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Antisense inhibition of myoD expression in regenerating rat soleus muscle is followed by an increase in the mRNA levels of myoD, myf-5 and myogenin and by a retarded regeneration

Ernő Zádor [a,*], Sándor Bottka [b], Frank Wuytack [c]

[a] *Institute of Biochemistry, Albert Szent-Györgyi Medical Center, University of Szeged, H-6701 Szeged, Hungary*
[b] *Institute of Plant Biology, Biological Research Center of the Hungarian Academy of Sciences, H-6701 Szeged, Hungary*
[c] *Laboratory of Physiology, Katholieke Universiteit Leuven, Campus Gasthuisberg, Leuven, B-3000, Belgium*

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Abstract

It has been reported that muscles of myoD−/− mice present a lower potential to regenerate, but there are no reports on the effect of acute interference with myoD expression limited in space and time to only a particular regenerating muscle. Here we relied on antisense inhibition of this factor. Four different oligos were tested. The suppression of regeneration indices (the expression of desmin, the formation of myotubes and the initiation of endplates) was the most pronounced, with the oligomer targeting a region encompassing the translation start site of myoD. A mixed backbone phosphorothioate–phosphate diester oligo (200 μl at 20 μM) was still detectable in the muscles 1 h after its administration and reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the level of the targeted 5′ end of the myoD mRNA was selectively decreased. The level of myoD protein was also lowered. Four hours after the antisense treatment, when the oligos were no longer detectable, the myoD mRNA level was restored and 24 h later it exceeded controls together with that of myf-5 and myogenin. After 4 weeks, the antisense-treated soleus muscles were similar to the control-treated and the untreated regenerated soleus with respect to fiber types and motor endplates, however, they contained smaller fibers which reflected the asynchronity of regeneration. This shows that successfully targeted simple antisense oligonucleotides can be used as selective tools for inhibition of individual factors in studying the process of muscle regeneration.

Keywords: Antisense oligonucleotide; myoD; Muscle regeneration

1. Introduction

It has been suggested that muscle regeneration is controlled by myoD, a nuclear protein, member of the group of myogenic regulatory factors (MRF: myoD, myf-5, myogenin, MRF4, reviewed by Ref. [1]). The myoD knock-out mice upregulate myf-5 [2], but otherwise are grossly normal, however their muscles contain increased levels of morphologically normal satellite cells and in spite of this, they show a lower potential to regenerate compared to the wild type [3]. The decreased regeneration potential is documented by a number of independent experiments on myoD null mice both in vitro and in vivo. First, primary cultures of myoD−/− myogenic cells showed a clear differentiation deficit compared to the wild type. Thus, these myogenic cells were considered as representing an intermediate stage between quiescent satellite cells and myogenic precursor cells [4,5]. In line with these observations, also satellite cells obtained from single muscle fiber cultures of myoD(−/−) mice were found to be more reluctant to differentiate, to show molecular and cellular characteristics different from those of the wild type and to present an altered expression pattern of the other MRF genes [6]. Classical in vivo regeneration models, like regeneration following crushing of muscle [3] or grafting muscle into another location [7] also indicated a delayed regeneration in myoD(−/−) mice. All these data strongly corroborate to the view that myoD is important for proper muscle regeneration. However, it should be stressed that muscles of myoD(−/−) mice do not express this regulatory factor and have never expressed it during their development. Therefore, their muscles differ from the wild type. This may

* Corresponding author. Laboratorium voor Fysiologie, Katholieke Universiteit Leuven, Campus Gasthuisberg, Leuven, B-3000, Belgium. Fax: +32-16-345-991.
E-mail address: erno@bioch.szote.u-szeged.hu (E. Zádor).

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critically affect the background determining the conditions of muscle regeneration [3–7]. Hence, experiments in which inhibition of myoD expression is limited in place and time to a particular muscle entering the initial phase of regeneration could provide further information on the role of this factor. According to our knowledge, such an experiment has not been performed yet.

Antisense nucleic acids are potential useful tools to provoke such a spatio-temporal inhibition of gene expression [8], and they can be easily introduced both into myoblasts and myotubes [9,10]. Here, we injected antisense oligonucleotides in soleus muscles which were regenerating from notexin-induced necrosis [11]. This regeneration model has been extensively characterised both in our laboratories [12–15] and by others [16,17] and found to be remarkably reproducible. We injected antisense oligomers targeted to different parts of the myoD mRNA 3 days after the induction of necrosis—i.e. at the time when the mRNA level for this transcription factor was at its maximum in regenerating muscle. The results show that injection of oligos targeted to a sequence encompassing the translational start site temporarily blocked muscle regeneration. The transcript and protein levels of myoD were initially suppressed but shortly thereafter rebounded and increased above control. The augmentation of myoD mRNA level was accompanied by similar increases in the levels of the myf-5 and myogenin transcripts confirming that in vivo the expression of these factors is closely regulated. Thereby, oligo injection disturbed the synchronization of the early steps of regeneration, delaying the expression of desmin, the formation of myotubes and the reinnervation of the fibers. After 4 weeks of regeneration, similar proportions of the fibers expressed the different MyHC isoforms in antisense-treated muscles as in the controls or in the untreated regenerated muscles, but the antisense-treated muscles showed increased numbers of small fibers, a sign of asynchronous regeneration [18].

2. Materials and methods

2.1. Selection and synthesis of oligodeoxynucleotides

Oligonucleotides containing naturally occurring phosphodiester bonds are subject to quick nucleolytic decomposition in cells. In order to stabilize our molecules, phosphorothioate internucleotide linkages were incorporated in a sequence-specific manner according to the principle of minimal modification [19]. Two thioate substituents were built in, on both the 5′ and the 3′ terminus, and one was positioned in the vicinity of each pyrimidine nucleotide, on either the 5′ or the 3′ side. The selection of target sites for antisense oligonucleotides was based on functional and structural considerations. We chose first the transcriptional start site as one of the potentially most vulnerable positions as shown by statistical evaluations [20]. Further candidate target sites were selected based on a prediction of the most probable secondary structures of the myoD mRNA computed by free energy minimization. For this purpose, the computer software *mfold* (version 3.0) of Zuker and Turner was applied [21]. Single-stranded regions were selected as potential target sites. The chosen fragments were further screened on the basis of the general criteria of primer planning and the free energy of duplex formation.

Oligodeoxynucleotides were synthesized on an Expedite 8909 synthesizer (Perkin-Elmer Biosystems, USA) on a scale of 1 μmol by the solid-supported β-cyanoethyl phosphoramidite method. Phosphorothioate linkages were built in at given positions by substituting the iodine oxidation with 0.4 M dibenzoyl tetrasulfide treatment for 2 min in the standard protocol. [22]. Production, purification, purity control and the measurement of the degree of thioation were performed as described elsewhere [23]. Purity of the products was >97%, the phosphorothioate/phosphodiester ratio corresponded to the expected theoretical ratio of phosphorothioate and phosphodiester groups within the experimental error. The sequences of the oligos are shown in Table 1.

