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Involvement of myogenic regulator factors during fusion in cell line C_2C_{12}

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ABSTRACT The myogenic factors, MyoD, myogenin, Myf5 and MRF4, can activate skeletal muscle differentiation when overexpressed in non-muscular cells. Gene targeting experiments have provided much insight into the *in vivo* functions of MRF and have defined two functional groups of MRFs. MyoD and Myf5 may be necessary for myoblast determination while myogenin and MRF4 may be required later during differentiation. However, the specific role of these myogenic factors has not been clearly defined during one important stage of myogenesis: the fusion of myoblasts. Using cultured C_2C_{12} mouse muscular cells, the time-course of these proteins was analyzed and a distinct expression pattern in fusing cells was revealed. In an attempt to clarify the role of each of these regulators during myoblast fusion, an antisense strategy using oligonucleotides with phosphorothioate backbone modification was adoped. The results showed that the inhibition of myogenin and Myf5 activity is capable of significantly preventing fusion. Furthermore, the inhibition of MyoD can wholly arrest the engaged fusion process in spite of high endogenous expression of both myogenin and Myf5. Consequently, each MRF seems to have, at this defined step of myogenesis, a specific set of functions that can not be substituted for by the others and therefore may regulate a distinct subset of muscle-specific genes at the onset of fusion.

KEY WORDS: *myoblast, fusion, MRFs, antisense*

Introduction

Differentiation of mammalian skeletal muscle cells is regulated by members of the MyoD family of myogenic regulatory factors (MRFs). These include MyoD (Pinney *et al.*, 1988), myogenin (Wright *et al.*,.1989), Myf5 (Braun *et al.*, 1989a) and MRF4/Myf6/Herculin (Braun *et al.*, 1990). Forced expression of any one of these will convert non-muscular cell lines into cells capable of myoblast fusion and muscle-specific gene expression (Davis *et al.*, 1987; Weintraub *et al.*, 1989; Lattanzi *et al.*, 1998). They are composed of a conserved basic DNA-binding domain and a helix-loop-helix (HLH) domain which allows heterodimerization with ubiquitous basic HLH proteins known as E-proteins (Murre *et al.*, 1989). MRF proteins can act as transactivators of muscle-specific genes by binding to a conserved consensus sequence termed E-box (CANNTG) present in the regulatory region of several muscle-specific genes. Recently, it has been suggested that the MRFs recruit chromatin-remodeling proteins to activate gene expression. The specific role of the different MRFs in skeletal muscle differentiation has not yet been completely defined essentially due to the existence of auto- and cross-regulatory loops between them (Thayer *et al.*, 1989). However, while it appears that both MyoD and Myf5 are well known to play an important role in myoblast determination and cell cycle regulation, it has been reported that myogenin may promote myotube formation and MRF4 may be involved at the latest stages of differentiation probably during myofibrillogenesis (Wright *et al.*, 1989; Hasty *et al.*, 1993; Nabeshima *et al.*, 1993; Rudnicki and Jaenisch, 1995; Lindon *et al.*, 1998).

The fusion of myoblasts is a key step of muscular differentiation generating elongated multinucleated muscle fibers from mononucleated myoblats. In culture, myoblasts proliferate, then migrate and align to fuse (Holtzer *et al.*, 1958; Kalderon and Gilula, 1979). As few results concerning MRFs at the time of fusion have been reported so far, we were interested in analyzing the chronology of appearance of these factors and studying their role in the phenomenon. Using mouse muscular cell line C_2C_{12}, it was shown that MyoD, Myf5 and myogenin are expressed in distinct expression patterns in fusing cells. In addition, an anti-sense strategy against

Abbreviations used in this paper: AS, antisense; BSA, bovine serum albumin; MCK, muscle creatine kinase; MD, MyoD; MF, Myf5; MG, myogenin; MRFs, myogenic regulatory factors; NT, non-transfected; R, random; SEM, standard error of the mean.

