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Myogenin binds to and represses c-*fos* promoter

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Abstract Myogenin (a member of the myogenic basic helix-loop-helix transcription factor family) seems to be the main effector of proliferation repression, a crucial step which precedes muscle cell terminal differentiation during muscle development. Proliferation repression most likely occurs through inhibition of proliferation-associated genes such as the proto-oncogene, c-*fos*. Here, we demonstrate that myogenin binds to an E-box located in the main element of the c-*fos* promoter, the serum response element (SRE). Results from co-transfection experiments indicate that myogenin acts as a repressor for the SRE. Our data suggest that myogenin could play a role in c-*fos* inhibition at the onset of muscle cell terminal differentiation.

Key words: Myogenin; c-*fos*; Serum response element

1. Introduction

Proliferation and differentiation are mutually exclusive processes. The balance between the two pathways is controlled both by proteins involved in the cellular proliferative response, such as proto-oncogenes, and by proteins involved in differentiation. This is best illustrated in the muscle model system. In the process of muscle cell terminal differentiation, proliferation inhibition is a crucial step which precedes muscle-specific gene expression and cell fusion into myotubes [1]. Indeed, terminal differentiation of myoblastic cell lines in vitro is triggered by the accumulation of the precursor cells (myoblasts) in a G_0 state [1]. This step is a prerequisite, and a number of mitogens [2–5] or oncogenes [6–10] inhibit terminal differentiation. The factors which regulate the balance between proliferation and differentiation in vivo during embryonic development or adult muscle regeneration are poorly understood at present. Recent data, however, have shed some light on the molecular mechanism involved in the control of this delicate balance, which seems to be, at least in part, regulated by myogenic factors of the bHLH family. Transcription factors from the bHLH family, which are instrumental in the muscle cell differentiation process, include MyoD [11–13], myogenin [14,15], Myf5 [16] and MRF4/herculin/myf6 [17]. They are all able to elicit in vitro a muscle determination program in a number of non-muscle cell types [18,19]. These muscle-restricted proteins share a domain of homology, the bHLH, which is also common to ubiquitous transcription factors such as the products of the E2A gene, E12 or E47 [20,21], with which myogenic factors form heterodimeric complexes. These heterodimers [22–26] bind to upstream regulatory sequences of the form CANNTG (E-boxes; [27]) in muscle-specific gene promoters. MyoD, myogenin and Myf5 are all able to transactivate these promoters efficiently [28], a function which involves a common motif in the basic domain [29].

In vitro, MyoD, myogenin, MRF4 and Myf5 seem to be largely interchangeable, although some differences are observed (essentially for MRF4; [30–32]). However, in vivo, they display largely distinct kinetics and patterns of expression during the course of embryonic development [33–35], suggesting that their multiplicity does not reflect a simple redundancy of the system. A key role in cell proliferation inhibition had been attributed to MyoD. Indeed, MyoD is a target for mitogenic differentiation inhibitors [2,7,10]. Moreover, MyoD acts as a negative regulator for cell proliferation in vitro [36,37]. Part of the mechanism by which MyoD blocks cell proliferation in vitro seems to be the repression of proliferation-associated genes. In particular, one of the first events associated with terminal differentiation is the disappearance of proto-oncogene mRNA such as the nuclear proto-oncogene c-*fos* [38]. c-*fos* is an immediate early, growth factor responsive gene which is part of the AP1 transcriptional complex [39,40] and which is necessary for bone development and haematopoiesis but not for muscle differentiation [41]. In fact, c-*fos* rather inhibits myogenesis, and when a high level of Fos protein is artificially maintained in myoblasts, terminal differentiation is severely impaired [7,10].

MyoD acts as a repressor of the c-*fos* promoter, most likely through the specific recognition of an E-box, located in the c-*fos* main regulatory sequence, the serum response element (SRE [42]). This E-box is in close proximity to the CArG box, a binding site for the serum response factor (SRF). However, recent gene knock-out experiments in mice (reviewed in [28,43]) suggested that, although MyoD represses proliferation in vitro, it is not a proper target for external (and unknown) anti-proliferative signals in vivo, and that myogenin is a more appropriate candidate for this function.