2.2. Animal treatment, administration of notexin and the antisense oligomers

Muscle injury was induced by the in situ injection of notexin, a snake venom [11]. The optimal notexin dose for inducing muscle necrosis had been established previously and the ensuing regeneration process in the soleus muscle was found to be remarkably reproducible as assessed by histological inspection and by a number of biochemical criteria [12–15]. Notexin was administered as described previously [12]. Shortly, male 300–360 g Wistar rats were narcotised by intraperitoneal injection of 1 ml 0.5% Na-pentobarbital per 100 g body weight. An incision was made through the skin on the lateral side of the left hind leg, the m. gastrocnemius was gently pulled aside and the soleus was slightly lifted up from its bed. Twenty micrograms of notexin in 200 μl of 0.9% NaCl was injected into the soleus. Finally, the wound in the skin was suture closed. On the third day after the injection of the toxin, the wound was opened again, and various concentrations of antisense DNA oligomers or adequate controls dissolved in 200 μl of 0.9% NaCl were injected into the regenerating muscle. At 1, 4, 24, 48 h or 25 days after oligo injection, the soleus muscles were removed

Table 1
Sequence of oligomers used in the experiments

Name	Sequence
Anti translational start[a]	5′ gtagctccatg*tccc*agttc3′
Anti cap site	5′ tgtcctggcccctagctta3′
Anti 245–264	5′ gccgtcggggcctagcaa3′
Random control	5′ tgaaggcgacgagaaggt3′
Scrambled antistart control[b]	5′ taccgtcgttcagatgctcc3′

[a] Used in both phosphorothioate and phosphate diester forms.
[b] Used only in phosphorothioate oligomer form.

Table 2
Primers and PCR conditions

Name	Sequence	Fragment length (bp)
MyoD5′ +	5′ ggttgagcgagaagcagga3′ (nt 258–276[a])	724
MyoD5′ −	5′ tgtagtaggcggcgtcgtag3′ (nt 3710–3730[a])	
Id1 +	5′ catgaaggtcgccagtagcag3′ (nt 67–87[b])	475
Id1 −	5′ gtccatctggttcttcctcagtgc3′ (nt 521–544[b])	
Id3 +	5′ aaggcgctgagcccggtg3′ (nt 18–35[c])	387
Id3 −	5′ ctgcgttcgggaggtgcc3′ (nt 387–404[c])	

The temperature profile used for the PCR was 95, 60, 72 each for 1 min, for myoD5′ 28 cycles and for the Id1 and Id3 25 cycles were applied. The other primers and PCR conditions for myoD, myf-5, myogenin, MRF4 and α-actin have been described in Ref. [13] and for GAPDH and SERCA1b in Ref. [12].

[a] Positions in the sequence of the rat myoD gene [26].

[b] Positions in the sequence of the rat Id1 cDNA [27].

[c] Positions in the sequence of the rat Id3 cDNA [28].

and the animals were sacrificed by an overdose of sodium pentobarbital. Each time point combined data from at least three animals, unless it is otherwise indicated. The earliest time point (1 h after antisense injection) was chosen considering the high turnover rate of myoD mRNA in myogenic cells [24]. The dissected muscles were tied onto a wooden stick in an extended position, frozen in isopentane cooled by liquid nitrogen and kept at − 70 °C until use.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was isolated according to the method of Ref. [25]. The total RNA content of muscles was not different from those of the non-antisense-treated ones. The RT reaction and the quantification by RT-PCR were done as described earlier [12]. The primers and PCR conditions are shown in Table 2. The number of PCR cycles was carefully adjusted to the linear dynamic range of the test. The identity of the amplified fragments of myoD, myf-5, myogenin, MRF4, Id1, Id3, actin, SERCA1b and GAPDH was confirmed by subcloning in pGEMT easy (Promega, Leiden, Netherlands) and sequencing. To radiolabel the PCR fragments for quantification, 5 μl (i.e. one-tenth of the volume) of the primary PCR mixture was transferred to a new tube and made up to 50 μl with the same amplification mix, except that [α-^{32}P] dCTP was added. Two additional PCR cycles were executed with

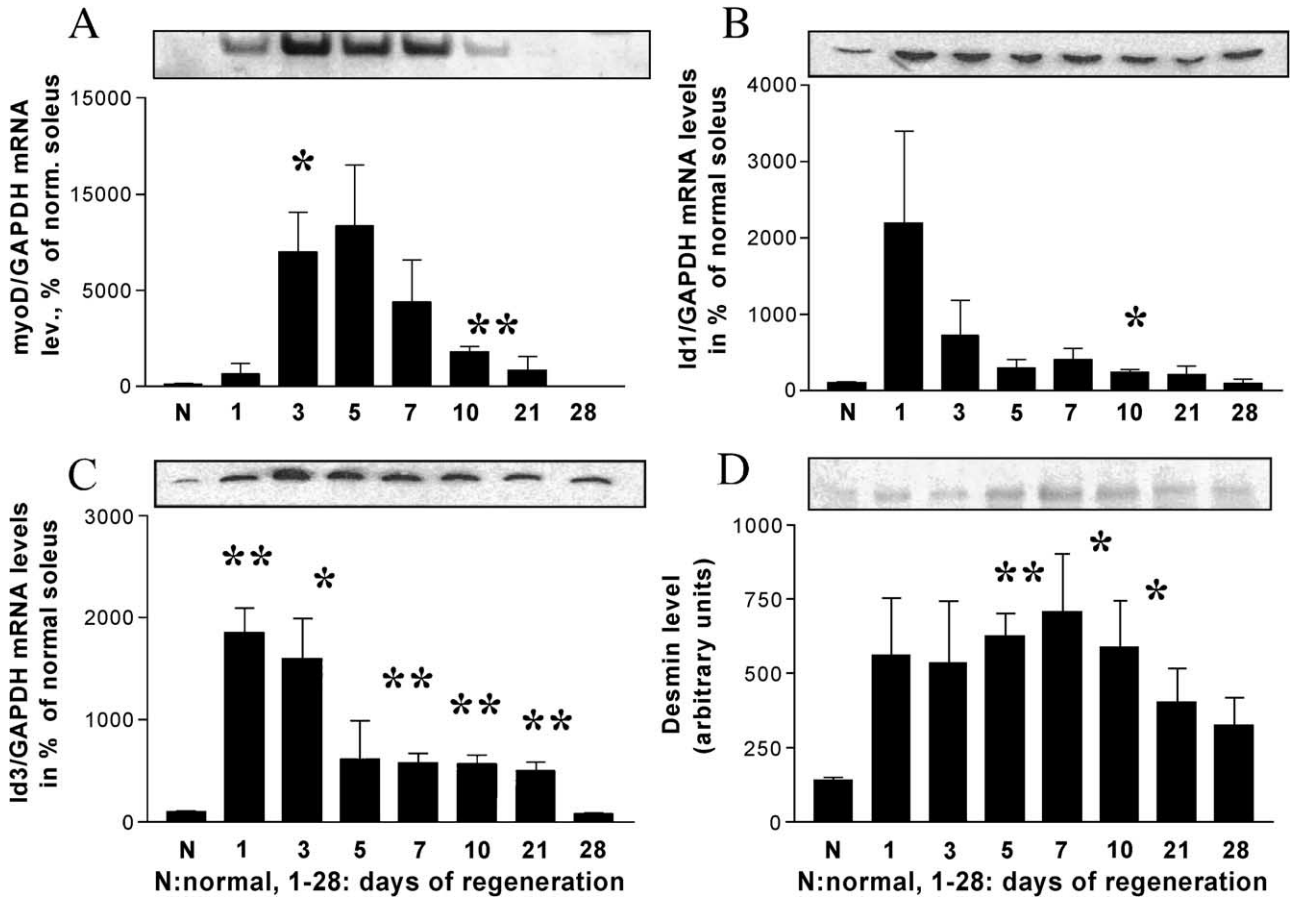

Fig. 1. The levels of markers of the progress of muscle regeneration. The levels of (A) myoD mRNA, (B) Id1 mRNA, (C) Id3 mRNA and (D) desmin in regenerating soleus muscles. The indicated values for mRNA levels are normalized to the GAPDH mRNA level of the same sample. For the protein immunoblots, extracts representing equal parts of the muscles (20–30 μg proteins) were loaded onto each lane. The inserts show an example of the three repetitions of the RT-PCR or the immunoblots. The vertical bars on columns represent ± S.E., $N=3$, $*P<0.05$, $**P<0.01$ compared to the normal values.

