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The Utrophin Gene Is Transcriptionally Up-regulated in Regenerating Muscle*

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The utrophin gene codes for a large cytoskeletal protein closely related to dystrophin, the gene mutated in Duchenne's muscular dystrophy. Although utrophin could functionally substitute for dystrophin, in Duchenne's muscular dystrophy patients it did not compensate for the absence of dystrophin because in adult muscle utrophin was poorly expressed and limited to subsynaptic nuclei. However, increased levels of utrophin have been observed in regenerated muscles fibers suggesting that utrophin up-regulation in muscle is feasible. We observed that utrophin mRNA was transiently up-regulated at early time points after muscle injury with a peak already 24 h after muscle damage and utrophin induction in activated satellite cells before fusion into young regenerated fibers. Injection of utrophin *lacZ* constructs into regenerating muscle demonstrated that the utrophin upstream promoter under the control of its intronic enhancer activated the transcription that leads to the expression of the reporter gene in the newly formed fibers, which was not limited to neuromuscular junctions. Utrophin enhancer activity was dependent on an AP-1 site, and in satellite cells of regenerating muscle the AP-1 factors Fra1, Fra2, and JunD were strongly induced. These results establish that utrophin was induced in adult muscle independently from neuromuscular junctions and suggest that growth factors and cytokines that mediate the muscle repair up-regulate utrophin transcription.

Duchenne's muscular dystrophy (DMD)[1] is caused by mutations or deletions of the dystrophin gene that lead to muscle wasting and affect about 1/3500 newborn males. The dystrophin gene codes for a large cytoskeletal protein that accumulates at the sarcolemma of muscle fibers and forms a complex that links the muscle cytoskeleton with the sarcolemma (1–6). The utrophin gene (also named dystrophin-related gene) is an autosomal homologue of dystrophin (7, 8). Utrophin is a 395-kDa protein with a high degree of amino acid identity with dystrophin (9, 10). Transgenic models demonstrated that constitutive expression of utrophin in muscle can functionally replace dystrophin and alleviate the muscle pathology, suggesting that up-regulation of the endogenous utrophin gene in patients represents a strategy to explore for the cure of DMD (11–13).

The large degree of protein identity as well as the ability to bind many of the same cytoskeletal proteins suggest that differences between dystrophin and utrophin are mostly due to their different regulation. Whereas the dystrophin gene is mostly expressed in cardiac and skeletal muscle, and its expression is induced by muscle differentiation, utrophin gene is expressed ubiquitously (8, 14–16). In adult skeletal muscle utrophin expression is low and limited to neuromuscular junctions (NMJ) (17–19). Utrophin is transcribed by two independently regulated promoters that give rise to two transcripts (A- and B-utrophin) that code for utrophins with different N termini (20, 21). Both promoters are active in several tissues. The upstream utrophin promoter is more active in kidney, whereas the downstream promoter, which is localized about 50 kb downstream, is more active in heart (17, 21). The upstream promoter is CpG-rich, TATA-less, and contains a consensus N box that enhances utrophin transcription at sub-synaptic nuclei. Similarly to the nicotinic acetylcholine receptor δ and ϵ subunit genes, it responds to heregulin (22–25).

This promoter is recognized by Sp1 and Sp3 factors that activate transcription synergistically with GABP (GA-binding protein) (26). The upstream promoter is also under the control of a *d*ownstream *u*trophin *e*nhancer (DUE) located at about 9 kb downstream within the second intron (27). DUE enhances transcription driven by the upstream promoter in muscle cells *in vitro*. Nothing is known of the nuclear factors binding to this enhancer and of its role in muscle *in vivo*.

Utrophin has been detected at extra junctional sarcolemma of regenerated muscles fibers (28–31). Although regenerating muscles showed higher levels of utrophin, no increase of utrophin mRNA was observed with respect to undamaged fibers leading to the suggestion that post-transcriptionally regulatory mechanism take place (31). However, the mechanism of this up-regulation is poorly understood. Due to the importance of understanding gene expression during muscle regeneration and the relevance of utrophin modulation for the cure of DMD pathology, we further investigated this regulation. Early time point of muscles repair is characterized by the activation of satellite cells: upon muscle damage quiescent satellite cells start proliferating, migrate to the injury site, and fuse together to repair the damaged fibers (see Ref. 32 and references therein). Different growth factors and cytokines released from muscle cells and from invading inflammatory cells are thought to mediate this regenerative process.

We investigated utrophin regulation in damaged muscle from early time points after muscle damage. Following the

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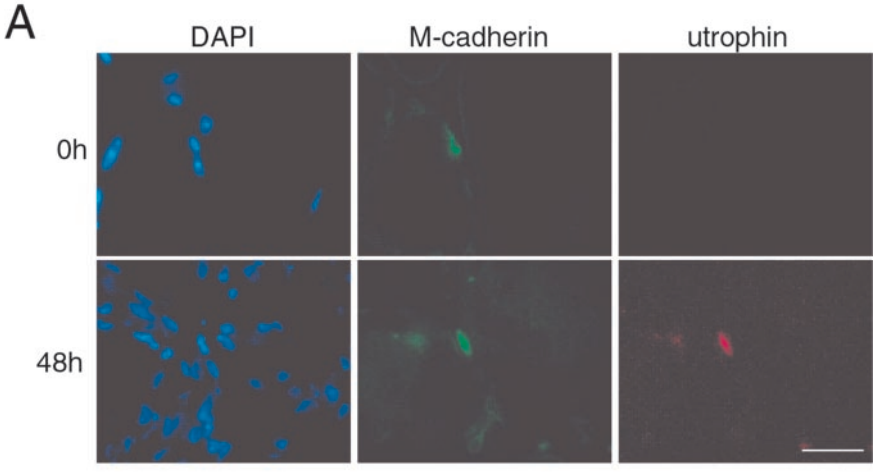
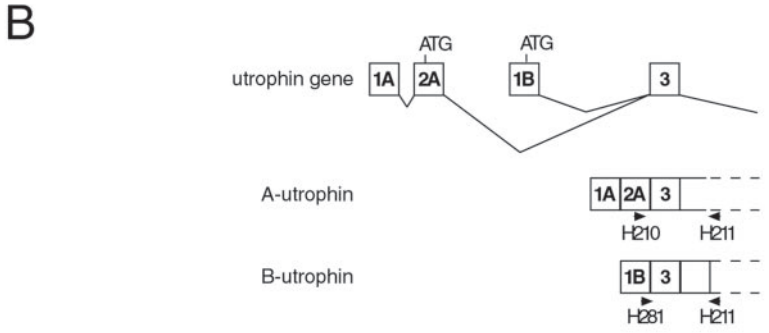
[1] The abbreviations used are: DMD, Duchenne's muscular dystrophy; NMJ, neuromuscular junctions; DUE, downstream utrophin enhancer; RT, reverse transcriptase; DAPI, 4,6-diamidino-2-phenylindole; TRITC, tetramethylrhodamine B isothiocyanate; FITC, fluorescein isothiocyanate; Upr, utrophin promoter; CAT, chloramphenicol acetyltransferase; BAC, bacterial artificial chromosome.