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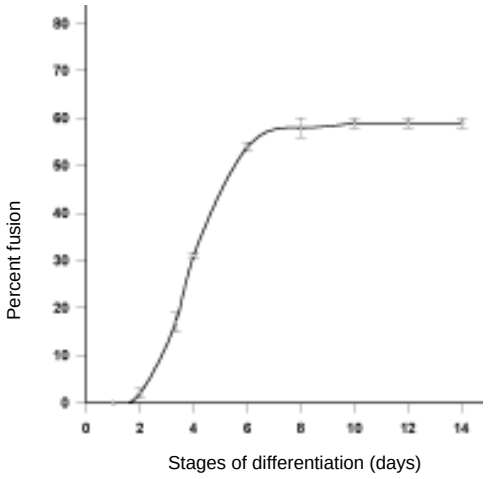

Fig. 1. Myoblast fusion during C_2C_{12} myoblast myogenesis. *Fusion was measured at various times after inducing differentiation and expressed as % fusion as already described (Brustis* et al.*, 1993). Vertical bars denote standard errors of the mean (SEM) for at least three different sets of cultures.*

each was used at different stages of the phenomenon and the consequences of such treatments on fusion progression were analyzed. The results clearly showed that inhibition of MyoD, Myf5 and myogenin is able to significantly prevent the process of fusion; supporting the idea that each member of the myogenic family has a specific and non-redundant role during the step of myotube formation and consequently may regulate a distinct subset of muscle-specific genes at the onset of fusion**.**

Results

Characteristics of C_2C_{12} Myoblast Fusion

Proliferating muscular cells placed in differentiation medium began to fuse after 2 days of culture with a maximal rate of fusion on the 4th day. The phenomenon progressively increased until day 8 and reached a maximum level of fusion when 60% of the nuclei were in multinucleated myotubes (Fig. 1).

Expression of MRFs at the Protein Level

The quantitative variations of the MRFs was analyzed by western blot during the C_2C_{12} fusion process. As noted in Fig. 2, MyoD, myogenin and Myf5 were present during the fusion process while MRF4 was undetectable even during the first 10 days of differentiation (data not shown). The level of MyoD (Fig. 2 A,B) exhibited a steady state level throughout the progression of the phenomenon. On the other hand, the level of Myf5 (Fig. 2 C,D) increased progressively to 40% until day 4 when the rate of fusion was maximal. At day 6, the amount decreased to about 30% compared to day 2 and presented a steady state level until day 10. As soon as the fusion process was initiated, the level of myogenin (Fig. 2 E,F) increased continuously until the absolute level of fusion was reached. During the exponential phase of the phenomenon, between the 2nd and the 6th day, the level of this MRF increased about 400%. Then, when fusion ceased the level of myogenin decreased slowly until day 10.

Antisense Treatment Efficiency

In order to evaluate the efficiency of the antisense strategy conducted against each MRF, the reporter plasmid pMCK-Luc which contains E-boxes of the mouse creatine kinase gene capable of binding myogenin, MyoD and Myf5 (data not shown) was used. At day 0, the reporter plasmid pMCK-Luc was co-transfected in cell line C_2C_{12} with synthetic oligodeoxyribonucleotides supposed capable of preventing MRFs synthesis. The transfection assay was standardized using the pSVβgal control plasmid. As reported in Fig. 3A, the data showed that each antisense oligomer was able to significantly reduce the luciferase signal confirming the efficiency of the antisense strategy and demonstrating that each protein is truely significantly reduced. Concerning controls, the three random sequences did not alter the signal of luciferase and consequently did not interfere with endogenous MRFs. The effect of antisense sequences on MRF expression was clearly and directly confirmed by immunoblotting at two different stages of differentiation (days 0 and 4) because MyoD was reduced in both cases by about 50% (Fig. 3B).