the same cycle parameters used in the primary PCR. The amplification products were analyzed by electrophoresis on 6% polyacrylamide gels. The gels were airdried and ^{32}P bands quantified by means of a Storm model 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

2.4. Immunoblotting procedures

myoD Proteins were measured by immunoblotting as in Ref. [29]. Muscles were homogenized in 31 volumes of lysis buffer (HEPES 10 mM pH7.5, KCl 5 mM, $MgCl_2$ 10 mM, mercaptoethanol 5 mM, sucrose 0.32 M) supplemented with a protease-inhibitor cocktail (Sigma) at 4 °C, vacuum-filtered through four layers of fine gauze and subsequently centrifuged for 8 min at $1000 \times g$ resulting in a supernatant fraction and a rough nuclear pellet. The pellet was dissolved in a buffer containing HEPES 20 mM pH 7.9, NaCl 0.42 M, EDTA 1 mM, DTT 2 mM glycerol 25% and subsequently used for gel electrophoresis and Western blotting. For each muscle, 5% of the total nuclear fraction or 0.25% of the total extract was loaded onto a 12% Laemmli-type acrylamide gel. Electrophoresis and semi-dry blotting onto PVDF membranes were carried out under standard conditions. For immunodetection, we used a 1:250 dilution of the myoD1 antibody of Novocastra Laboratories (Newcastle, UK), a 1:500 dilution of the myoD rabbit antiserum of Santa Cruz Biotech. (Santa Cruz, CA, USA) and a 1:300 dilution of the monoclonal desmin antibody of Dako (Glostrup, Denmark). After incubation with an HRP-coupled secondary antibody Dako (anti mouse, 1:1000) a Ni-enhanced DAB/peroxidase reaction was carried out [30] followed by densitometric quantification. In some experiments, the immunoblots were evaluated with the Vistra ECF system (Amersham Life Science, Little Chalfont, England, UK) in which case alkaline phosphatase-coupled secondary antibodies (1:10,000) were used and the bands were quantified on the Storm FluorImager (Molecular Dynamics, Sunnyvale, CA, USA).

2.5. 5-Bromo-2′-deoxyuridine (BrdU)-labelling and detection

One milligram of BrdU in 200 μl 0.9% NaCl was injected into the soleus in the same manner as the snake venom at various days after the notexin treatment. Sixteen hours later, the soleus was removed, fixed overnight at 4 °C in 4% paraformaldehyde in PBS and kept at −20 °C until freeze sectioning. Before making cross-sections, the soleus was cut into six equally sized blocks from the proximal to the distal tendon. Three sections from each block were randomly chosen to be processed for immunostaining. To denature the nuclear DNA, the sections were treated with 2 N HCl for 30 min and washed for 3×10 min in 50 mM Tris–HCl pH 7.6, 100 mM NaCl. The endogenous peroxydase was quenched with 3% H_2O_2 in PBS for 5 min, followed by 2×10 min washes in PBS. Sections were blocked for 20 min in 1% BSA and 5% normal goat serum. The primary antibody (mouse monoclonal anti-BrdU, Sigma, incubated for 1 h at 1:100) and the secondary antibody (biotinylated goat anti-mouse, Vector Laboratories, incubated for 30 min at 1:300) were diluted with the blocking serum. For intermittent washings, the blocking solvent was used after the primary antibody and PBS after the secondary. The immunocomplexes were visualised by the Vectastain ABC Elite kit (Vector Laboratories). The labelled nuclei were counted under the light microscope. For background correction, BrdU was also injected into control soleus muscles of untreated animals and the average number of labelled nuclei in three controls subtracted from the number of BrdU-labelled nuclei of notexin-regenerated muscles.

2.6. Histochemistry and immunostainings

A 2-mm-thick block was taken from the central part of each frozen soleus muscle and cryosections were stained

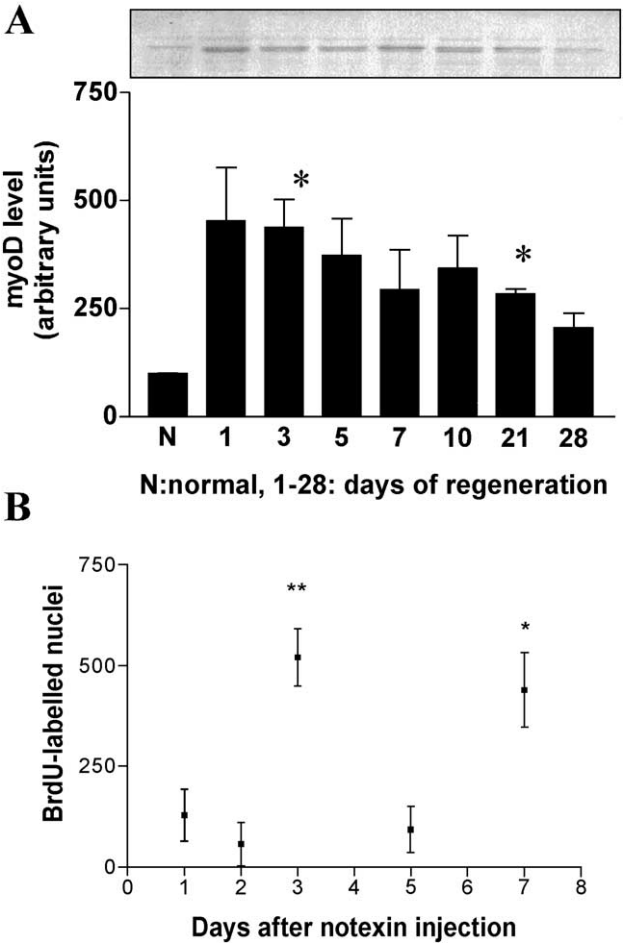

Fig. 2. The myoD level (A) and the incorporation of BrdU into the nuclei (B) during regeneration of soleus muscles. (A) The myoD protein in the first part of regeneration largely followed its transcript level (see Fig. 1A). (B) The incorporation of BrdU reflects two mitotic waves in the first week of regeneration. Note that the first mitotic wave on day 3 coincides with the significant increase of myoD. These events mark the activation of myogenic cells. $N = 3$. Symbols as in Fig. 1.

with hematoxylin–eosin to monitor the extent of the necrosis and the subsequent regeneration. Acetylcholinesterase staining for motor endplates was done as in Ref. [13].