TABLE I
Oligonucleotide primers used in the RT-PCR reactions

Gene	5′ primer	3′ primer
A-utrophin	ATGGCCAAGTATGGGGACCTTG	GTGGTGAAGTTGAGGACGTTGAC
B-utrophin	GCAGCCACCACATTTCGTTGG	GTGGTGAAGTTGAGGACGTTGAC
Dystrophin	GGTGGGAAGAAGTAGAGGACTG	GACATTGTTCAGGGCATGAACTC
c-fos	GATGTTCTCGGGTTTCAACGCC	GATTCCGGCACTTGGCTGCAG
fosB	CAAGCTTTTCCCGGAGACTACGAC	GCCGTCTTCCTTAGCGATGTTG
fra-1	ATGTACCGAGACTACGGGGAAC	CTGCAGCTCTTCAATCTCTCGCTG
fra-2	ATGTACCAGGATTATCCCGGGAAC	GCAGCTCAGCAATCTCTTTCTGCAC
c-jun	TCTACGACGATGCCCTCAACG	GATCTGTTGGGGCAAGTGGTGC
junB	GATGTGCACGAAAATGGAACAGCC	GCGTCACGTGGTTCATCTTGTGCAC
junD	ATGGAAACGCCCTTGTATGGCG	AGCTGGCTTTGCTTGTGCAGGTC
GAPDH	GGTCACCAGGGCTGCCATTTG	TTCCAGAGGGGCCATCCACAG





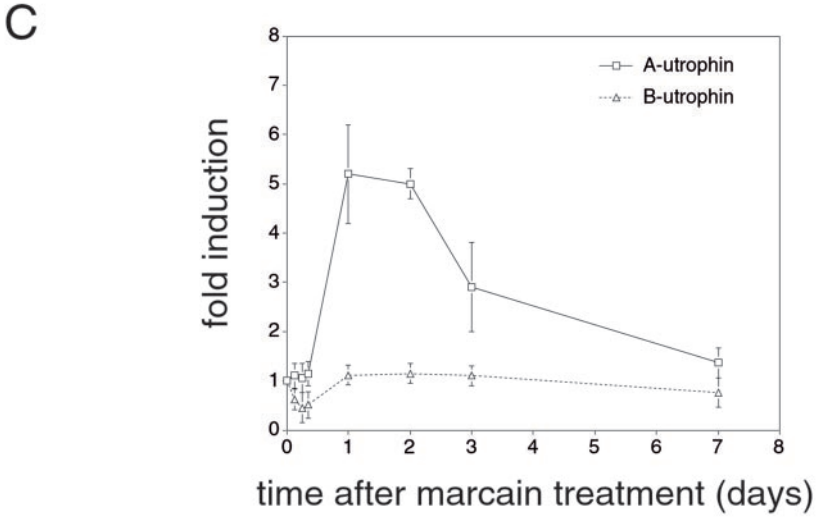


FIG. 1. **After injury utrophin is expressed in satellite cells, and its mRNA transcribed from the upstream promoter is up-regulated.** *A*, triple immunofluorescence staining visualizing the nuclei (DAPI), M-cadherin (FITC), and utrophin (TRITC) in cryostat sections of healthy muscles or in muscles after 48 h from the marcain treatment as indicated. M-cadherin stains non-activated and activated satellite cells, whereas utrophin is up-regulated only in activated satellite cells. *Bar,* 5 μm. *B*, schematic view of the alternative utrophin transcripts including either exons 1A and 2A (A-utrophin) transcribed from the upstream promoter or exon 1B (B-utrophin) transcribed from the downstream promoter as described previously (21). *C*, quantitative evaluation of A-utrophin transcripts (*open squares*) or B-utrophin transcripts (*open triangles*) in regenerating muscle performed with one-step quantitative RT-PCR. Specific 5′-oligonucleotides were used to amplify the transcripts containing the exon 1A or exon 1B from total RNA of mouse muscles at 0, 3, 6, and 8 h and 1, 2, 3, and 7 days after marcain treatment. The values normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA levels represent the mean of three independent experiments.

injury we observed that utrophin mRNA is strongly induced during the muscle regeneration process. Our data lead to the conclusion that utrophin is already activated in satellite cells before fusion into young regenerated fibers. By injection of reporter constructs within the damaged muscles, we observed that this induction is due, at least in part, to transcriptional activation and is under the control of utrophin upstream promoter and enhancer DUE. The enhancer sequence contains an AP-1 site perfectly conserved between mouse and human which is necessary for the enhancer function. Analysis of the AP-1 expression factors after muscle damage showed that these factors are induced with kinetics that correlate with utrophin mRNA in active satellite cells. Because soluble factors play an important role in satellite cell activation (33–35), these results suggest that cytokine(s) might specifically induce a pathway that activates utrophin transcription in mononucleated muscle cells.