Indeed, whereas inactivation of MyoD activity, which requires inactivation of both MyoD and Myf5 genes [44,45], results in a total absence of muscle cells [46], myogenin gene disruption results in apparently normal myoblastic precursor cells [47] which, however, do not mature into muscle [47,48]. Interestingly, myoblasts obtained from mice in which the myogenin gene has been disrupted do form myotubes in vitro when deprived of growth factors [48]. Taken together, these data suggest that the main step in which myogenin is involved in vivo is the terminal step of the process (terminal differentiation is also, in vitro, the step at which myogenin is induced and required [49,50]). They also indicate that a function for which myogenin is not replaced by other members of the family in

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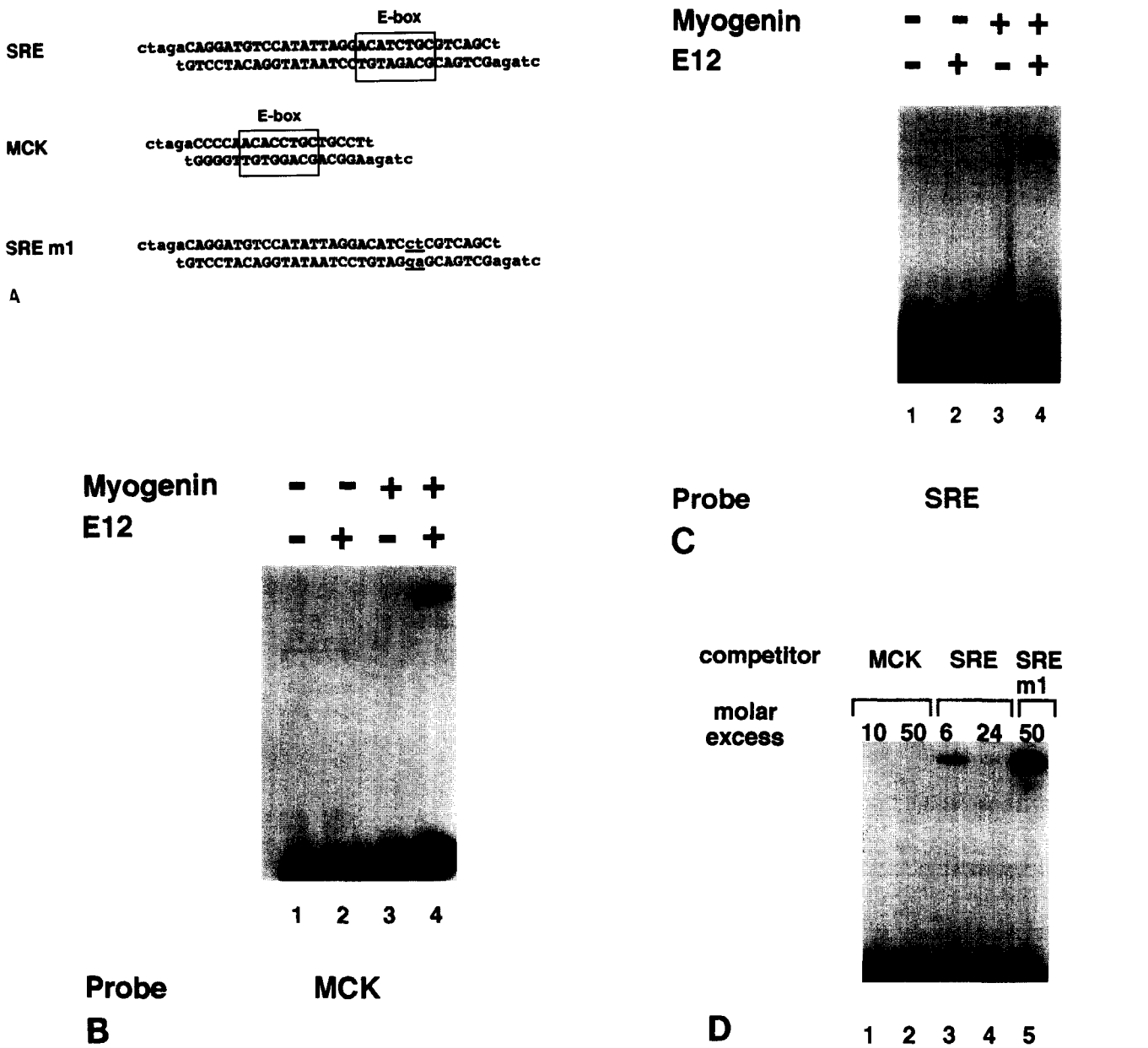