For immunostaining, cryosections were pretreated with 0.3% H_2O_2 and blocked in 10% rabbit serum, 1% BSA for 30 min. Primary antibodies for desmin (1:50 antibody, Dako), for MyHC1 (BA-D5) or MyHC2a (SC-71) (1:10) were used as in Refs. [14,29]. Immunoreactions were visualized with an HRP-coupled secondary antibody (1:150, Dako) followed by a DAB/peroxidase reaction (0.05 mg/ml DAB, 0.006% H_2O_2).

2.7. Measurement of the stability of oligodeoxynucleotides in the muscle tissue

Phenol-chloroform washed total RNA extracts also containing the oligonucleotides were prepared from the oligomer-injected regenerating muscles and one-tenth of the volume of these was subjected to 5′-end labelling with [γ-^{32}P] ATP using T4 polynucleotide kinase as described by Ref. [31]. The reaction mix was denatured at 65 °C for 5 min and loaded onto a 20% urea acrylamide gel. The dried gel was subjected to phosphorimage analysis. The oligos were identified by electrophoretic migration.

2.8. Fiber area and statistics

The fiber area was obtained by measuring light micrographs of transversal muscle sections with a digitizer interfaced to a computer. For each muscle, at least 100 fibers were measured.

To assess the significance of differences for the BrdU incorporation, the Newman–Keuls test and for the RT-PCR, the immunoblot and the fiber area, Student's unpaired *t*-test were used. $P < 0.05$ was considered as significant, $P < 0.01$ as very significant and $P < 0.001$ as extremely significant level of difference.

3. Results

3.1. The changes in mRNA levels of myoD, ID1 and ID3 and the level of desmin during normal muscle regeneration

The level of myoD mRNA, which is normally very low in adult muscle, peaked on day 3 after administration of notexin and declined gradually through the 4 weeks of regeneration (Fig. 1A). We have also measured the transcript levels of the helix–loop–helix transcriptional inhibitors of DNA binding

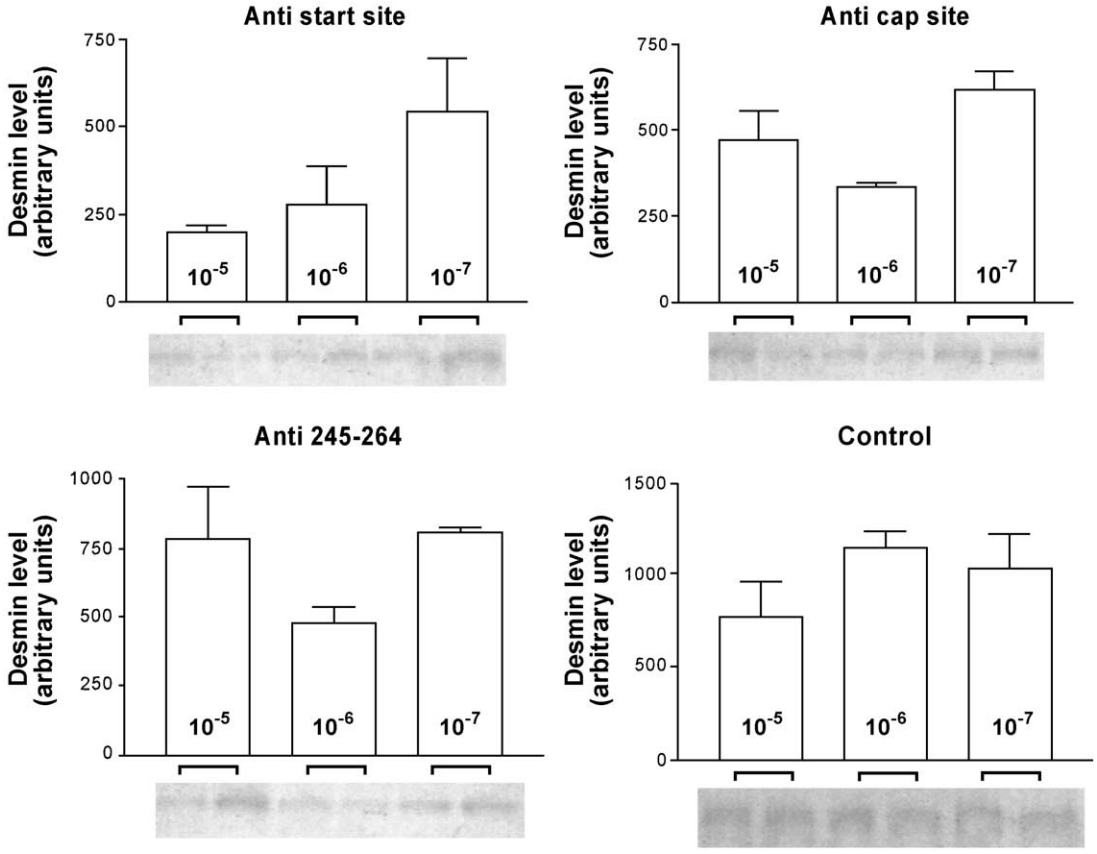

Fig. 3. The effect of phosphodiester oligonucleotides on the expression of desmin. Oligonucleotides were injected after the third day of regeneration and the desmin level was measured after the fifth day. The different doses were tested in duplicate. The loadings were representing equal parts of the muscles also not differing by protein content (20 μg protein per lane). Symbols as in Fig. 1.

Id1 and Id3 which are known to inhibit myogenic differentiation [32,33]. Both Id1 and Id3 increased dramatically on the first day and they also declined to the normal level by day 28 of regeneration (Fig. 1B,C). The level of desmin, a differentiation marker for myoblasts, was low in the normal soleus and it gradually increased to a maximum on days 5–10, then decreased back to the normal level (Fig. 1D). We reported earlier that desmin is expressed uniformally over the whole transverse section from day 4 onward [13].

3.2. The myoD protein level and BrdU-labelling

The myoD protein level was low in the normal soleus, but it began to rise already on the first day after notexin injection and, similar to its transcript, became significantly higher than the resting value on day 3 (Fig. 2A). Later in regeneration, this factor remained well above the initial resting value up till day 21 in spite of the fact that at this time the myoD transcript was already clearly declining.