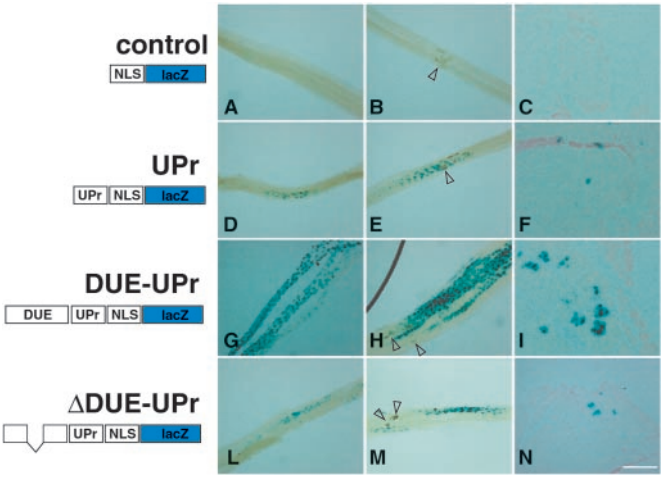


FIG. 2. **Utrophin upstream promoter and enhancer are active during muscle regeneration.** Tibialis anterior muscles were injected with marcain and *lacZ* reporter plasmid containing a nuclear localization signal (*NLS*) with utrophin regulatory sequences as indicated. Fibers were isolated from regenerating muscle 10 days after treatment and were stained for β-galactosidase and AcChoEase activities. *A–C*, control performed by injecting the *lacZ* mock vector in marcain-treated muscle. *D–F*, muscle injected with a plasmid containing *lacZ* under the control of the utrophin promoter (−896 + 103) indicated as UPr. *G–I*, injections of plasmid containing the *lacZ* under the control of the utrophin promoter and a *Bgl*II genomic fragment containing the utrophin enhancer DUE. *L–N*, injections of plasmid containing the *lacZ* under the control of the utrophin promoter and a *Bgl*II genomic fragment deleted of the 128 bp corresponding to enhancer DUE. The synapses are indicated by *open arrowheads.* To detect the nuclei position in the cross-sections of the muscle, fibers were stained with eosin (*C*, *F*, *I*, and *N*). *Bar*, 50 μm.

MATERIALS AND METHODS

Plasmid Construction and Plot Analysis—To isolate the correspondent mouse sequence of the enhancer DUE, we obtained the BACs containing the 5′ end of mouse utrophin gene from the Genome Systems. The screening of the BACs library was performed using the oligonucleotides G296, GGTCAGCACCAACACTATTTG, and G297, GCCGGGCAACTTTGTTCTCC, for the PCR amplification of the mouse utrophin promoter and G298, ATGGCCAAGTATGGGGACCTT, and G299, CAGATCTGGACTTAATGATGTC, for the amplification of the exon 2. We sequenced the positive BACs and derived plasmids from the mouse utrophin exon 2 for 4774 bp. The mouse utrophin second intron sequence has been deposited in GenBank™ under accession number AJ278913.

lacZ reporter plasmids were generated starting from the promoterless plasmid pNSlacZ (control), in which *lacZ* cDNA was fused with the nuclear localization signal of SV40 (36). The utrophin promoter sequence for the UPr in front of the *lacZ* construct was obtained from the CAT reporter construct UPr described previously (27). DUE-UPr was obtained by cloning the 3-kb *Bgl*II-*Bgl*II genomic fragment containing the enhancer DUE in front of the utrophin promoter. ΔDUE-UPr was obtained by deleting the 128 bp corresponding to the minimal enhancer sequence (27) by PCR using the following oligonucleotides: H133, GAGAAGATCTAAATTAACTGTCTTAAAATACAC; H134, GAGAAGATCTGAAGAGTGACATTAGGCC; H135, GAGAGGATCCAATCTTTAAAAATATAAGAACTCAGTAATG; and H136, GAGAGGATCCAATTATGTTGCAAAAGCCAGTAGATAAATT. DUE128-UPr was obtained by cloning the 128 bp corresponding to the minimal enhancer sequence wild type in front of the utrophin promoter. DUE128mAP1-UPr was obtained by mutating the AP-1 site as described previously (27).

CAT reporter plasmids UPr, DUE, DUEmAP-1 have been described previously (27). The mutant DUEmGATA was generated by polymerase chain reaction using the following primers: G392, GAGAAAGCTTCAAATTGCTTAGAGTGTT; G367, CGCGGATCCAGCCAAAAGAAT-

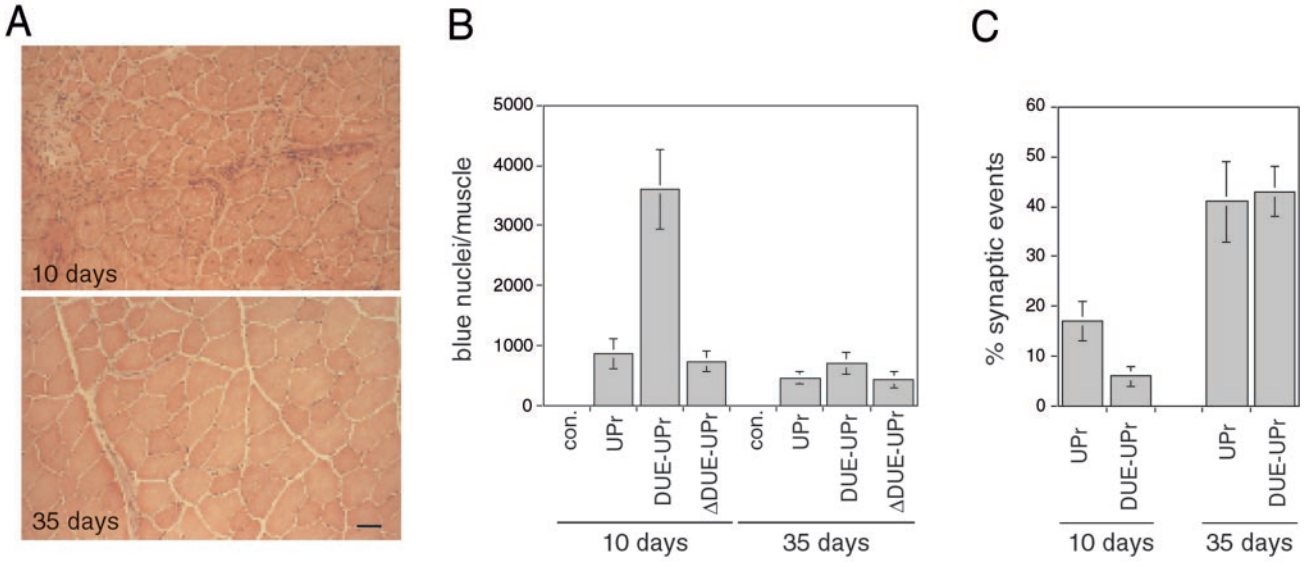


FIG. 3. **The enhancer DUE increases the extra-synaptic activity of the utrophin promoter in young muscle fibers.** *A*, eosin-hematoxylin staining of cross-sections from tibialis anterior muscle at day 10 (*upper panel*) and day 35 (*lower panel*) of regeneration. At day 10 young fibers are recognizable for the centered nuclei, whereas only mature fibers are visible at 35 days from injury. *Bar*, 10 μm. *B*, number of β-galactosidase (*blue*)-positive nuclei in muscles injected with the indicated *lacZ* constructs as described in Fig. 2. β-Galactosidase activity was measured in muscles collected at 10 or 35 days of muscle regeneration. *C*, percentage of synaptic groups of blue nuclei (events) with respect to the total events obtained with constructs containing the utrophin promoter (*UPr*) and utrophin promoter and enhancer (*DUE-UPr*) at 10 and 35 days of regeneration. The average of four independent experiments is reported.