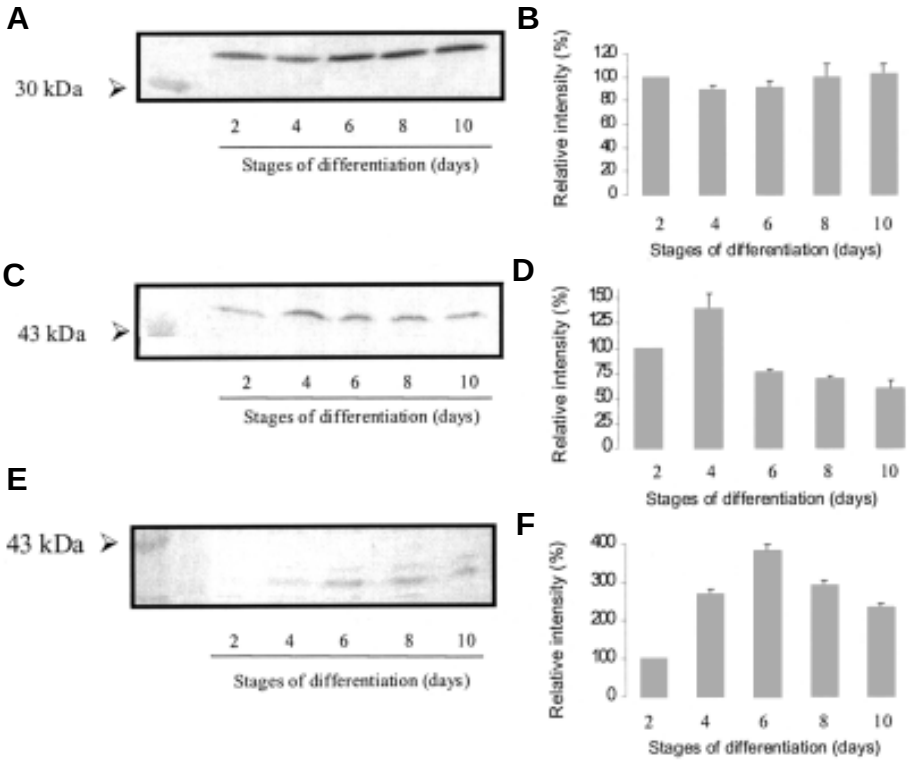

Fig. 2. Expression of MyoD (A and B), Myf5 (C and D) and myogenin (E and F) during C_2C_{12} differentiation. *Protein extracts obtained from cells cultured for various periods were resolved in a 10 % polyacrylamide gel and transferred onto a PVDF membrane. Blots were developed with specific monoclonal antibodies as described in Materials and Methods. The apparent density of the bands was estimated by scanning with a video densitometer. Results were expressed as percentage of MRFs level at day 2 (B, D and F). Bars on the graph represent the standard deviation for at least three different sets of cultures.*

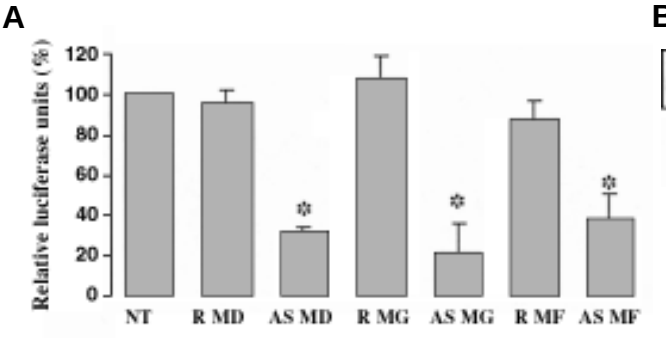

Fig. 3. Analysis of the efficiency of the antisense treatment against MRFs. **(A)** *The reporter plasmid MCK-luc was co-transfected at day 0 in C_2C_{12} myoblasts with an antisense oligomer against myogenin (AS MG), MyoD (AS MD) or Myf5 (AS MF) or a random sequence (R MG, R MD or R MF). After 36 hours, proteins were extracted and luciferase activity was determined using a luminometer. The obtained signal was standardized using the pSVβgal control plasmid. Results were expressed as percentage of luciferase activity in non-transfected cells (NT). Bars on the graph represent the standard deviation for at least three different sets of cultures.*Significantly different from corresponding control.* **(B)** *Cultured cells were transfected by an antisense oligomer against MyoD (AS MD) or by a random sequence (R MD). The transfection was conducted at days 0 and 4. The total proteins were harvested 36 hours after transfection and a Western-blot analysis was performed using a specific antibody against MyoD.*

Effect on Myoblast Fusion of Antisense Treatments against MRFs

Taking into account the results reported above, the antisense strategy was conducted at different appropriated stages of differentiation for each myogenic factor because their expression patterns are clearly distinct. As myogenin expression seems to be closely correlated to the process of fusion, the antisense treatment against myogenin was initiated at different stages of myoblast differentiation (Figs. 4A, 5). In each case, the antisense strategy had a significant effect on myoblast fusion with a maximal inhibition (44%) when the treatment was applied at the beginning of fusion (day 3.5). Because MyoD presented a steady state level during C_2C_{12} myoblast differentiation, the treatment against this factor was conducted on day 3, when expression of myogenin was still lower and Myf 5 was highly maintained; and at day 4.5, when Myf5 levels were significantly reduced and myogenin was in its exponential phase of expression. The results are presented in Figs. 4B, 5. Using antisense oligomers against MyoD, the extent of fusion was inhibited by at least 80% compared to random sequence whatever the stage of the treatment. Concerning Myf5, the treatment was performed at day 3 before its maximal level was reached. The data are presented in Figs. 4C, 5 and show a significant inhibition of the fusion phenomenon by 57% compared to the random sequence.