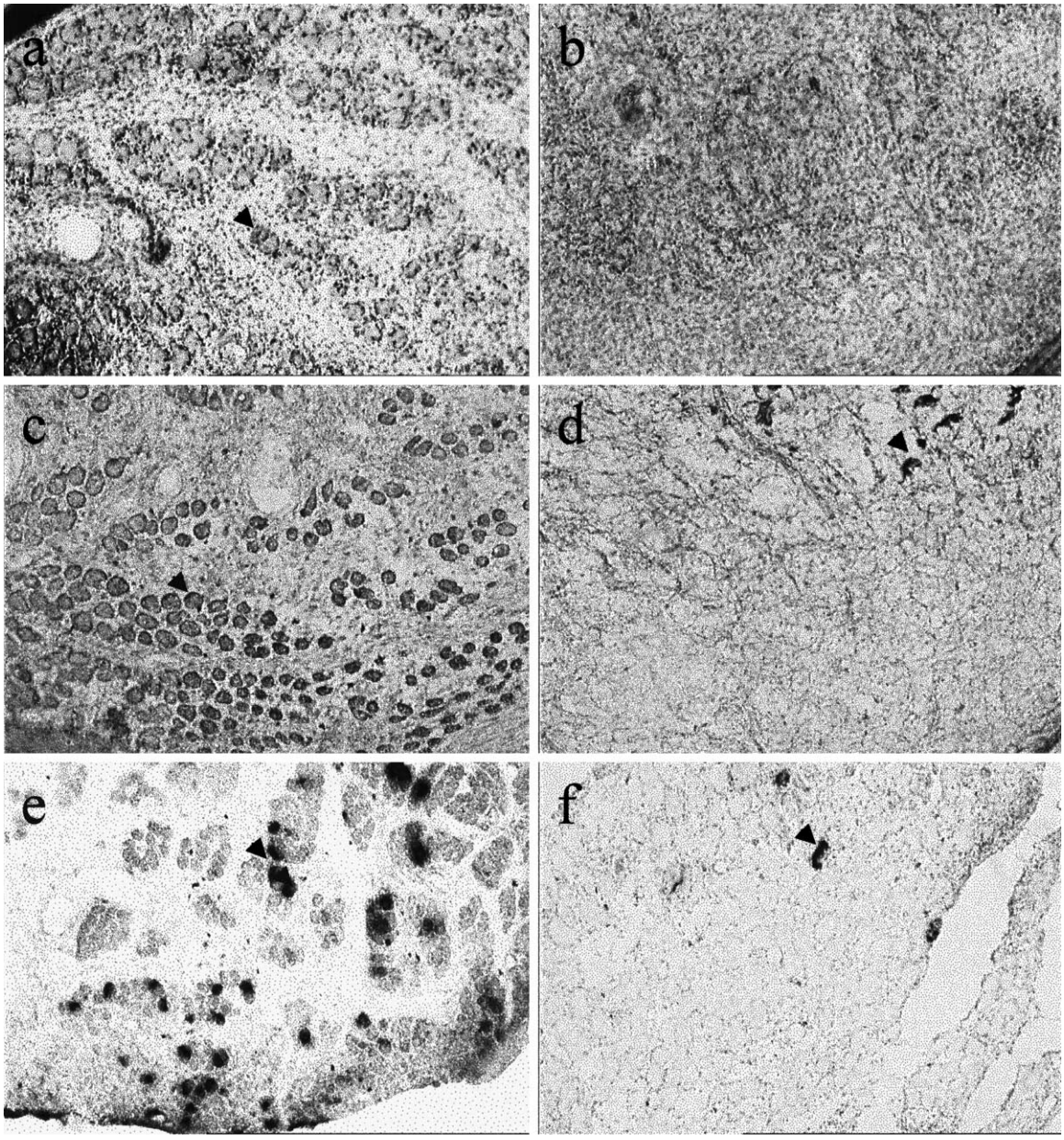

Fig. 4. The effect of anti start on muscle morphology during regeneration. The effect 2 days after injecting 2×10^{-5} M antistart phosphodiester oligo on (b) the myotube formation, (d) the desmin expression and (f) the endplate formation into a soleus 3 days after notexin injection. Control slides of the myotubes, desmin and endplates in muscles treated by the random oligomer are shown on (a), (c) and (e), respectively. The arrows point to (a) the myotubes, (c) the desmin-positive myotubes and (d) myoblasts and the acetylcholinesterase stained endplates (e and f).

In order to monitor cell proliferation, which sets in immediately upon activation of satellite cells, we administered BrdU, a thymidine analog, to the soleus ($n=3$ for each time point, results indicated $\pm$ S.E.). The BrdU is incorporated into the DNA during the S phase of the cell cycle, and it therefore labels nuclei involved in DNA replication. More nuclei were labelled during the first 2 days following muscle injury compared to the uninjured controls (423 ± 89 nuclei labelled in untreated controls; 132 ± 66 extra nuclei on day 1 and 57 ± 55 on day 2 after notexin injection, Fig. 2B). However, these differences were not statistically significant. But after the third day, the increase became highly significant (532 ± 71 above the controls, $P<0.01$). This suggested that a first wave of cell proliferation took place 3 days after notexin administration. After 5 days, the incorporation was again not significantly different from that of the control muscles. After 7 days, there appeared to be a second wave of mitosis (453 ± 91 positive nuclei above the controls, $P<0.05$).

3.3. myoD Antisense oligos inhibit the early steps of muscle regeneration

Since the transcript levels of myoD, myf-5 and myogenin show a maximum around the third day of regeneration in soleus muscle (see above and in Ref. [13]), we chose this time for the intramuscular administration of three different myoD antisense oligos (phosphodiester oligos or phosphorothioate oligos). In order to assess the impact of this treatment, we monitored the maturation of the newly formed myotubes by determining the expression of desmin, the increase in the cytoplasm/nuclear volume ratio of the fibers, and the number of endplates after day 5, i.e. 2 days after the injection of the oligomers. In the absence of antisense treatment, the cytoplasm/nuclear volume ratio of the newly formed myotubes begins normally to rise on days 3–4 [13,16], and the formation of new endplates on the fibers becomes apparent on day 5 [13,34]. Of three different anti-sense oligos tested, only the oligomer targeting the translational start site (anti-start) exerted a clear dose-dependent effect on the desmin expression. In contrast, the oligomers targeting the mRNA cap site (anti-cap), a sequence stretching from nt 245–264 (A-245) of myoD, or a control oligomer (corresponding to a random sequence of four nucleotides), when applied in concentrations between 20 nM and 20 μM, caused a variable or less striking effect (Fig. 3). The formation of new endplates on the myotubes was inhibited similarly as the expression of desmin. Only the effect of the highest concentration of anti-start is shown in Fig. 4. Based on the above dose–response relationship, 20 μM of this oligomer was chosen to investigate the effect on muscle regeneration.

3.4. The stability of antisense oligos in the regenerating muscle

Since injected oligonucleotides are subject to rapid degradation, we tested the stability of the antisense oligodeoxynucleotides in the muscle tissue (Fig. 5). The phosphorothioate version of anti-start and its corresponding scrambled oligo were well detectable 1 h after injection, and even after 4 and 24h trace amounts were left. Oligomers containing the naturally occurring phosphodiester linkage are more prone to biodegradation and disappeared from the muscle after 24h (see last lanes on Fig 5). We have therefore used phosphorothioate oligos in our further experiments.

3.5. myoD Antisense phosphorothio oligomer initially suppressed the mRNA level of myoD but thereupon caused a coordinated augmentation of the mRNA levels of myoD, myf-5 and myogenin in regenerating muscle

We monitored the effect of the phosphorothioate–phosphate version of anti-start on the transcript levels of myogenic regulatory factor mRNAs and used thereby glyceraldehyde-3-phosphate dehydrogenase transcripts as an internal control. The level of myoD mRNA, measured by RT-PCR with primers encompassing the translation start site, was found to be decreased already 1 h after injecting anti-start into the muscles, meanwhile the mRNA levels of myf-5 and myogenin were unchanged (Fig. 6). Interestingly, when we also tried to measure the level of the myoD transcripts by amplifying a fragment stretching from nt 726–946, which corresponds to the central part of myoD's cDNA, no such decrease was observed after 1 h.