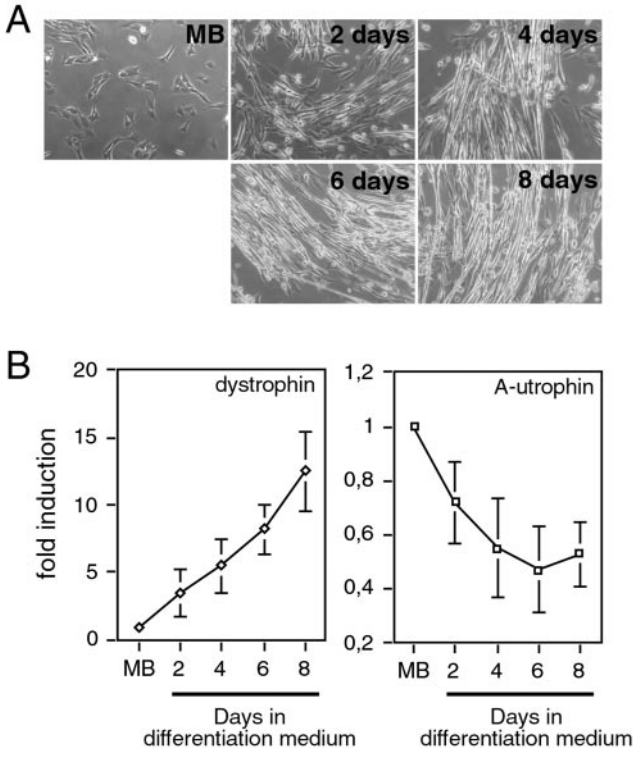


FIG. 4. **A-utrophin transcript is down-regulated during muscle differentiation *in vitro*.** *A*, C2C12 myoblasts (*MB*) and myotubes after 2, 4, 6, and 8 days in differentiation medium are as indicated. *B*, one-step quantitative RT-PCR was done with 200 ng of total RNA from myoblasts and myotubes using specific primers for dystrophin or A-utrophin transcripts. Dystrophin and A-utrophin levels in myoblasts were referred as 1. The values normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA levels represent the mean of three independent experiments.

GTGATTC; mGATA forward, GAGATCTAGAATGAATCACATTCTTTTGG; and mGATA reverse, GAGATCTAGAGCTATGTAACAACTAAA.

All reporter constructs sequences were confirmed by automated dideoxynucleotide sequencing (Applied Biosystems, Inc., model 377).

The graphical representation of the human-mouse sequences alignment of 5′ end of utrophin intron 2 was obtained with BLAST 2 Sequence (37).

Nuclear Extracts and Gel Mobility Shift Assay—Nuclear extracts from RD rhabdomyosarcoma cells (ATCC CCL-136) were obtained as described previously (14). Probes and competitors for gel mobility shift assay were obtained by annealing of the following oligonucleotides: H309, GTGTATATGAATCACAT, and H310, GTGTATGTGATTCATAT, for probe DUE AP-1; G410, AGCTTAGGAGTCCCGGAAGCAGGGAGGGGGGTGGGGGGATGGGCCG, and G411, GATCCGGCCCATCCCCCCACCCCCCTCCCTGCTTCCGGGACTCCTA, for aspecific competitor; B7, AGCTAAGCATGAGTCAGACAC, and B8, GATCGTGTCTGACTCATGCTT, for TRE; H44, GATCTAATTTAGTTGTTACATAGCTCAGATATGTCTAGAATTCTTA, and H45, GATCTAAGAATTCTAGACATATCTGAGCTATGTAACAACTAAATTA, for mAP-1; H42, GATCTAATTTAGTTGTTACATAGCTCCACAATGAATCACATTCTTA, and H43, GATCTAAGAATGTGATTCATTGTGGAGCTATGTAACAACTAAATTA, for mGATA.

The probes were labeled by Klenow fill-in reaction in presence of [α-^{32}P]dATP and [α-^{32}P]dCTP.

Binding reactions (20 μl) contained 10 μg of nuclear extracts and 2 μg of poly(dI-dC) in 10 mM Tris (pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol, 4% Ficoll. Complexes were allowed to form for 20 min on ice and resolved on 5% 39:1 acrylamide/bisacrylamide gels in 0.5% Tris borate/EDTA.

Cell Culture and Transfections—Mouse C2C12 and human RD rhabdomyosarcoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. C2C12 myoblasts were differentiated in 2% horse serum changing medium every 2 days. Transfection experiments were performed as described previously (26).

In Vivo Expression of the lacZ Constructs—Three-month-old CD1 mice were anesthetized, and 25 μl of 0.5% Marcain (bupivacaine) was injected into the tibialis anterior muscles. DNA solution (25 μl of 2 μg/μl) in phosphate-buffered saline solution was injected as described previously (38, 39). At the indicated times, mice were killed, and tibialis anterior muscles were removed, fixed for several hours in phosphate-buffered saline, 4% paraformaldehyde, rinsed, and stained overnight at 37 °C for β-galactosidase activity. Fibers presenting blue nuclei were removed, stained for AcChoEase activity (40), and analyzed under the microscope.

mRNA Analysis in Regenerating Muscles—Three-month-old CD1 mice were anesthetized, and 25 μl of 0.5% Marcain (bupivacaine) solution was injected into the tibialis anterior muscles. At the indicated times, injected and non-injected mice were killed, and tibialis anterior muscles were removed and quickly frozen in liquid nitrogen, and the total mRNA was extracted by guanidine thiocyanate standard method.

0.4 μg of total mRNA was used for the one-step quantitative RT-PCRs using LightCycler-RNA Amplification Kit SYBR Green I (Roche Molecular Biochemicals) according to the manufacturer's description. The reactions were run in the LightCycler instrument (Roche Molecular Biochemicals). The identity of each amplified product was controlled by sequence. The oligonucleotides used for the one-step RT-PCRs are listed in Table I.

Histological Analysis—7 μM frozen cryostat sections were simultaneously immunostained with the goat polyclonal anti-utrophin sc-7450 (Santa Cruz Biotechnology) and anti-JunD sc-74 (Santa Cruz Biotechnology) and one of the indicated rabbit polyclonal antibodies anti-M-cadherin, anti-Fra-1 sc-605 (Santa Cruz Biotechnology), and anti-Fra-2 sc-604 (Santa Cruz Biotechnology) and counterstained with DAPI to identify the cell nuclei. The MyoD and anti M-cadherin antibodies were kindly provided by G. Cossu and L. De Angelis.