To confirm these results an antisense strategy was performed using different combinations of paired antisense oligomers (Fig. 6). All the antisense combinations were capable of preventing completely and immediately the fusion process, confirming our previous results.

Discussion

Time-course of MRF protein expression was followed in myoblasts differentiation and more particularly at the time of fusion. Myogenic cells expressed three of the myogenic factors, MyoD,

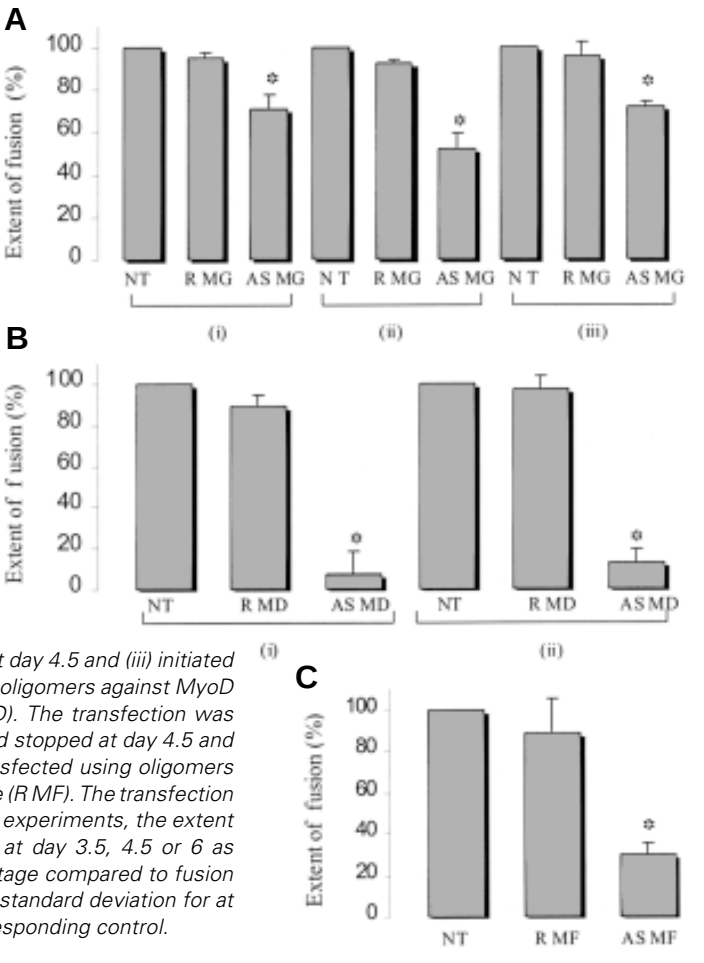

Fig. 4. Antisense treatment against MRFs in fusing C_2C_{12}. *Cultured cells were transfected during 36 hours using 0.50 μM antisense oligomers. Controls were done using adapted random sequences.* **(A)** *Cultured cells were transfected using oligomers against myogenin (AS MG) and control was done using the myogenin random sequence (R MG). The transfection was conducted at three different stages of differentiation: (i) initiated at day 2 and stopped at day 3.5; (ii) initiated at day 3 and stopped at day 4.5 and (iii) initiated at day 4.5 and stopped at day 6.* **(B)** *Cells in culture were transfected using oligomers against MyoD (AS MD) and control was done using the MyoD random sequence (R MD). The transfection was conducted at two different stages of differentiation: (i) initiated at day 3 and stopped at day 4.5 and (ii) initiated at day 4.5 and stopped at day 6.* **(C)** *Myogenic cells were transfected using oligomers against Myf5 (AS MF) and control was done using the Myf5 random sequence (R MF). The transfection was initiated at day 3 and stopped at day 4.5 of differentiation. For all these experiments, the extent of fusion was measured immediately after transfections were stopped, at day 3.5, 4.5 or 6 as described in Materials and Methods. Results were expressed as a percentage compared to fusion progression in non-transfected cells (NT). Bars on the graph represent the standard deviation for at least three different sets of cultures. *Significantly different from the corresponding control.*