Four hours after anti-start oligo injection, the transcript levels of myoD, myf-5 and myogenin were not different from the control, but after 24h the levels of the three myogenic regulatory factors were increased by 60–100% above controls. The transcript levels of MRF4, skeletal actin and the neonatal sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pump did not differ significantly from the controls

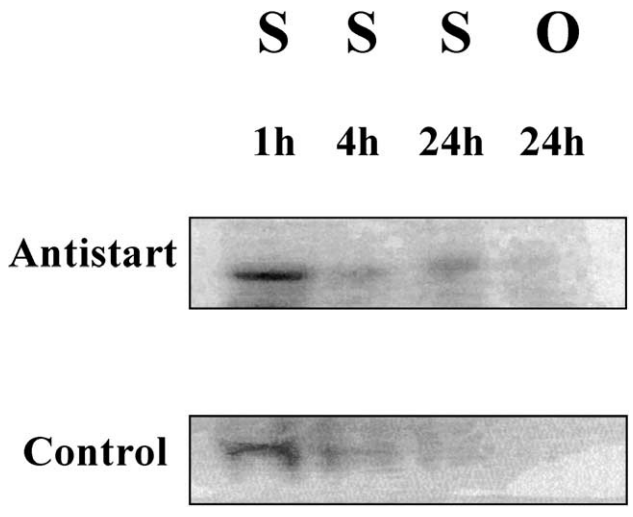

Fig. 5. The stability of 2×10^{-5} M antistart and its scrambled control oligomer in the regenerating muscle at various times after the injection. The last lanes show phosphodiester antistart oligo with the random control. S—phosphorothioate, O—phosphodiester.

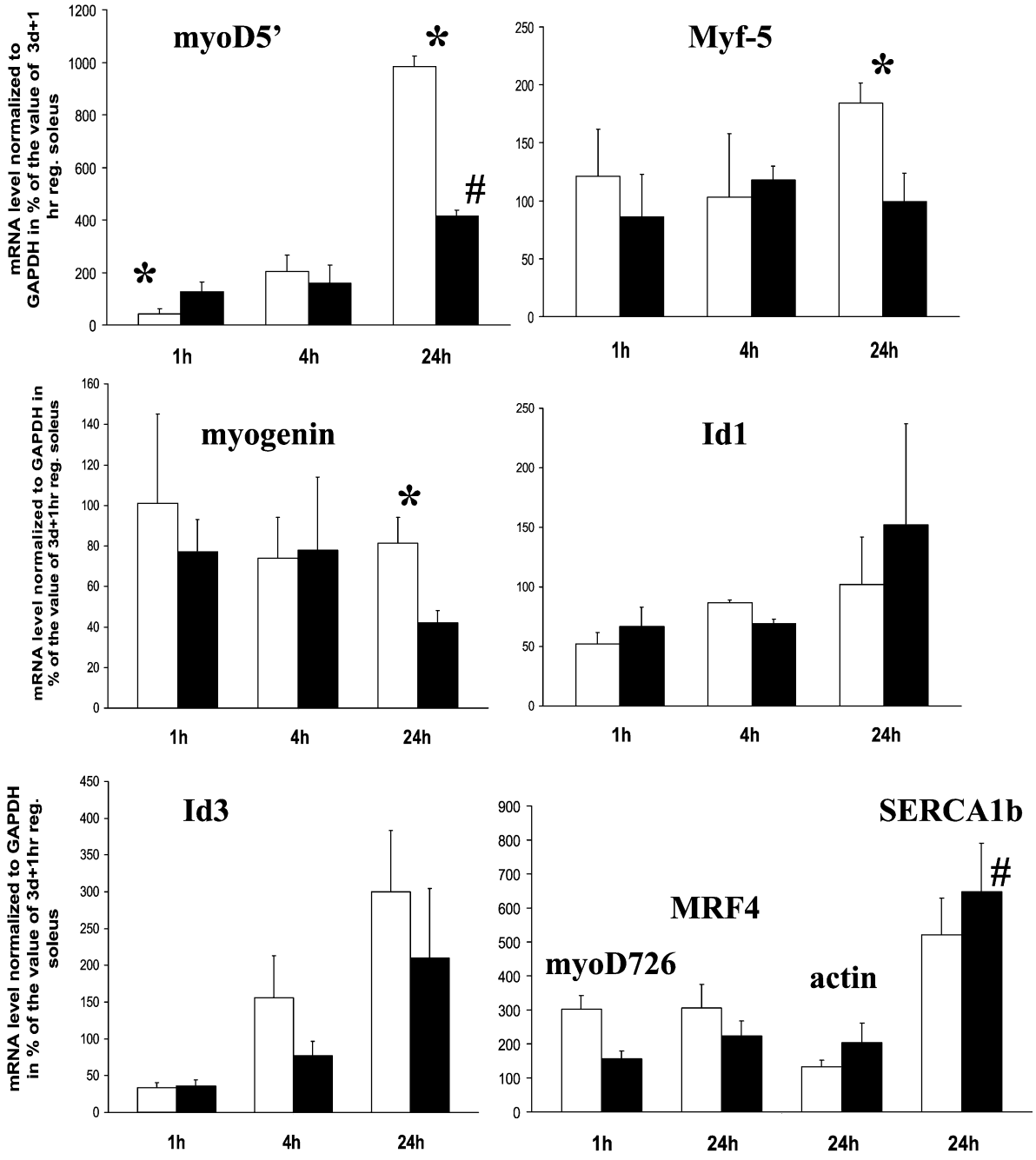

Fig. 6. mRNA levels of regenerating soleus muscles at different times after the injection of 2×10^{-5} M antistart phosphorothioate oligonucleotides on the third day of regeneration. The 100% is set to the mean mRNA values of 3 days + 1 h regenerated non-antisense treated soleus muscle. White columns show values from antisense treated and black columns from control oligo treated muscles. Note that the PCR analysis of fragment 726–946 of the myoD cDNA was done only on samples obtained at 1 h after oligo injection. The levels of MRF4, SERCA1b and α-actin were measured only at 24 h after the oligomer injection. $N = 3$. Symbols as on Fig. 1. * indicates a significant difference to the control oligo treated muscle and # indicates a significant difference in the control oligo treated muscles compared to the non-antisense treated ones.

(24h after oligo treatment), and neither did the levels of the inhibitors of DNA binding Id1 and Id3 (1, 4 and 24h after oligo treatment, Fig. 6). It should however be remarked, as shown above, that the levels of the myogenic regulatory factors and of the Ids themselves (set at 100% in these experiments) were upregulated during muscle regeneration compared to the uninjured muscle (Fig. 1B,C). It is interesting to note that after 24h in the control-oligo-treated regenerating muscles myoD and SERCA1b were also upregulated. The former probably resulted from the repeated injection of the regenerating muscle and was found to be lower than in the antisense-treated muscles. The latter,

reflecting the sudden uprise of this neonatal calcium pump on days 3–5 of regeneration, did however not differ from what was seen in antisense-treated counterparts. We also must note here that the effect of the phosphorothioate–phosphate oligos (antistart and control) was not distinguishable from that of the phosphate ester oligos on the morphology and markers of regeneration as shown on Fig 4.