RESULTS

Utrophin Transcription Was Induced in Muscles after Injury—It has been reported previously (29–31) that in regenerated muscle after damage utrophin was transiently present in the sarcolemma of regenerated fibers. However, in regenerated muscles utrophin mRNA was not increased suggesting that post-transcriptional regulation mechanisms regulate utrophin in regenerated muscle cells (31).

Muscle regeneration required the activation of satellite cells that proliferate and fuse to form new muscle fibers. We therefore analyzed utrophin expression at early time points after muscle damage performed by degeneration/regeneration experiments treating mouse tibialis anterior muscles with marcain as myotoxic agent (41, 42). Double staining of M-cadherin, a marker for satellite cells (42), and utrophin revealed a strong utrophin signal in satellite cells already at 48 h after injury (Fig. 1*A*). Thus, we found that utrophin up-regulation was already activated at early stages of the muscle-regenerative process, and the newly synthesized protein was already present in satellite cells before fusion into newly regenerated fibers. The presence of newly synthesized utrophin in satellite cells could explain the discrepancy observed between the presence of utrophin in the regenerated fibers and the levels of utrophin mRNA (31). We therefore analyzed utrophin mRNA levels by quantitative RT-PCR analysis at early time points of muscle regeneration. Because utrophin was transcribed by two independent promoters, we used two sets of primers that discriminate between the RNA transcribed from each promoter (21) (Fig. 1*B*). Analysis of utrophin transcripts at different times revealed that the utrophin mRNA transcribed from the upstream promoter was induced in regenerating muscle with a peak of 5–6-fold induction 24 h after marcain treatment (Fig. 1*C*). The utrophin transcripts remained high for the next 2 days and then started to decline and reached almost base-line levels at 7 days after treatment. Under the same conditions transcription driven from the downstream promoter was not induced. Thus, utrophin mRNA driven by the upstream promoter was up-regulated at early time points after muscle damage, and at the time point where new muscle fibers are formed, it was already down-modulated. This implies that during the muscle-regeneration process utrophin was induced transcrip-

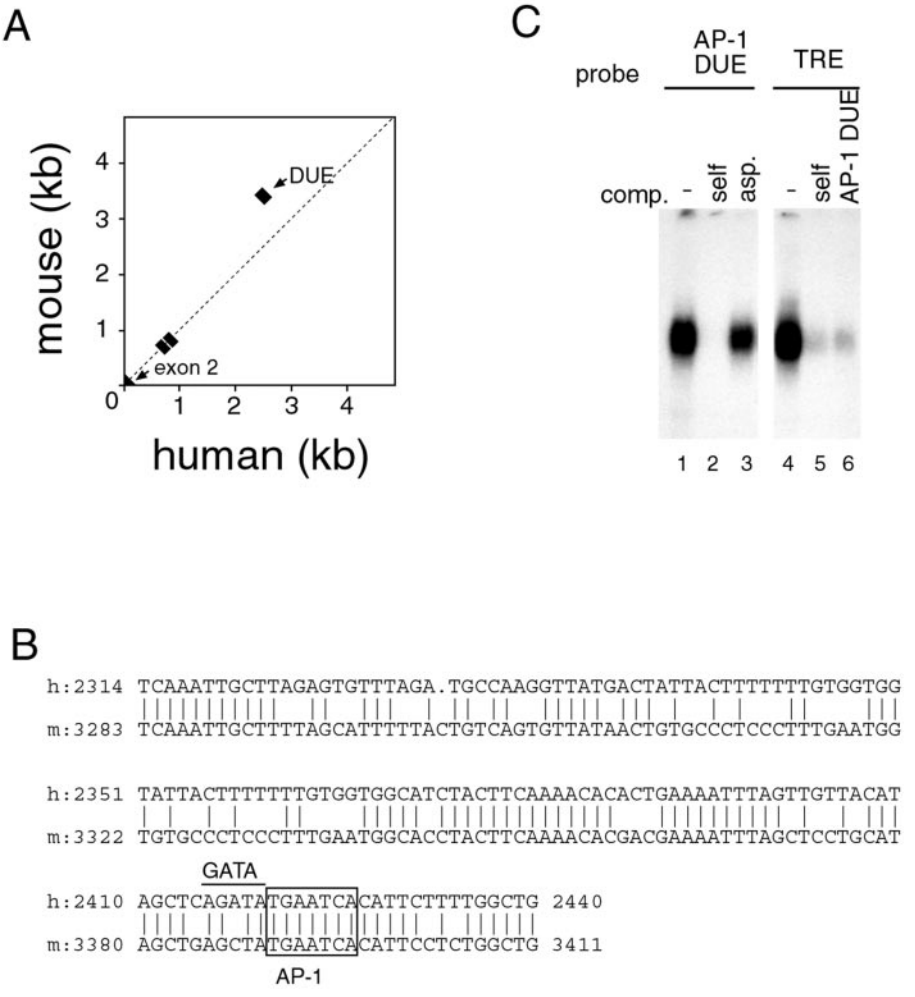


FIG. 5. **DUE sequence is conserved in mouse and contains a functional AP-1 site.** *A*, graphical representation of the human-mouse sequences alignment of 5′ end of utrophin intron 2 obtained with BLAST 2 Sequence (37). Mouse intron sequence was deposited under GenBank™ accession number AJ278913. The *boxes* indicate matching regions. Sequences corresponding to exon 2 and enhancer DUE are indicated by *arrows*. *B*, sequence alignment of human (*upper*) and mouse (*lower*) enhancer DUE. Numbering corresponds to the distance from the ATG start codons contained in the exon 2. The putative AP-1 (*boxed*) site is 100% conserved, and a putative GATA (*overlined*) site is not conserved in mouse. *C*, nuclear extracts obtained from RD cells were incubated with a DNA-labeled probe containing the putative AP-1 site of the enhancer DUE (*lanes 1–3*). The complex (*comp.*) obtained is indistinguishable from the complex shifted with a canonical TRE sequence (*lane 4*). The complex on DUE AP-1 was specifically competed by incubation with 100-fold molar excess of itself (*lane 2*) but not with a nonspecific (*asp.*) unlabeled probe (*lane 4*). The complex formed on TRE was specifically competed with 100-fold molar excess of itself or with the DUE AP-1 oligonucleotide (*lanes 5* and *6*).

tionally, and this activation was due to the upstream promoter.