Fig. 1. Myogenin binds to the c-*fos* E-box with a high affinity. The sequences of the various oligonucleotides used as probes or competitors are shown in A; the E-box is indicated and the nucleotides mutated in SRE m1 are underlined. Myogenin and E12 proteins were in vitro translated separately or together, as indicated, and analysed by EMSA using a probe including the MCK canonical E-box (B and D) or the c-*fos* E-box (C). B and C were run simultaneously on the same gel and the myogenin–E12 complexes migrated to the same position. In D, the product of a co-translation experiment was analysed using an MCK probe and various molar excesses of an MCK (lanes 1 and 2), an SRE (lane 3 and 4) or an SRE mutated in the E-box (lane 5).

vivo seems to be the block of proliferation which precedes the induction of muscle structural proteins and the fusion into myotubes. Our recent data suggested that proliferation is blocked, at least in part, through the repression of proliferation-associated genes such as c-*fos* [38]. Here, we have tested the hypothesis that myogenin could be as good a repressor of the c-*fos* promoter as MyoD. We demonstrate that myogenin, provided that it is in a heterodimeric form, recognises the c-*fos* E-box with a high affinity. Furthermore, forced expression of myogenin in NIH 3T3 cells results in c-*fos* SRE inhibition. This function requires the myogenin bHLH region. These results suggest that myogenin could be the actual effector of c-*fos* repression at the onset of muscle cell terminal differentiation.

2. Materials and methods

2.1. *In vitro translation and electrophoretic mobility shift assay (EMSA)*

pEMSV E12 [38] and pEMSV myogenin [51] (a kind gift of Dr. E. Olson) plasmids were in vitro transcribed using T3 RNA polymerase and translated using a Promega translation kit, according to the recommendations of the manufacturer. Double-stranded oligonucleotides including an SRE element (top strand: 5′ CTAGACAGGATGTCCAT-

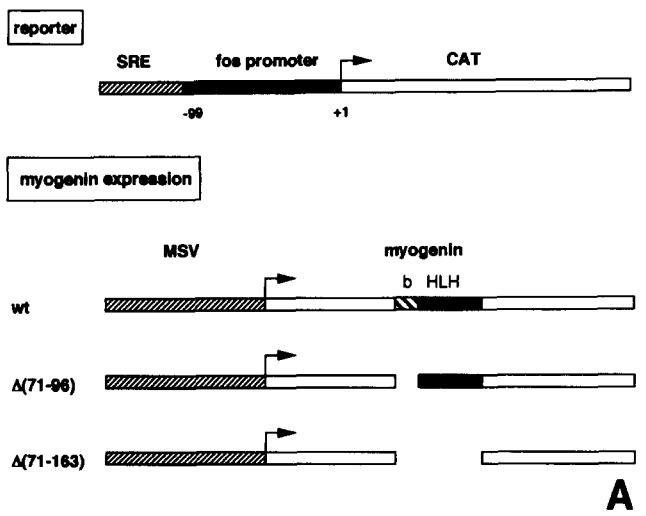
ATTAGGACATCTGCGTCAGCT) or an MCK downstream canonical sequence (top strand: CTAGACCCCAACACCTGCTGCCTT) were purified and radiolabeled with γ-^{32}P as previously described [52]. For EMSA analysis, 2 μl of lysate were diluted in 5 μl of buffer D [53] and incubated for 10 min at 4°C with 1 μg of poly(dG:dC) and then for 15 min at room temperature with 2 ng of radiolabeled oligonucleotide in the presence, when indicated, of an excess of unlabeled oligonucleotide as a competitor. Samples were analysed on a 4% non-denaturing gel, dried and autoradiographed as described [38]. Results were quantified by densitometry analysis of adequate exposures of autoradiograms.