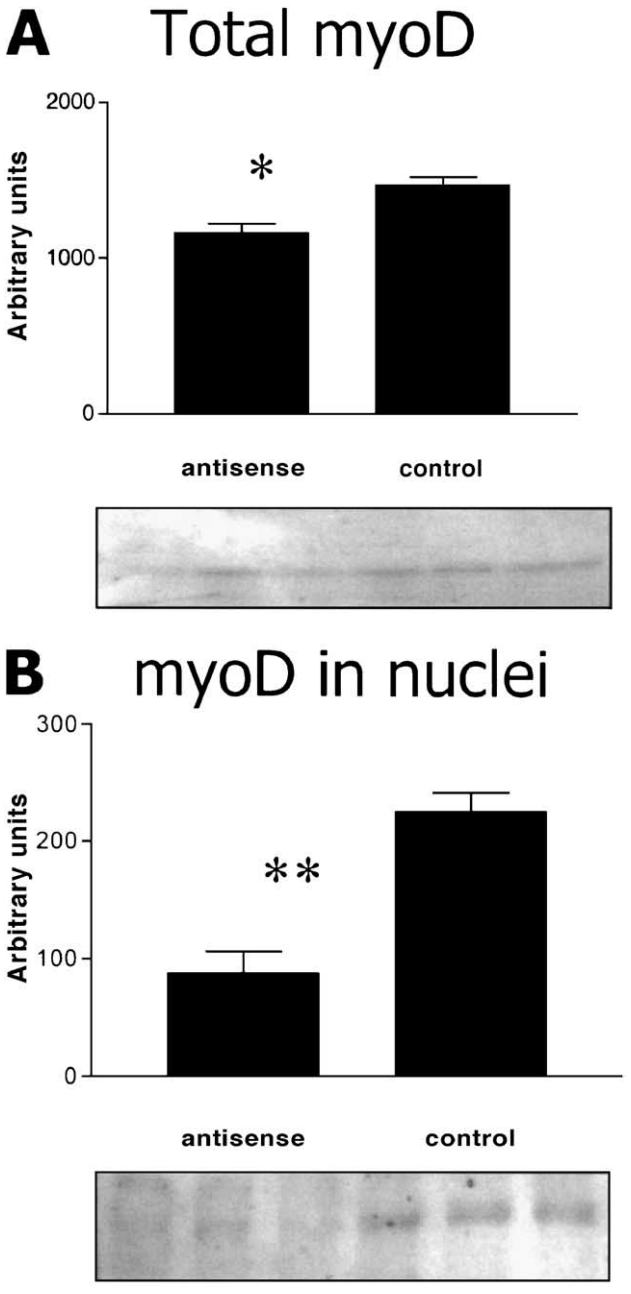

Fig. 7. Inhibition of myoD level by the antistart phosphorothioate oligomer. (A) Total extract and (B) the nuclear extract of the antistart-treated muscle. The samples were collected on the third day of regeneration and 1 h after the injection of oligos. The loadings were normalized to muscle weight and protein content. The insert shows the immunoblots. $N=3$. Symbols as in Fig 1.

3.6. Injection of the antistart oligo decreases the myoD protein level in the regenerating soleus

Next we assessed the effect of injection of 200 μl of 2×10^{-5} M antistart phosphorothioate oligo on the protein level of myoD in the total muscle and the nuclear extract. One hour after administration of the oligo, the levels of myoD protein were decreased ($P<0.05$) in total muscle (Fig. 7A) and even more lowered ($P<0.01$) in the nuclear fraction (Fig. 7B) compared to the control.

3.7. Antisense inhibition of myoD does not affect the expression pattern of MyHC isoforms, but increases the number of small fibers in regenerating soleus

After 4 weeks of regeneration, 98% of the fibers in the regenerated soleus muscles treated with antistart and control phosphorothioate oligo expressed the myosin heavy chain of the type I (slow-oxidative) fibers (MyHC1), and 12% of the fibers expressed the myosin heavy chain of the type IIA (fast-oxidative) fibers (MyHC2a) as it is shown on Fig 8. This situation is not different from what is seen in non-treated regenerating muscles [16,14]. However, in the anti-start-treated regenerated soleus muscles $41 \pm 2.1\%$ of the fibers showed areas under 2000 μm^2, compared to only $16.5 \pm 2.1\%$ in the controls ($N=3$, $P<0.05$). The average fiber areas, 3080 and 3350 μm^2, subsequently, were not significantly different from each other. Therefore, the anti myoD treatment resulted in more small immature fibers suggesting a delayed and incomplete regeneration as is also observed in dystrophic muscles [18].

4. Discussion

A new finding of our work is that inhibition of myoD does not immediately upregulate myf-5 or the other myogenic regulatory factors although it delays regeneration. Myf-5 is known to be increased in myogenic cells cultured from myoD −/− mice [4,6] or in myoblast cell lines expressing myoD antisense from a vector [35]. To our knowledge, our experiments demonstrated for the first time that in the regenerating muscle the temporal decrease in myoD is not directly coupled to the increase of myf-5 mRNA, a marker of quiescent adult skeletal muscle satellite cells [36]. This suggests that the upregulation of myf-5 is more dependent on extra cellular signals and developmental decisions than on the selective downregulation of myoD. A single injection of a mixed backbone phosphorothioate–phosphate diester antisense oligo directed against the translational start codon of myoD decreased this factor's transcript and protein levels already after 1 h. The inhibition depended on the stability of the oligo that we have documented in this study and on the turnover of myoD. Such a fast decrease of the myoD level under inhibition is not unexpected in view of the high turnover rate of myoD mRNA and protein in myogenic cells