To localize utrophin regulatory elements that drive utrophin expression in newly formed fibers, we injected marcain-treated muscles with various constructs in which the *lacZ* gene containing a nuclear localization signal was under the control of utrophin regulatory elements. β-Galactosidase activity was measured by counting blue nuclei in isolated newly formed muscle fibers at 10 days after injury when new fibers were formed (Fig. 2). Histological analysis of β-galactosidase-positive fibers showed that injected utrophin promoter drives blue staining in regenerated muscles (Fig. 2*D*). The presence of DUE induced a strong increase in the number of blue nuclei (Fig. 2*G*). Double staining of β-galactosidase-positive nuclei and NMJ by acetylcholinesterase activity revealed staining in both synaptic and extra-synaptic nuclei indicating that β-galactosidase was present in both sites (Fig. 2, *D* and *E*). Injection of the construct containing the utrophin enhancer DUE showed an increased number of stained nuclei within a single positive fiber and an increased number of events in extra-synaptic regions with respect to the promoter alone (Fig. 2, *G* and *H*). Transverse sections of injected muscles revealed the presence of muscle fibers with central nuclei positive for β-galactosidase, which demonstrated that the *lacZ* was expressed in cells forming new fibers (Fig. 2, *C*, *F*, *I*, and *N*).

Quantitative analysis was performed by counting blue nuclei at 10 days after injury when newly formed muscle fibers show central nuclei and, at 35 days, when injured muscles are fully regenerated with nuclei located at the periphery of the fibers (Fig. 3*A*). At 10 days after injury the utrophin promoter drove weak but measurable transcription. Injection of the plasmid containing the utrophin enhancer DUE upstream of the promoter resulted in about a 4-fold increase in activity (Fig. 3*B*). Evaluation of synaptic *versus* non-synaptic staining of β-galactosidase positive nuclei at 10 days after injury revealed blue staining in about 20% of post-synaptic nuclei muscle injected with the utrophin promoter, whereas only 5–6% were positive when we used the construct containing the utrophin enhancer. Thus, in young regenerating fibers transcription driven by the utrophin promoter was not limited to NMJ nuclei. In addition, DUE determined a significant increase of the total number of events and non-synaptic β-galactosidase-positive nuclei.

Little staining was observed 35 days after marcain treatment. Moreover, at this time the β-galactosidase post-synaptic expression was increased to 50% with no significant differences between the promoter alone and the promoter with the enhancer. This suggests that at 35 days from injury utrophin transcription driven by the upstream promoter was reduced to low levels and limited to NMJ, thus behaving like the endogenous promoter in non-injured muscles. Under these conditions the utrophin enhancer was not active.

The above data demonstrated that utrophin upstream promoter was transiently induced in activated myoblasts and suggest that its transcription was down-modulated in myotubes. To test whether utrophin was expressed in myotubes, we analyzed utrophin mRNA transcript levels in C2C12 muscle cells before and after differentiation by quantitative RT-PCR (Fig. 4). In accordance with data published previously (15, 43, 44), during myoblasts to myotubes differentiation, dystrophin mRNA was up-regulated. Instead, analysis of A-utrophin mRNA expression at different times after differentiation re-

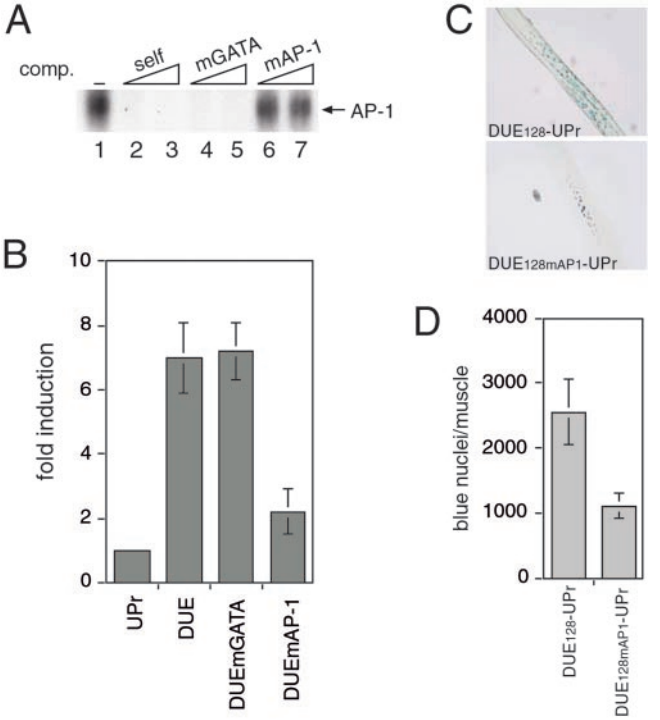


FIG. 6. **AP-1 plays an important role for DUE activity.** *A*, in bandshift assay the AP-1 containing complex (*comp.*) on the enhancer DUE is competed by incubation with 50- and 100-fold molar excess of double-strand oligonucleotides containing the sequence wild type (*lanes 2* and *3*) or mutated in the putative GATA (*lanes 4* and *5*) site but not with unlabeled probe mutated in the AP-1 site (*lanes 6* and *7*). *B*, CAT reporter plasmids were transfected in RD rhabdomyosarcoma cells. The mutation of the AP-1 site, but not of the putative GATA site, significantly decreases the activity of the enhancer DUE. *C*, fibers of tibialis anterior muscle of mouse injected with marcain and *lacZ* reporter plasmids containing the A-utrophin promoter and the minimal enhancer sequence (128 bp) wild type (DUE128-Upr, *upper panel*) or mutated in the AP-1 site (DUE128mAP1-UPr, *lower panel*). *D*, number of β-galactosidase-positive nuclei in muscles injected with the indicated *lacZ* constructs; the AP-1 mutation decreases the DUE activity i*n vivo*.