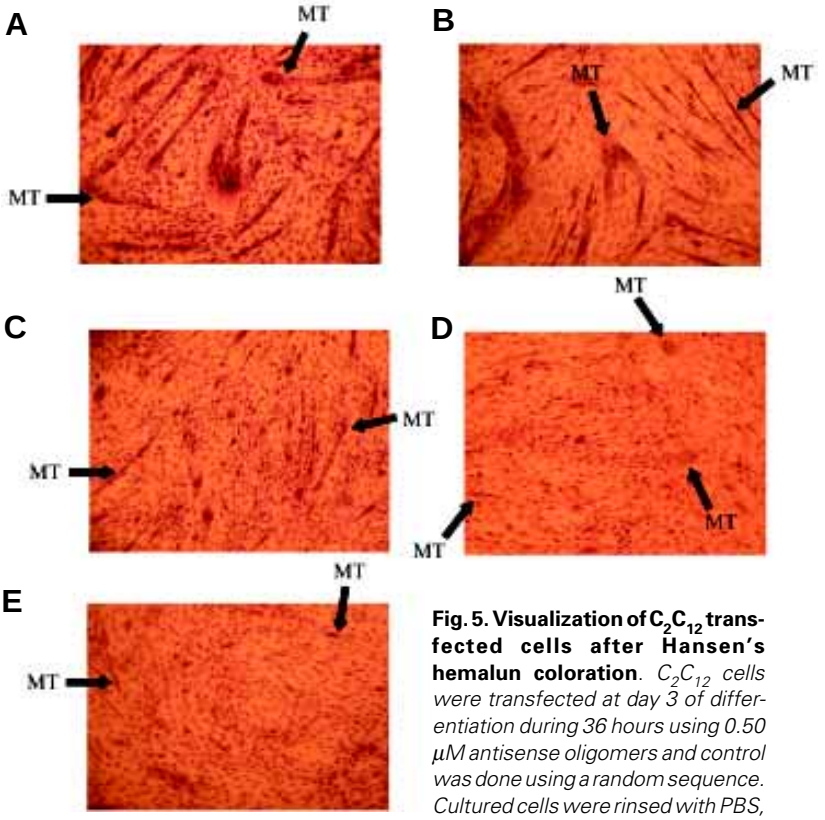

Fig. 5. Visualization of C_2C_{12} transfected cells after Hansen's hemalun coloration. *C_2C_{12} cells were transfected at day 3 of differentiation during 36 hours using 0.50 µM antisense oligomers and control was done using a random sequence. Cultured cells were rinsed with PBS, fixed in 4% paraformaldehyde and stained in Hansen's hemalun. The cells were visualized using light microscopy (X20). Cultured cells were transfected using liposome only* **(A)**, *an adapted random sequence* **(B)**, *oligomers against myogenin* **(C)**, *oligomers against Myf5* **(D)** *or oligomers against MyoD* **(E)**. *MT,* myotubes.

myogenin and Myf5, but did not express MRF4 even in the latest stages under the present culture conditions. Indeed, MRF4 is preferentially expressed in adult muscle fiber, probably due to its late function during myogenesis pathway.

We observed that MyoD was present at a steady state level during the extent of fusion while the levels of Myf5 and myogenin varied significantly during the stages of differentiation. The expression of myogenin was highly correlated to the fusion curve while the expression of Myf5 protein increased until day 4 then droped abruptly and did not vary until day 10. These results associated with a recent study concerning the time-course of mRNA levels of these proteins in the same cell line (Shimokawa *et al.*, 1998) suggest that these myogenic factors are essentially regulated at the transcriptional level during myotube formation. Such an evolution for myogenin protein is in agreement with many studies indicating a close correlation between this factor and the process of fusion (Wright *et al.*1989; Shimokawa *et al.*, 1998). Previous work concerning MyoD has indicated a constitutive expression at the myoblast stage (Montarras *et al.*, 1989; Laurent *et al.*, 1997). Our results complete these studies and show that MyoD is expressed throughout the process of fusion and even at the later stages of differentiation. On the other hand, some investigations conducted in our laboratory using primary myoblast culture from rat embryos revealed different expression patterns for these because MyoD and Myf5 increases progressively throughout myotube formation (submitted publication). Consequently, the distinct pattern of the three myogenic regulators during earlier C_2C_{12} myogenesis may suggest that each member of the family regulates a distinct subset of muscle-specific genes at the onset of fusion.