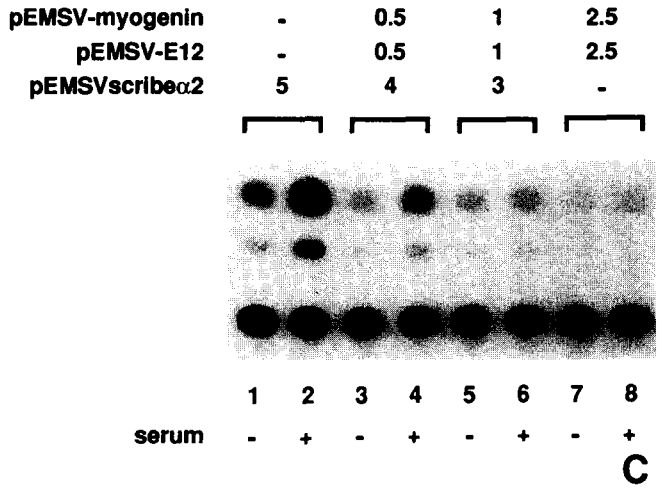
2.2. Plasmids

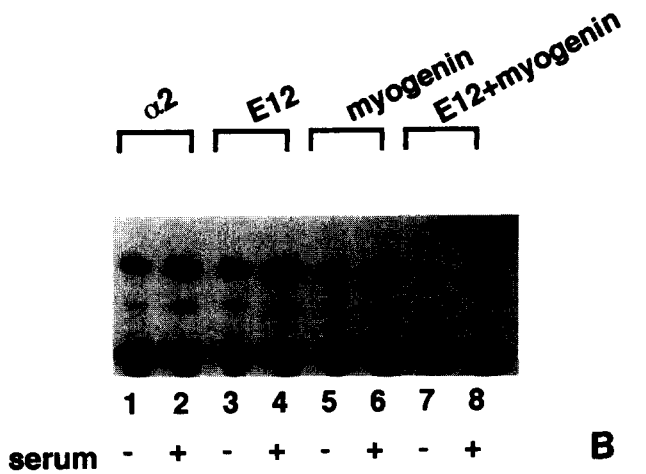
pEMSV E12 and pSRE CAT were as described in [38]. pEMSV-myogenin was a kind gift of Dr. E. Olson. pEMSV Δ(71–96)-myogenin and pEMSV Δ(71–163)-myogenin were constructed using a PCR (polymerase chain reaction) amplified insert. The internal primers were TGCAAGGTGCACAGCGCCTCCTGCAG and GGCGCTGTGCACCTTGCATGCCCACG for pEMSV Δ(71–163)-myogenin and TGCAAGGTGGTGAATGAGGCCTTCGAGG and CTCATTCACCACCTTGCATGCCCACG for pEMSV Δ(71–96)-myogenin. The forward external primer included a consensus translation start site [54], and both forward and reverse external primers included an *Eco*RI restriction site for cloning convenience. The sequences of these primers were: GGAATTCACCATGGAGCTGTATGAGACATCCC (forward) and GGGGGGAATTCAGTTGGGCATGGTTTCG (reverse). These constructs were controlled by partial sequencing (which did not show any mutations), and results obtained with these constructs were confirmed using two independent clones.

2.3. Cells and transfections

NIH 3T3 cells were maintained in DMEM supplemented with antibiotics (a mixture of penicillin and streptomycin from Gibco, used according to the manufacturer's recommendations) and 5% FCS (fetal calf serum). Cells were transfected by electroporation as described previously [38]. Briefly, cells were harvested by scraping, washed and resuspended in 150 μl of DMEM (Dulbecco's minimal essential medium) supplemented with 0.5% FCS. 2 μg of SRE-CAT, indicated doses of pEMSV-E12, pEMSV-myogenin or mutants, and 1 μg of RSV-luc (a construct including the luciferase reporter gene under the control of the Rous sarcoma virus long terminal repeat), as an internal control for transfection efficiency, were added. After electrical shock (using a Bio-Rad apparatus at 960 μF and 200 V), each sample was divided into two aliquots and cells were maintained in DMEM 0.5% FCS for 48 h, after which one of the aliquots was treated with 20% serum for 4 h. Cells were harvested and extracts were standardized based on the luciferase activity of the non-serum-treated sample (samples from the same transfection were standardized based on the protein content, as measured by a Bio-Rad assay). CAT activity was measured using [^{14}C]chloramphenicol and standard procedures, with a 4 h assay.