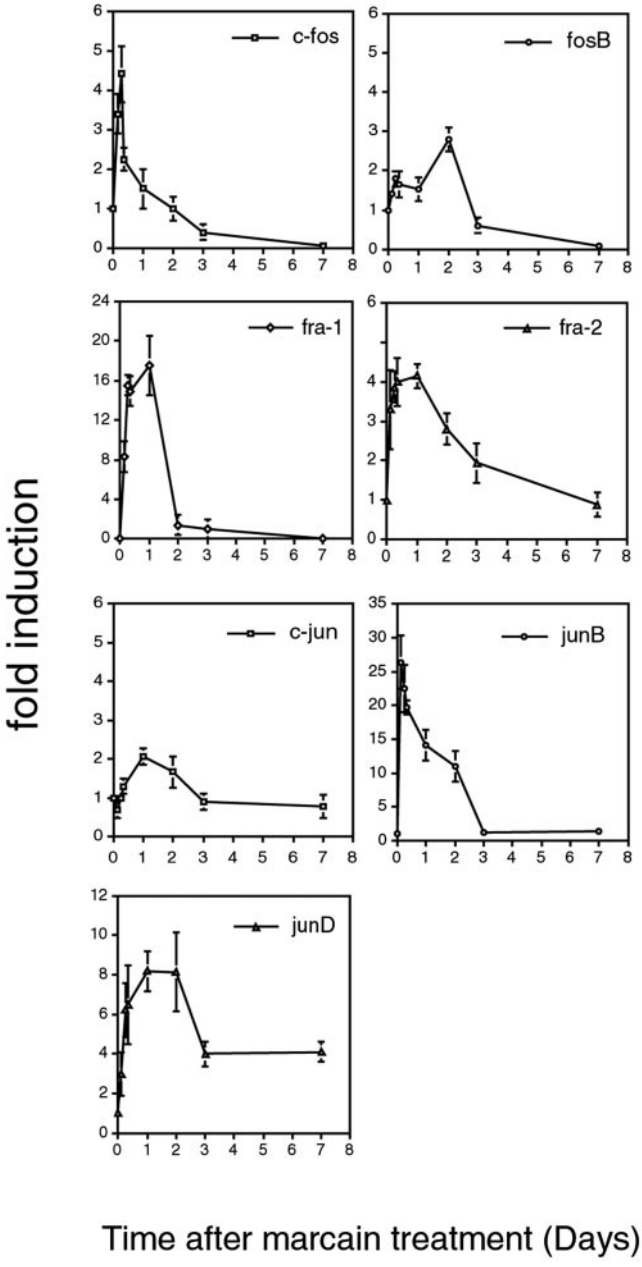


FIG. 7. **AP-1 factors are induced during muscle regeneration.** One-step quantitative RT-PCR to quantify expression of AP-1 factors transcripts in regenerating muscle. Oligonucleotides specific for each AP-1 family member were used to amplify the transcripts from total RNA of mouse muscles collected at 0, 3, 6, and 8 h and 1, 2, 3, and 7 days after marcain treatment. The values were normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA levels. The average of three independent experiments is reported.

vealed that this transcript was expressed at higher levels in undifferentiated myoblasts than in differentiated myotubes.

DUE Activity Was Dependent on AP-1 Site—Taken together the above experiments suggest that during muscle regeneration utrophin transcription was transiently activated, and this activation was strongly enhanced by DUE. We cloned and sequenced the mouse utrophin second intron. The comparison of the mouse sequence with the human intron revealed that although this intron was not conserved (Fig. 5, *A* and *B*), the enhancer DUE showed 74% identity (Fig. 5*A*). This enhancer contained a perfectly conserved AP-1 site. Binding analysis with nuclear extracts from RD rhabdomyosarcoma cells revealed that on the AP-1 site of DUE a specific retarded complex was formed. Cold DUE AP-1 specifically competed with itself as well as with a canonical TRE element suggesting the binding of AP-1 factors (Fig. 5*C*). Previous analysis revealed that DUE activates utrophin transcription in human RD rhabdomyosarcoma and in mouse muscle C2C12 cells (27). Point mutations of the putative AP-1 of human DUE inhibited the binding of AP-1 factor and strongly impaired the enhancer activity in RD muscle cells, whereas mutations within the non-conserved putative GATA element did not affect DUE activity (Fig. 6, *A* and *B*). Transfections and binding experiments of the mouse DUE demonstrated a conserved functional equivalence between human and mouse utrophin regulatory elements (data not shown).

Comparison of blue nuclei staining between a construct containing the wild type enhancer (DUE128-UPr) and a construct carrying a mutation within the AP-1 site (DUE128mAP1-UPr) demonstrated that the AP-1 site of DUE played an important role in the enhancer activity *in vivo* (Fig. 6*C*). Quantitative analysis counting blue nuclei at 10 days after injury showed that mutation of the AP-1 site significantly reduced the transcriptional efficiency of the wild type enhancer (Fig. 6*D*).

AP-1 Factors Were Induced in Satellite Cells after Muscle Damage—To measure whether AP-1 factors were induced in activated satellite cells, we analyzed the transcripts of each member of the AP-1 family at various times after injury. Although all AP-1 members were up-regulated under these conditions, each factor responded with different kinetics and intensity (Fig. 7). Whereas *junB* and c-*fos* were induced at early

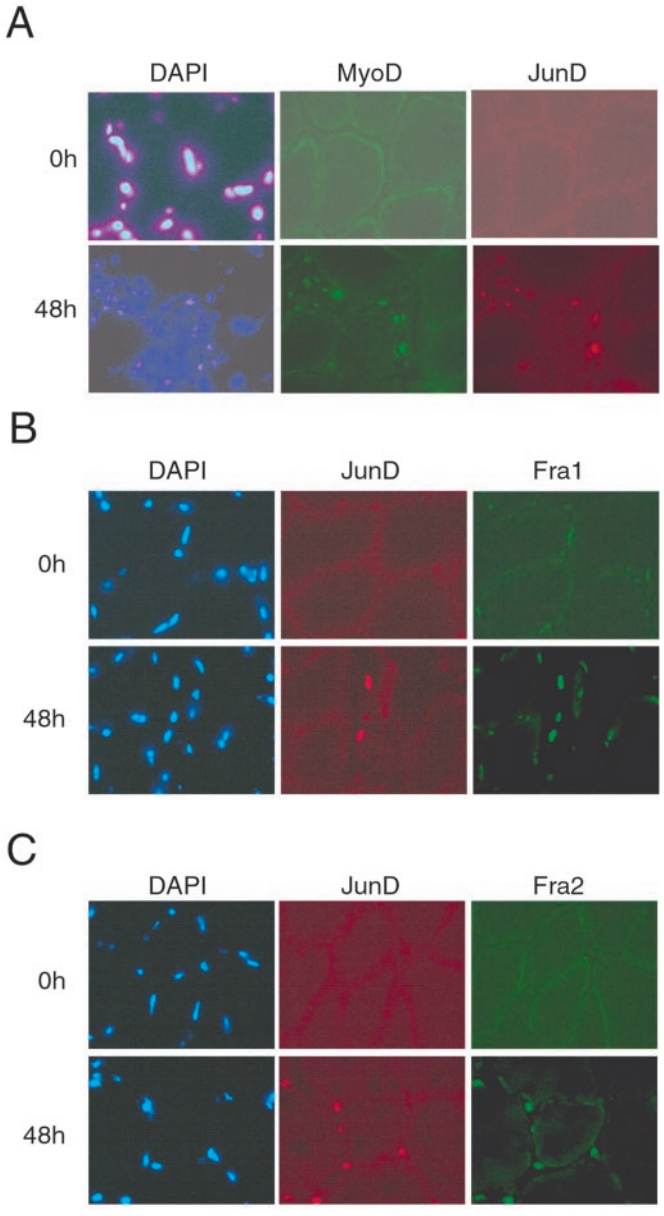


FIG. 8. **JunD, Fra1, and Fra2 are expressed in activated satellite cells.** *A*, triple immunofluorescence staining visualizing the nuclei (DAPI), MyoD (FITC), and JunD (TRITC) in cryostat sections of healthy muscles or in muscles after 48 h from the marcain treatment as indicated. *B* and *C*, triple immunofluorescence staining the nuclei (DAPI), JunD (TRITC), Fra1 (FITC), or Fra2 (FITC) in cryostat sections of healthy muscles or in muscles after 48 h from the marcain treatment as indicated. *Bar*, 5 μm.

