated from expressing (G-8 myoblasts and hind limb skeletal muscle cells) and non-expressing cells (C3H/10T1/2 fibroblasts). A plasmid was constructed (pMO50, fig.1), that transcribes part of the 5' end of structural MyoD1 gene fused to a unique sequence of cauliflower mosaic virus. Primer extension by an oligonucleotide complementary to the unique sequence allows the detection of the transcribed RNA. As shown in figure 6 a correct initiation of transcription when compared with the *in vivo* transcription was obtained by using nuclear extracts from G-8 myoblasts (lane 4) and hind limb skeletal muscle cells (lane 1). In addition, sensitivity of transcription to low concentrations of α-amanitin indicates that the MyoD1 gene is transcribed by RNA polymerase II (lane 2).

By using nuclear extracts from C3H/10T1/2 fibroblasts (lane 3), we detect also specific transcriptional activity of plasmid pMO50. These results indicate that either cell-type specific regulatory factors or their DNA targets are missing in our cell free system, or that other mechanisms such as DNA methylation may play an important role in the cell type specific expression of MyoD1.

Promoter activity in transfected myoblasts and fibroblasts

The activity of the mouse MyoD1 gene promoter was assayed by transient transfection of MyoD1-β-galactosidase fusion constructs into G-8 myoblasts and C3H/10T1/2 fibroblasts. By using part of the first exon of MyoD1 in our construct (pMO10, fig.1) we include both translational efficiency and potential functional and structural effects associated with the CpG island on MyoD1 promoter activity. Plasmid pMO10 transfected into G-8 myoblasts yielded about 10% of the activity detected with the SV40 early promoter of plasmid pCH110 (data not shown). A similar promoter activity was detected when deleting most of the first exon containing the MyoD1 CpG island (plasmid pMO22 (fig.1) data not shown). Therefore we conclude that there are no major positive regulatory elements in the MyoD1 CpG island. Interestingly, both plasmids (pMO10, pMO22) were also active in fibroblasts where the endogenous gene is inactive. These results indicate that either our constructs have no fibroblast specific negative regulatory elements or that as already stated above DNA methylation may play a central role for the cell-type specific silencing of the MyoD1 gene.

Inactivation of MyoD1 promoter activity by CpG methylation

The ability of 5-azacytidine to reactivate a silent MyoD1 gene (11) suggests, that DNA methylation may play a specific role in regulating MyoD1 gene expression. In order to test the effect of methylation on the activity of our constructs a sensitive promoter test vector (pMO29, fig.1) expressing the luciferase gene controlled by the MyoD1 promoter was transfected into myoblasts and fibroblasts in the methylated and unmethylated form. Figure 8a shows that methylation of plasmid pMO29 by CpG methylase SssI resulted in a complete block of MyoD1 promoter activity both in myoblasts and fibroblasts. By comparison, methylation of plasmid pPALU containing the SV40 early promoter reduced its activity to 15% and did not lead to a complete block of transcription (data not shown). It has been suggested (51) that the decrease of SV40 early promoter activity by DNA methylation is mainly a result of inactive chromation formation over the entire plasmid. When compared to the inhibition of the methylated SV40 promoter the complete block of MyoD1 promoter activity indicates additional silencing mechanisms. MyoD1 DNA methylation, therefore, may either prevent the binding of important activating factors or enhance the binding of a repressor(s) necessary for the silencing of the gene.

In contrast to the *in vivo* data, *in vitro* transcription with the same methylated DNA and muscle extracts resulted only in a 50% reduction of specific promoter activity (figure 8b). Similar results were obtained using nuclear extracts from C3H/T101/2 fibroblasts and G-8 myoblasts (data not shown).

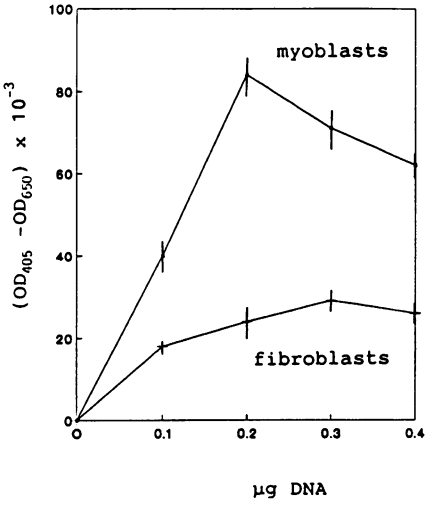

Figure 7: Expression of MyoD1-β-galactosidase fusion gene in transfected cells. Increasing concentration of plasmids (pMO10 fig.1) were transfected into G-8 myoblasts and C3H/10T1/2 fibroblasts according to Lucibello and Mueller (38). The difference in optical density OD_{405}-OD_{650} was measured using a microtiter plate reader and corrected for cell-type specific background (38). Each point represents the average of four different transfection series with a standard deviation below 15%. A control plasmid with the MyoD1 promoter in the inverse orientation didn't show any promoter activity (data not shown).