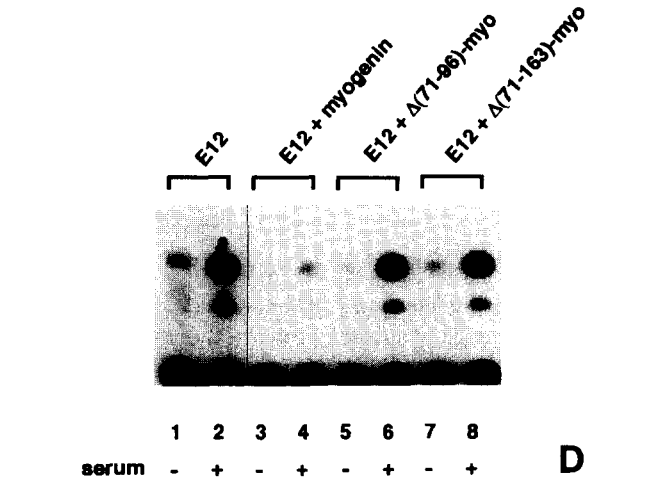


Fig. 2. Myogenin represses the c-*fos* promoter. NIH 3T3 cells were co-transfected with an SRE-CAT reporter construct together with an EMSV-driven expression vector for myogenin, E12 or a mixture of both, as indicated (the constructs used are described in A; the vehicle pEMSV scribe α2 was used in the controls). Cells were cultured in low serum for 48 h and then treated (lanes +) or not (lanes −) with 20% serum for 4 h. (B) Myogenin and E12 act synergistically (dose of each expression vector used was 1 μg); (C) inhibition by myogenin/E12 is dose dependent; (D) inhibition requires integrity of the bHLH domain of myogenin (dose of each expression vector used was 1 μg).

3. Results and discussion

3.1. Myogenin/E12 heterodimers bind to an E-box in c-fos SRE

In order to test whether myogenin can recognise an E-box in the c-*fos* promoter, we have analysed in vitro translated proteins by an electrophoretic mobility shift assay (EMSA; Fig. 1). Myogenin and E12 proteins were in vitro translated separately or together and analysed for E-box recognition using an E-box from muscle creatine kinase (MCK) or the c-*fos* promoter (Fig. 1A). As expected [26], myogenin did not bind to the MCK E-box with a high affinity, and binding was detectable only when E12 and myogenin were co-translated (Fig. 1B). Similar results were obtained with the c-*fos* E-box (Fig. 1C). The lysates programmed with both E12 and myogenin formed a complex with c-*fos* SRE oligonucleotide, whereas no complex was detectable when each protein was translated separately. Competition experiments (Fig. 1D) indicated that the affinity of myogenin/E12 heterodimers for MCK and c-*fos* E-boxes were of the same order of magnitude. Indeed, when used as unlabeled competitor, similar molar excesses of both probes were needed to observe a significant inhibition. Quantification of competition results suggested that myogenin/E12 heterodimers display an affinity for the c-*fos* E-box which is about 2–5 fold smaller than their affinity for the MCK canonical E-box. Taken together, these results indicate that myogenin acquires a high affinity for the c-*fos* E-box upon hetero-dimerization with E12, as was demonstrated for MyoD [38].

3.2. Myogenin/E12 hyper-expression results in c-fos promoter repression

In order to assess whether myogenin can exert an inhibitory effect on the c-*fos* promoter, as we have demonstrated for MyoD [38], myogenin was assayed for c-*fos* SRE modulation in a transient co-transfection assay (Fig. 2). NIH 3T3 fibroblasts, which do not express any of the myogenic bHLH proteins, were used as recipients for these experiments. Cells were transfected with a reporter construct including the c-*fos* SRE in front of a c-*fos* minimal promoter (Fig. 2A) [38], together with pEMSV-E12 (an expression vector for E12), pEMSV-myogenin (an expression vector for myogenin), or both; pEMSV-scribe α2, the vehicle, was used as the control. Results (Fig. 2) were in good correlation with data obtained by EMSA analysis. Co-transfection of each expression vector independently did not have any effect on the reporter CAT (chloramphenicol acetyl-transferase) gene expression, whereas co-transfection of both plasmids virtually abolished the reporter response to serum (Fig. 2B). Note that a significant inhibitory effect could be observed with pEMSV-myogenin alone when 5 times more expression vector was used (data not shown). The inhibition was dose dependent (Fig. 2C). Similar to what has been demonstrated for MyoD [38], the inhibition required the integrity of the bHLH domain: two mutants (described in Fig. 2A) in which the basic domain (Δ71–96) or the whole of the basic helix-loop-helix domain (Δ71–163) had been deleted (and which had lost the ability to dimerise and to bind to DNA; data not shown), did not inhibit c-*fos* SRE activity (Fig. 2D). Repression by myogenic proteins thus involves the bHLH domain. The precise mechanism of this repression is currently under investigation and our data suggest that this mechanism might be complex. The CArG-box (binding site for SRF) and the E-box (binding site for myogenic bHLH proteins) overlap, and binding of these factors is mutually exclusive [38]. However, our recent data suggest that a physical interaction between SRF and myogenic bHLH could also be involved (Trouche et al., submitted).

Taken together, these data demonstrate that myogenin binds to and represses the c-*fos* promoter, a function which, in vitro, myogenin shares with MyoD. In vivo, however, myogenin seems to be a better candidate as an effector molecule of c-*fos* inhibition during muscle cell terminal differentiation.

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