In an attempt to clarify the role of each MRF during this phenomenon, an antisense strategy was used during myoblast differentiation particularly during the fusion period. Our strategy used synthetic lipid as a carrier and modified oligomers with a phosphorotioate backbone to prevent endonuclease degradation. Fluxes and localization of such oligomers in living cells are similar to those of phosphodiester oligonucleotides (Marti G. *et al.*, 1992). Moreover, the transfection system used was optimized permitting significant reduction in the amount of oligonucleotides by 30 to 60 times as compared to previous studies (Florini and Ewton, 1990; Balcerzak *et al.*, 1995; Williams *et al.*, 1996; Balcerzak *et al.*, 1998). In addition, the present experimental procedure using random sequence showed that the lipid carrier did not significantly perturb myoblast membrane fusion.

According to the present results, each treatment significantly affects myoblast fusion with less perturbation concerning myogenin inhibition because fusion is prevented by 44%. However, when performed at the beginning of the phenomenon, the level of fusion is only reduced by 24% suggesting that myogenin activity is not maximal at this time. When performed at the end of the fusion process, progression of fusion is reduced by about 25% compared to the control; these last results could be, in part, explained by the fact that the uptake of cationic lipid was progressively reduced during fusion progression (Hebling-Leclerc *et al.*, 1999). An analogous quantitative effect on fusion using myogenin antisense has been noted using rat primary myoblast (unpublished data). Although myoblasts from myogenin-null mice are well known to be incompetent to initiate fusion (Hasty *et al.*, 1993; Rawls *et al.*, 1995; Venuti *et al.*, 1995; Hirayama *et al.*, 1997), our data confirm that myogenin is crucial for the progression of normal fusion and more particularly at the onset of fusion.

Concerning MyoD, the strategy used was capable of almost completely blocking myotube formation. Although the strategy was conducted at two different stages when Myf5 and myogenin are differentially expressed, the consequences on fusion are similar and myoblasts lose the ability to continue the fusion process engaged. Consequently, because fusion is totally blocked, it could be hypothesized that the functions of Myf5 and myogenin are not redundant

TABLE 1

CHARACTERISTICS OF THE ANTISENSE SEQUENCES

Name	Sequence (5'-3')	Position
Antisense myogenin (AS MG)	ATAGGGGGATGTCTCATACAG	60-80
Random myogenin (R MG)	GCAGTCTAGATGGACGATGAT	
Antisense MyoD (AS MD)	GTCCCGGAGTGGCGGCGATAG	201-221
Random MyoD (R MD)	TGGCGAGTCACGAGCGCGTGG	
Antisense Myf5 (AS MF)	GAAGGGGAGAACTGGCAGCCG	15-35
Random Myf5 (R MF)	GGCGCGAAAGACGCGTGAAGG	

Characteristics of the antisense sequences. Underlined nucleotides designate phosphorothioate oligos.

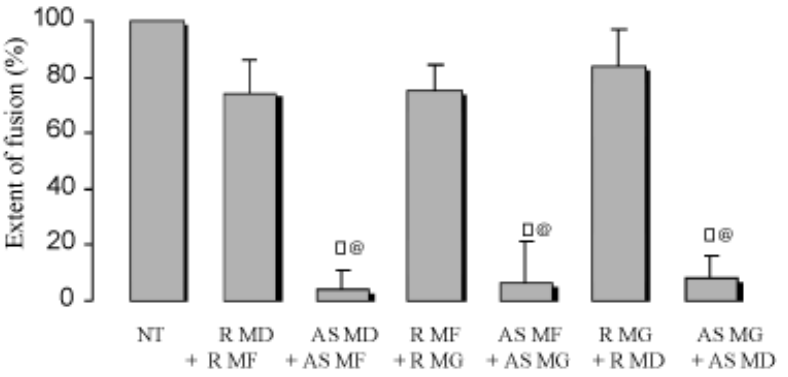

Fig. 6. Paired antisense treatment against MRFs in fusing C_2C_{12}. *Myogenic cells were transfected by each paired combination at day 3 during 36 hours using 0.50 μM oligomers against each MRF and control was done using an adapted paired random sequence. The extent of fusion was measured as described in Materials and Methods. Results were expressed as a percentage compared to fusion progression in non-transfected cells. Bars on the graph represent the standard deviation for at least three different sets of cultures. *Significantly different from the corresponding control.*