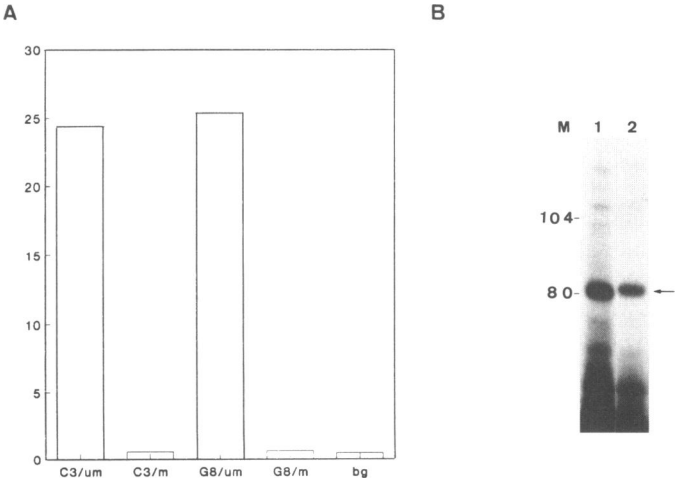

Figure 8: CpG methylation inhibits MyoD1 promoter activity both *in vivo* and *in vitro*. A) Plasmid pMO29 unmethylated (um) and methylated (m) was cotransfected with plasmid pCH110 as internal control into C3H/10T1/2 fibroblasts (C3) and G-8 myoblasts (G8) and normalized luciferase activity was measured according to de Wet et al. (39). bg; background of untransfected cells. B) *In vitro* transcription assay using muscle nuclear extracts and plasmid pMO50 unmethylated (lane1) and methylated (lane 2) by CpG methylase (SssI). The arrow indicates the position of the full size transcripts.

DISCUSSION

Using an oligonucleotide probe corresponding to the 5′ end of the mouse MyoD1 cDNA, we isolated genomic clones containing both the 5′ upstream region and the full coding part of the mouse MyoD1 gene interrupted by two introns. A conversion of fibroblasts into myoblasts was obtained by transfection of the coding part of MyoD1 linked to the SV40 early promoter thus proving that we have isolated a fully functional MyoD1 gene. We identify within 650 bp of the 5′ flanking region various potential target sequences for ubiquitous and muscle specific factors. Among them is a potential cAMP response element that could also be the target for the previously reported inhibition of MyoD1 gene expression by the oncogenes fos and jun (29). In addition we find four consensus sequences for MyoD1 (16), which are possibly involved in autoregulation and regulation by other helix-loop-helix proteins. Using S1 mapping we localize the major transcriptional start site of the MyoD1 promoter 3 bp upstream of the previously published 5′ end of the MyoD1 cDNA (7). We show by *in vitro* transcription and by transient transfection into myoblasts, that the 5′ upstream region contains a functional promoter. The α-amanitin sensitivity of *in vitro* transcription and the organisation of several potential regulatory elements similar to other promoters (42–47) indicate, that the MyoD1 promoter is most likely transcribed by RNA polymerase II.