time points with a peak between 3 and 6 h after injury and were rapidly down-modulated, *fra-1*, *fra2*, and *junD* showed a slower kinetics with their mRNA still present at 48 h after injury. Double staining of regenerating muscle at 48 h after damage with MyoD, which is a marker for actively proliferating satellite cells (42), showed that MyoD-positive cells also stained for JunD (Fig. 8*A*), thus demonstrating that JunD was expressed in activated satellite cells. JunD did not form stable homodimers although it heterodimerized with members of the Fos family, which do not homodimerize among themselves (45). We therefore performed double staining of JunD with either Fra1 or Fra2, and the AP-1 members were induced with comparable kinetics (Fig. 8, *B* and *C*). We observed JunD co-staining with both Fra1 and Fra2 at 48 h after muscle injury. Thus, in activated satellite cells both JunD/Fra1 or JunD/Fra2 functional heterodimers can be formed.

DISCUSSION

In the present study we describe the regulation of the utrophin gene during muscle regeneration. We demonstrate that during muscle regeneration utrophin gene is activated, at least in part, at the transcription level. Analysis of utrophin mRNA demonstrated that its messenger is transiently up-regulated at early time points after muscle damage. By analysis of transcription driven from injected DNA constructs, we identified the regulatory elements necessary for utrophin mRNA up-regulation. Higher activation levels were observed with constructs in which the promoter was linked to the intronic enhancer DUE. Within few weeks after damage *lacZ* expression drops and blue nuclei became mainly restricted to NMJ. Thus our data strongly suggest that following muscle injury the utrophin promoter, activated by the DUE intronic enhancer, responds with a transient increase of utrophin transcription. Importantly, we observe that in regenerated muscles utrophin regulatory elements drive *lacZ* expression not only to NMJ nuclei but also to nuclei distributed along the young regenerated fibers. To date, this is the first demonstration that utrophin transcription can be induced in the whole fibers of adult muscle. Our results show that the increase in utrophin mRNA is transient and relatively rapid and, at 1 week after damage, utrophin mRNA returns to low levels. This induction kinetics correlates with satellite cell activation. In these cells utrophin expression could be detected with specific antibodies. Thus, utrophin transcription is already activated in satellite cells before or at the time of fusion into young fibers, suggesting that most of the utrophin present along the newly formed fibers is already synthesized at the time of myoblast fusion to myotubes. The higher expression of A-utrophin in myoblasts then in myotubes was also observed *in vitro* in C2C12 cells. Interestingly, the down-regulation of utrophin from myoblasts to myotubes is accompanied by the increase of dystrophin expression corroborating the hypothesis that during development, and in muscle regeneration, utrophin expression precedes dystrophin behaving as a developmental precursor of dystrophin (18, 30, 46, 47). Although a functional role of utrophin in myoblasts is not yet clear, it is possible that utrophin might be necessary for the correct assembly and stabilization of the dystroglycan complexes before the appearance of dystrophin in fully differentiated myotubes.

In newly regenerated fibers utrophin should functionally substitute for dystrophin. However, in DMD patients this expression is not able to inhibit muscle wasting likely because of the down-modulation of utrophin, and following the early stages of muscle regeneration it is not replaced by dystrophin. Different from dystrophic patients, the mutant mice of the dystrophin gene (*mdx*) show a mild phenotype probably because of higher expression of utrophin that partially compensates for lack of dystrophin (28, 48). Indeed, double mutant mice *mdx*/*utr*$-/-$ lacking both dystrophin and utrophin develop a severe muscular dystrophy and die prematurely (10, 49). Marked increase in the severity of skeletal myopathy is also obtained with double mutant mice *mdx*/*myoD*$-/-$ (50, 51). These mice show impaired differentiation of satellite cells toward muscle progenitors suggesting that in man the severity of the pathology could also be due to the limited muscle regeneration potential. Thus, in DMD patients repeated cycles of degeneration-regeneration would exhaust the regenerating potential of satellite cells leading to a massive activation of connective tissue that results in muscle fibrosis.

Our experiments demonstrate the relevance of the DUE intronic enhancer with its conserved consensus AP-1 site for utrophin up-regulation in skeletal muscle. Activation of signaling cascades that induce these AP-1 factors may contribute to

utrophin induction in muscle cells. Fra-1 has been shown to be activated by c-Fos in different cell types (52, 53). JunD is not induced by mitogenic growth factors; however, its promoter contains several known inducible elements (54). Further studies should help clarify which pathways are involved in utrophin activation via AP-1 factors.

It is likely that constitutive activation of utrophin in muscle of DMD patients would cure or ameliorate the pathology. The results presented in this report demonstrate that the induction of utrophin expression in extra-synaptic regions of the dystrophic muscle is feasible. Which specific factors play a role in utrophin transcriptional up-regulation has not yet been identified, and it has not been verified whether these specific factors could also work in fully differentiated myotubes. It is worth noting that utrophin up-regulation in mature fibers following local inflammation mediated by virus has been observed (55). Several cytokines have been implicated in satellite cells activation and differentiation during muscle regeneration. Increasing evidence points out the role of soluble factors released by infiltrating cells into the damaged muscle (32). Among these, macrophages play an important role as these cells are the prominent infiltrating cells in damaged muscles within the first 48 h and secrete soluble factors with mitogenic effects on myoblasts, and their depletion impairs muscle regeneration (33, 34, 56). In addition, factors like FGF-6 and HGF/SF have shown to be potent activators of satellite cells (57, 58). As soluble factors involved in the muscle regeneration process induce utrophin expression, a challenge for the future will be the identification of specific factors that mediate utrophin transcriptional activation in adult muscle.

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