with those of MyoD concerning myotube formation. However, numerous previous authors have reported differing results concerning MyoD. For example, White *et al*., 2000, reported that muscle regeneration is delayed but not impaired in MyoD(-/-) mice and Rudnicki *et al*., 1992, suggested that MyoD is dispensable for skeletal muscle development. In addition, L6 myogenic cells are capable of fusion but do not express MyoD (Braun *et al*., 1989b) suggesting that the genes mediating myoblast fusion are not activated by MyoD in this rat model. However, although our results are different from those showing that MyoD is dispensable for muscle development, they are in agreement with many recent studies (Sabourin *et al*., 1999; Cornelison *et al*., 2000) which have demonstrated that MyoD(-/-) satellite cells failed to form multinucleated elongated myotubes. Moreover, characterization of the BC3H-1 cell line (Schubert *et al*., 1974) has revealed that myoblast express myogenin and Myf5 but do not express MyoD and although these myogenic cells express many skeletal muscle-specific genes, they do not fuse. These cells gain the ability to fuse only when MyoD is expressed from a transfected cDNA (Taubman *et al*., 1989; Brennan *et al*., 1990). However, even though the studies cited have shown a relation between the lack of MyoD and the inability to initiate fusion, our study demonstrated for the first time that MyoD inhibition is capable of blocking the engaged fusion process. Consequently, MyoD may thus play a specific and pivotal role in regulating genes that mediate myoblast fusion initiation and progression. Numerous studies have shown evidence of a positive link between MyoD and IGFII, which could explain the loss of autonomous differentiation when MyoD is lacking (Montarras *et al*., 1996, Pinset *et al*., 1997). Another hypothesis is that MyoD activity may be required to down-regulate Myf5 or IGFI at the onset of fusion, factors which promote more proliferation than differentiation (Rudnicki *et al*., 1992; Montarras *et al*., 1996; Sabourin *et al*., 1999).

The extent of fusion is significantly decreased using only Myf5 antisense although MyoD and myogenin are highly expressed at this stage of the treatment. Our analysis suggested that in C_2C_{12} myoblast cells, fusion of mononuclear cells into multinucleated myotubes is also dependent on Myf5 activity and cannot be wholly substituted for by the two other MRFs. Such a role of Myf5 during earlier myogenesis at the onset of fusion has never been described. Indeed, this factor is known to act rather in myoblast determination and in cell cycle control. However, our results are in agreement with those obtained in L6 myogenic cells because it has already been suggested that fusion depends on high levels of Myf5 in this model (Braun *et al*., 1989b).

The role of each MRF at the time of fusion was confirmed by the results obtained using different combinations of paired oligomers. As a matter of fact, each co-transfection was capable of completely preventing the phenomenon of fusion. In conclusion, the present study suggests that in cell line C_2C_{12}, distinct aspects of the myogenic program and particularly the fusion process are dependent upon control of MRFs. Each of them may have a specific set of functions that cannot be wholly substituted for by the others. This hypothesis is supported by the multiplicity of the myogenic factors, their distinct expression pattern during fusion and by the fact that some promoters could be trans-activated by only a subset of MRFs (Yutzey *et al*., 1990; Chakraborty *et al*., 1991). On the other hand, the fact that myogenin or Myf5 inhibition does not result in an absolute arrest of the fusion process may be due to the existence of overlapping functions between these MRFs. These results support the idea of the existence of a threshold level of expression of MRFs at the fusion step.

Materials and Methods

Reagents

Chemicals and materials are obtained from the following sources. Dubelcco's modified Eagle medium (DMEM), fetal bovine serum (FBS), horse serum (HS) were from Gibco (Cergy-Pontoise-France). Culture dishes were from Fischer-Scientific (Elancourt-France) and gelatin from Becton-Dickinson (Le Pont de Claix-France). The DNA extraction kit was from Qiagen (Courtaboeuf-France). The β-Gal staining set was from Roche Diagnostics (Meylan-France). The PVDF (polyvinylidene fluoride) membrane was from Millipore (Saint-Quentin en Yvelines-France). The BCA protein assay kit was purchased from Pierce (Bezons-France). Primary antibodies against MRFs were from Tebu (Le Perray en Yvelines-France) and secondary antibodies were from Sigma (Saint-Quentin Fallavier-France). The NBT/BCIP and the luciferase assay system were from Promega (Charbonnieres-France). All other chemicals were from Sigma.