As indicated by the effects of various oncogenes (28–30), growth factors (26), serum (25) and electrical activity (27), MyoD1 gene expression in muscle cells is under multiple positive and negative control. Furthermore, regarding the dominant effect of the MyoD1 protein in converting various cell lines to the myogenic lineage (7,8), a mechanism is expected to prevent any expression of MyoD1 in non-muscle tissues. The experiments of Thayer and Weintraub on the silencing of the MyoD1 gene in specific somatic cell fusions suggest the presence of specific repressor(s) in cells not expressing MyoD1 (52). In addition, the initiation of the myogenic program in fibroblasts treated with the demethylating agent 5-azacytidine (11) strongly suggests, that at least in fibroblasts DNA methylation plays a role in preventing MyoD1 gene expression. Further experiments carried out by Lassar et al. have shown that the transfection of fibroblasts with total uncloned genomic DNA prepared from myoblasts (where MyoD1 is not methylated), but not from fibroblasts (where MyoD1 is methylated), was able to initiate the myogenic program (53). More evidence for a direct role of DNA methylation in promoter inactivation comes from the observation that the immortalisation of primary fibroblasts and the complementary silencing of the MyoD1 gene involved in terminal differentiation is correlated with the hypermethylation of the MyoD1 associated CpG island (41). We show here that for the inhibition of MyoD1 promoter activity both *in vivo* and *in vitro* the MyoD1 CpG island is not absolutely required. However, it is open whether the CpG island has at the chromosomal level a role in the formation of active or inactive chromatin or in the stabilisation of the methylated or unmethylated state at the MyoD1 promoter (54). It remains to be shown, whether DNA methylation inhibits the MyoD1 promoter by preventing directly binding of positive acting factors (9) or indirectly by means of a methyl DNA binding protein (55) or the formation of inactive chromatin (10). Recently the existence of such repressor proteins binding preferentially to methylated DNA has been described (55–58).

The isolation and identification of the MyoD1 promoter is one step towards a more detailed characterisation of specific elements and mechanisms responsible for cell-type and tissue specific gene expression.

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REFERENCES

1. Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, K., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y. and Lassar, A. (1991) *Science*, **251**, 761–766.
2. Emerson, C. P. (1990) *Cur. Op. in Cell Biol.*, **2**, 1065–1075.
3. Chen, J. and Jones, P. (1989) *Cur. Op. in Cell Biol.*, **1**, 1075–1080.
4. Olson, E. (1990) *Genes Dev.*, **4**, 1454–1461.
5. Rosenthal, N. (1989) *Cur. Op. in Cell Biol.*, **1**, 1094–1101.
6. Kingston, R. E. (1989) *Cur. Op. in Cell Biol.*, **1**, 1081–1087.
7. Davis, R. L., Weintraub, H., Lassar, A. B. (1987) *Cell*, **51**, 987–1000.
8. Weintraub, H., Tapscott, S. J., Davis, R. L., Thayer, M. J., Adam, M. A., Lassar, A. B. and Miller, D. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5434–5438.
9. Iguchi-Ariga, S. M. M. and Schaffner, W. (1989) *Genes Dev.*, **3**, 612–619.
10. Keshet, I., Lieman-Hurwitz, J. and Cedar, H. (1986) *Cell*, **44**, 535–543.
11. Konieczny, S. and Emerson, C. P. (1984) *Cell*, **38**, 791–800.
12. Tapscott, S. J., Davis, R. L., Thayer, M. J., Cheng, P., Weintraub, H. and Lassar, A. B. (1988) *Science*, **242**, 405–411.
13. Murre, C., Schonleber McCaw, P., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., Weintraub, H. and Baltimore, D. (1989) *Cell*, **58**, 537–544.
14. Murre, C., Schonleber McCaw, P. and Baltimore, D. (1989) *Cell*, **56**, 777–783.
15. Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L. and Weintraub, H. (1990) *Cell*, **61**, 49–59.
16. Davis, R. L., Cheng, P., Lassar, A. B. and Weintraub, H. (1990) *Cell*, **60**, 733–746.
17. Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., Hauschka, S. D. and Weintraub, H. (1989) *Cell*, **58**, 823–831.
18. Piette, J., Bessereau, J. L., Huchet, M. and Changeux, J. P. (1990) *Nature*, **345**, 353–355.
19. Lin, H., Yutzey, K. and Konieczny, S. F. (1991) *Mol. Cell. Biol.*, **11**, 267–280.
20. Sartorelli, V., Webster, K. A. and Kedes, L. (1990) *Genes Dev.*, **4**, 1811–1822.
21. Wentworth, B. M., Donoghue, M., Engert, J. C., Berglund, E. B. and Rosenthal, N. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 1242–1246.
22. Weintraub, H., Davis, R., Lockshon, D. and Lassar, A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5623–5627.
23. Thayer, M. J., Tapscott, S. J., Davis, R. L., Wright, W. E., Lassar, A. B. and Weintraub (1989) *Cell*, **58**, 241–248.
24. Yutzey, K. E., Rhodes, S. J. and Konieczny, S. F. (1990) *Mol. Cell. Biol.*, **10**, 3934–3944.
25. Peterson, C. A., Gordon, H., Hall, Z. W., Paterson, B. M. and Blau, H. M. (1990) *Cell*, **62**, 493–502.
26. Vaidya, T. B., Rhodes, S. J., Taparowsky, E. J. and Konieczny, S. F. (1989) *Mol. Cell. Biol.*, **9**, 3576–3579.
27. Eftimie, R., Brenner, H. R. and Buonanno, A. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 1349–1353.
28. Colmenares, C., Teumer, J. K. and Stavnezer, E. (1991) *Mol. Cell. Biol.*, **11**, 1167–1170.
29. Lassar, A. B. and Thayer, M. J. (1989) *Cell*, **58**, 659–667.
30. Miner, J. H. and Wold, B. J. (1991) *Mol. Cell. Biol.*, **11**, 2842–2851.
31. Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour University Press, Cold Spring Harbour.
32. Artelt, P., Grannemann, R., Stocking, C., Friel, J., Bartsch, J. and Hauser, H. (1991) *Gene*, **99**, 249–254.
33. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry*, **18**, 5294–5299.
34. Ley, T., Angnon, M., Pepe, G. and Nienhuis, A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4775–4779.