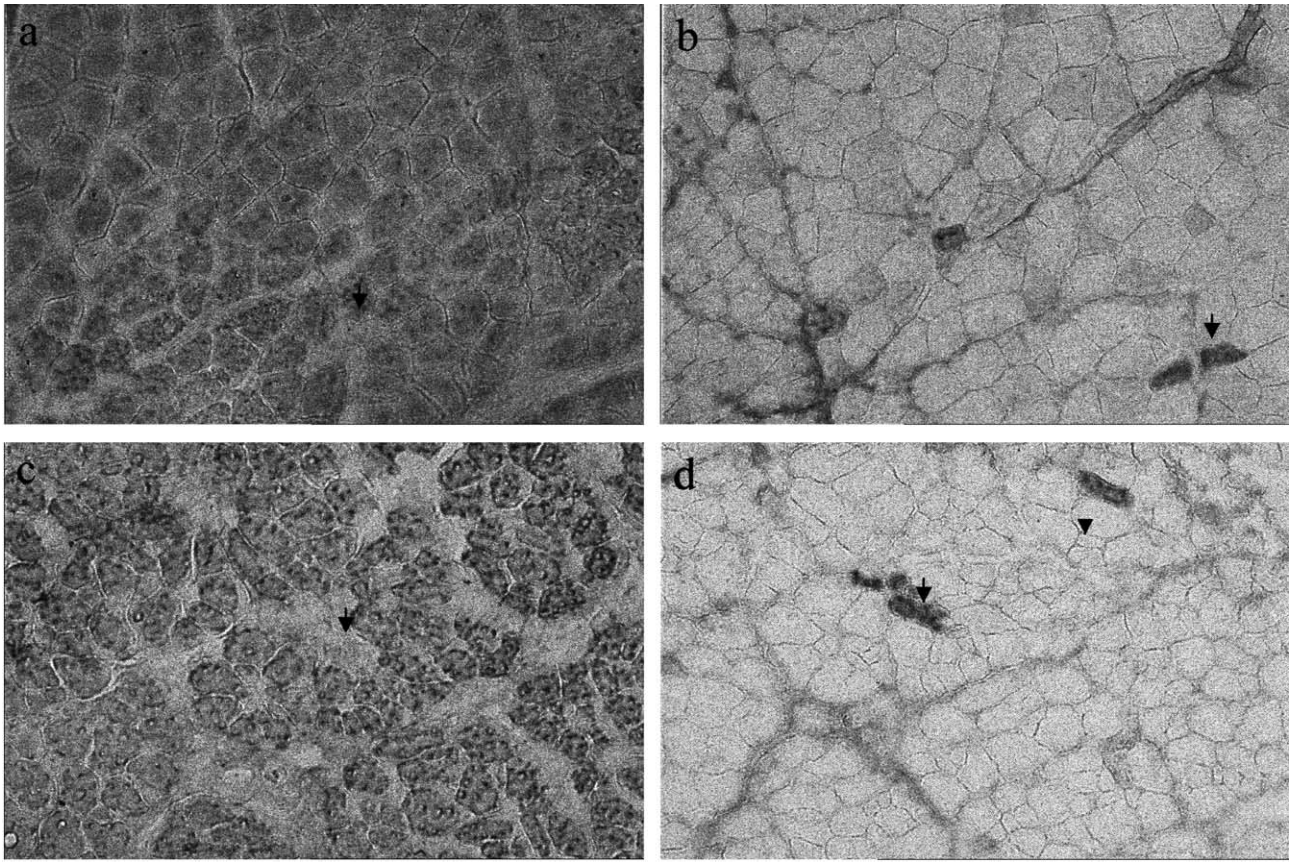

Fig. 8. The expression of myosin heavy chain proteins in the antistart thiooligo injected regenerating muscles after 28 days of regeneration. MyHC1 staining of (a) scrambled control and (c) antistart treated muscle, MyHC2a staining of (b) scrambled control and (d) antistart-treated regenerated muscle. The arrows point to the corresponding fibers on the parallel sections. Note arrow head on (d) indicating a small fiber.

[24,37]. The mechanism of this antisense inhibition probably includes heteroduplex formation between the antisense oligo and the targeted mRNA sequence. In the cytoplasm such an RNA/DNA heterodimer cannot be translated, moreover within the nucleus, it might act as a substrate of RNase H and be removed by degradation [8].

Separate PCR amplifications of different fragments of the myoD cDNA corresponding either to the targeted zone comprising the translational start site or to a non-targeted central part of the myoD cDNA showed that the antisense treatment preferentially destroyed the region complementary to the antisense probe. This is consistent with the suggestion that the effect of the antistart oligo might not only involve blocking of the translation but may also result from RNase degradation of the targeted sequence. The fact that the antistart oligo contained a TCCC sequence might have improved its efficiency. Indeed, 48% of the most effective antisense oligonucleotides contain such a sequence [20]. This motif, which was suggested to activate RNase was found to be the most effective when present in introns, thereby suggesting that the antisense action took place inside the nucleus at the level of the primary gene transcript. However, in our experiments this TCCC motif was clearly exonic.

The antisense oligo was almost totally destroyed in the regenerating muscle 4 h after its injection. The fact that the myoD mRNA level was back to normal at this time appeared to confirm this observation. To our surprise, however, we found that 24h after administration of the oligo, the mRNA levels of myoD were higher in the antisense-treated muscles than in the controls. This apparently compensatory augmentation of myoD transcript was paralleled by a simultaneous increase of myf-5 and myogenin mRNA levels. It is known that the mRNA levels of the myogenic regulatory factors in soleus muscle are moderately increased by denervation [38], which is not necessarily followed by a corresponding increase in their protein levels [39] and that the mRNAs are again decreased when reinnervation takes place in the regenerating muscles [13, 34]. Therefore it cannot be ruled out that the retarded onset of regeneration and reinnervation in our experiments is also a consequence of the compensatory mechanisms marked by the secondary augmentation of myoD, myf-5 and myogenin mRNAs rather than of the decrease of myoD itself.

The coordinated uprise of myoD, myf-5 and myogenin transcript levels can be explained by the mutual positive feedback MRFs exert on their gene transcription [40] rather than by an increased number of muscle precursor cells, since

the mRNA levels of MRF4, Id1, Id3, skeletal actin and SERCA1b were not elevated compared to the control. Such a close coregulation of myoD, myf-5 and myogenin mRNAs is also observed in vitro in many individual satellite cells in culture [41] and in regenerating muscle [13]. Further, it has been reported that increases in the stability of myoD mRNA also result in a parallel elevation of myoD and myogenin in C2 myoblasts [24].

The incorporation of BrdU showed that the first wave of mitosis took place on the third day of regeneration. The slightly earlier uprise of myoD levels therefore coincided with the G1 phase [1], immediately preceding the S-phase during which BrdU is incorporated. It is unlikely that before the third day of regeneration, many satellite cells have been activated into myogenic precursor cells and started to proliferate. The myoD protein level was low in the normal soleus, it began to rise already 1 day after injection and became significantly higher on day 3 (Fig. 2A). MRF production at that early time is most likely largely accounted for by myogenic precursor cells immigrating into the damaged muscle [42,43].

The observation that the levels of desmin did not decrease during the first 3 days of regeneration was at variance with the report of Ref. [44] which described a reduced level of this intermediate filament protein after notexin injection. This discrepancy must be explained by the different way in which the muscle necrosis was induced in both groups. Whereas Vater et al. [44] injected the venom subcutaneously, we injected it intramuscularly. As shown in several earlier reports of our group [12–14], necrosis elicited by intramuscular injection of notexin is complete. The increase in the levels of desmin before the first mitotic wave on the third day of regeneration may result either from activated satellite cells or from muscle precursor cells that migrated from the surrounding muscles into the injured soleus [42].

The precise timing of changes in the myogenic markers such as the myogenic factors [13], the incorporation of BrdU, the expression of desmin, the formation of myotubes and reinnervation enabled us to monitor the progress of muscle regeneration. The antisense treatment showed that the expression of myoD orchestrates muscle reconstruction. Even a relatively short-lasting inhibition of myoD expression was sufficient to disarrange the regenerative events with long-lasting consequences, as shown by the elevated numbers of small fibers [18] remaining 4 weeks after antisense injection. This disturbed regeneration was probably not only caused by delaying the activation of satellite cells and postponing the exit from the cell cycle [1], but also preventing myoblast fusion [5,7], a feature showed by myoD −/− myoblasts. It is know that myoD controls the expression of M-cadherin, which is required for cell cycle withdrawal and myoblast fusion (reviewed in Ref. [43]). The temporal decrease in the level of myoD elicited by myoD antisense injection in regenerating muscle did not result in a significant paralell upregulation of the level of myf-5 as reported to be the case in primary myogenic cell culture [4], in satellite cells of single fiber cultures [6] and in the developing muscles of myoD knockout mice [2]. Also at variance with our results is the observation that when myogenic cells were transfected with a vector expressing myoD antisense mRNA, the myf-5 mRNA was upregulated [35]. Therefore our work suggests that short lasting inhibition of myoD expression via antisense oligo injection exerts an effect different from that obtained by myoD gene ablation or by myoD antisense RNA transcribed from a transfected vector.

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