Antisense Oligodeoxynucleotides

Purified oligonucleotides were synthesized by Eurogentec (Seraing-Belgium) and were chosen because they were short enough to penetrate living cells and long enough to be sequence specific (Varga *et al.,* 1999). The three nucleotides in the 3'- and in 5'-ends are modified oligonucleotides with phosphorothioate backbone modification conferring exonuclease resistance. The characteristics of the antisense oligomers are summarized in Table 1.

Cross-examination of Genbank showed neither auto-hybridization between these oligonucleotides nor a complementary sequence in any other endogenous gene thus far entered into the database. For each MRF, the random sequence had the same composition of bases as the antisense sequence.

Plasmids

The reporter vector pMCK-Luc contained the promoter-enhancer from mouse muscle creatine kinase (Sternberg *et al.*, 1988). The pSVβgal control plasmid was driven by the simian virus 40 enhancer and promoter and served as internal standard for transfection.

Cell Culture

Mouse C_2C_{12} myoblasts were grown in DMEM supplemented with 10% FBS under 10% CO_2 atmosphere at 37°C using culture dishes pretreated with gelatin (1%). When the cells became nearly confluent the medium was replaced with DMEM containing 2% HS to induce differentiation (day 0).

Transfection Assay

For transfection, C_2C_{12} mouse myoblasts were seeded at a density of $2x10^4$ viable cells/cm^2. The antisense oligonucleotides were transfected using synthetic cationic lipid method: Tfx-50 (Promega-Charbonnieres-France). After optimization, 0.50 µg/ cm^2 were used as DNA concentration and transfection was performed with a charge ratio of Tfx-50 reagent to DNA determined at 3:1. The transfection mixture was maintained in the cultured cells for 36 hours (Monia *et al.*, 1996).

Luciferase Assay System

All culture samples and reagents were brought to room temperature before the assay was performed. Luciferase activity was determined using a luminometer (Lumat LB 9507-Berthold-Colombes-France). The luciferase assay was normalized on the basis of a β-galactosidase assay.

Electrophoresis and Western Blot

Cells were detached mechanically from culture dishes at 4°C, sonicated and placed in sample buffer (Tris HCl (0.05 M) pH 6.8, SDS (4%), bromophenol blue (0.01%), glycerol (30%), DTT (1%)) and boiled for 5 min. Samples were loaded on 10% polyacrylamide gel. The separated proteins were transferred electrophoretically ($2mA/cm^2$) onto PVDF membranes using semi-dry transfer. The membranes were blocked with TBS (tris-buffered saline) solution containing 5% BSA (bovine serum albumin) and incubated with specific antibodies against MyoD ($1/1000^e$), myogenin ($1/500^e$), Myf5 ($1/500^e$) or MRF4 ($1/100^e$). Secondary antibody conjugated to alkaline phosphatase dilutioned of 1:10,000 was used for detection with NBT/BCIP as substrate. Membranes were stained for total proteins to verify the quantity and to confirm equal loading.

Measurement of Myoblast Fusion

Cultured cells were rinsed twice with PBS (phosphate-buffered saline) at different stages of differentiation, fixed in 4% paraformaldehyde for 15 min at room temperature and stained in Hansen's hemalun for 9 min. The lack of significant contamination by fibroblasts was determined by light microscopy using Hansen's hemalun stain and by an immunohistochemical technique using specific antibodies against desmin. The extent of fusion was then determined by visual inspection of culture dishes as previously reported (Brustis *et al.*, 1993). Fusion (%) = (number of nuclei in myotubes)/(total number of nuclei in myoblasts and myotubes) x 100.

Protein Quantification

Protein concentration was determined using a BCA protein assay kit with BSA as standard.

Densitometric Analysis

The apparent density of the band on the membranes was estimated after scanning with a video densitometer (Bioprofile,Vilber-Lourmat-Marne La Vallée-France). The linearity and the reproducibility of such quantification were verified by repeated measurements using different concentrations of purified fibronectin (Euromedex-Souffelweyersheim-France).

Statistical Analysis

The statistical significance of the difference between multiple groups was determined using ANOVA. When f was associated with a probability $p<0.05$, inter-group comparisons were conducted using Student's t test. Data were expressed as means ±SEM (standard error of the mean) and were representative of at least three different experiments.

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