35. Zahradka, P., Larson, E. D. and Bruce, H. S. (1989) *Exp. Cell Res.*, **185**, 8–20.
36. Shapiro, J. D., Sharp, A. P., Wahli, W. and Keller, J. M. (1988) *DNA*, **7**, 47–55.
37. Andrisani, M. O., Hayes, E. T. Roos, B. and Dixon, J. E. (1987) *Nucl. Acids Res.*, **15**, 5715–5728.
38. Lucibello, F. C. and Mueller, R. (1989) *Methods in Mol. and Cell. Biol.*, **1**, 9–18.
39. De Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R. and Subramani, S. (1987) *Mol. Cell. Biol.*, **7**, 725–737.
40. Padgett, R. A., Grabowski, P. J., Konarska, M. M., Seiler, S. and Sharp, P. A. (1986), *Ann. Rev. Biochem.*, **55**, 1119- 1150.
41. Jones, P. A., Wolkowicz, M. J., Rideout III, W. M., Gonzales, F. A., Marziasz, C. M., Coetzee, G. A. and Tapscott, S. J. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 6117–6121.
42. Bucher, P. (1990) *J. Mol. Biol.*, **212**, 563–578.
43. Maniatis, T., Goodbourn, S. and Fischer, J. A. (1987)
Science, **236**, 1237–1245.
44. Mitchell, P. J. and Tjian, R. (1989) *Science*, **245**, 371–378.
45. Mar, J. H. and Ordahl, C. P. (1990) *Mol. Cell. Biol.*, **10**, 4271–4283.
46. Jones, N. C., Rigby, P. W. and Ziff, E. B. (1988) *Genes Dev.*, **2**, 267–281.
47. Baldwin, T. J. and Burden, S. J. (1989) *Nature*, **341**, 716–720.
48. Klein-Hitpass, L., Tsai, S. Y., Greene, G. L., Clark, J. H., Tsai, M. and O'Malley, B. W. (1989) *Mol. Cell. Biol.*, **9**, 43- 49.
49. Israel, A., Le Bail, O., Hatat, D., Piette, J., Kieran, M., Logeat, F., Wallach, D., Fellous, M. and Kourilsky, P. (1989) *EMBO*, **8**, 3793–3800.
50. Jones, K. A., Yamamoto, K. R. and Tjian, R. (1985) *Cell*, **42**, 559–572.
51. Adams, R. L. P., Bryans, M., Rinaldi, A., Smart, A. and Yesufu H. M. I. (1990) *Phil. Trans. R. Soc. Lond.*, **326**, 189- 198.
52. Thayer, M. J. and Weintraub, H. (1990) *Cell*, **63**, 23–32.
53. Lassar, A. B., Paterson, B. M. and Weintraub, H. (1986) *Cell*, **47**, 649–656.
54. Tazi, J. and Bird, A. (1990) *Cell*, **60**, 909–920.
55. Boyes, J. and Bird, A. (1991) *Cell*, **64**, 1123–1134.
56. Ehrlich, M., Zhang, X. Y., Asieda, C. K., Kahn, R. and Supakar, P. C. (1990) Eds. Clawson, D. B., Willis, Weinbach, A. and Jones, P. A., In Nucleic Acid Methylation, UCLA Symposium in Molecular and Cellular Biology, **128**, 351–365.
57. Pawlak, A., Bryans, M. and Jost, J. P. (1991) *Nucl. Acids Res.*, **19**, 1029–1034.
58. Vaccaro, M., Pawlak, A. and Jost, J. P. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 3047–3051.