Forced Expression of MyoD Creates a Muscle-Specific Test System for Exogenous Genes

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Abstract

A new approach free of time-consuming cloning procedures is described using MRF4 regulatory sequences acting as a physiological and muscular-specific promoter on human tissues; this method is useful for testing the expression of exogenous genes in cultured myoblasts and myotubes. pMRF4Z is a plasmid containing the regulatory sequences of Herculin (or MRF4, or MyF6), a gene of the MyoD family, driving the expression of a reporter gene *(E.Coli* β-galactosidase or "lacZ"). The DNAs were introduced into the cultured cells by the biolistic gun: mouse embryo-derived cells and human fibroblasts were bombarded with high velocity micro projectiles each carrying at the same time two different plasmids. The pair of plasmids were a MyoD construct together with pMRF4Z or pCMVlacZ, in the latter plasmid a viral promoter drives the expression of a reporter gene. The forced expression of MyoD family genes induced myogenesis and several days after the bombardment polynucleated myotubes were positive for lacZ activity.

Key words: MyoD, MRF4, biolistic, myogenesis, gene transfer, cell culture.

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Non-muscle cells, i.e., fibroblasts, amnyocites or chorionic-villus cells, which are not encumbered by near exhaustion of their proliferating potential and are easily obtained in significant quantities without time-consuming cloning procedures, were permanently converted into myoblasts by transfection with a MyoD construct [18]. The cloning of MyoD [5-22] and its three close relatives, myogenin [25-7], Myf-5 [4] and MRF4/ herculin/ Myf-6 [4-17-12] gave a very important contribution to the current view of skeletal myogenesis. Forced expression of any of these genes in non-muscle cultured cells can convert them to a skeletal muscle phenotype; myoD induced myoblasts fuse creating polynucleated myotubes and mature myofibers in innervated cultures [21], suggesting that these regulators play a significant role in determination and differentiation of skeletal muscle [23]. During muscle development *in vivo* MyoD and Myf-5 are responsible for mediating primary activation, defining the myoblast state, positioning the proper number of cells in the muscle-forming regions of the body and for receiving inhibitory signals from the environment. Myogenin and MRF-4, once activated by MyoD or Myf-5, are used for bypassing negative control *in vivo*, and for activating most downstream muscle structural genes by direct interaction with transcriptional enhancers [23].

MyoD acts forming heterodimers with E47 [5] (a myogenesis related protein) for the purpose of binding DNA for the activation of gene expression. In the presence of FBS, in proliferative medium, myogenic differentiation is inhibited through the intracellular expression of Id (Inhibitor of differentiation) proteins. Id bind E47 displacing MyoD, meanwhile if FBS (Fetal Bovine Serum) is withdrawn from the medium, E47 is released from Id and is free to bind MyoD and promote differentiation. Both these reactions are very fast. pECE.MyoD~E47 is a DNA construct where a SV40 promoter forces the expression of MyoD covalently bound to E47 by a peptide (tether). This construct is a dominating positive myogenic factor that cannot be inhibited in its action as a trigger of the differentiation program [13].

Patapoutian et al. [15] identified cis-regulatory sequences from the MRF4 locus that are able to direct the expression of the bacterial β-galactosidase gene (reporter gene, MRF4Z) in patterns reflecting that of endogenous MRF4.

In the present work, utilizing the Biolistic technique in tissue culture, we show how co-transfection of easily obtainable non-myogenic cells with a MyoD construct together with MRF4Z (figure 1) is an interesting and speedy approach for the development of a muscle-specific test system for exogenous genes. Keeping in mind the fact that there are no human myogenic cell lines, this approach could be very useful for studies involving DMD patients because dystrophic myoblasts senesce earlier than the normal ones [3] and they can hardly reach a sufficient number for non-viral gene transfer. The MyoD transfection system we are describing on human fibroblasts could facilitate studies leading to the transplantation of self dystrophin-competent myoblasts in dystrophic patients.

Material and Methods

Plasmids

The constructs utilized are described in figure 1 and are all generous gifts of Prof. Barbara Wold (California Institute of Technology). Every experiment was performed as co-transfection of two plasmids contemporarily: one of the two MyoD constructs together with one of the two reporter genes, the pMRFZ test plasmid and the pCMVlacZ as control. The plasmids were propagated in DH5 *E. Coli* bacterial strain after transformation by electroporation utilizing a Gene Pulser (Bio-Rad) apparatus according to standard protocols [2]. Plasmid DNA was purified by Plasmid Maxi Kit (Qiagen).

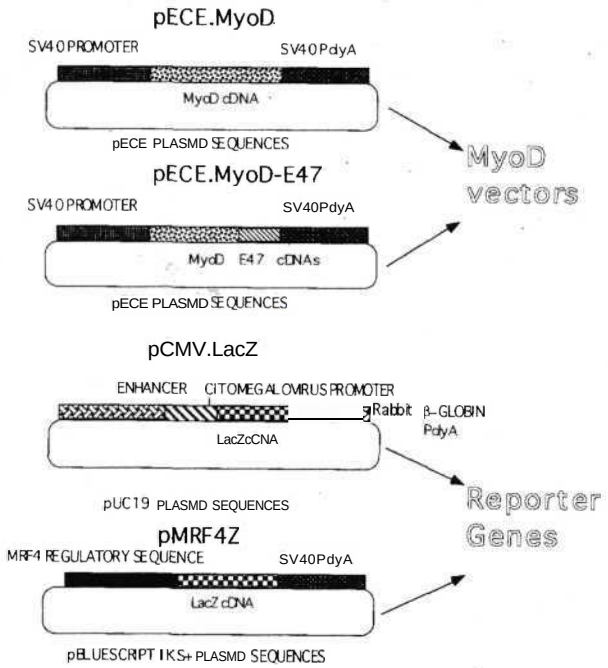

Figure 1. Detailed description of the DNA constructs utilyzed. Each transfection was performed with one MyoD expression vector together with one reporter gene contemporarily.

Transfections

M10 tungsten microprojectiles with a diameter ranging from 0.7 to 1 um were coated with plasmid DNA by precipitation with 1.25 M $CaCl_2$ and 0.02 M free base Spermidine according to the method described [19]. DNA coated particles are loaded onto a Kapton membrane ("macrocarrier") in an ethanol suspension and allowed to dry. Macrocarrier is mounted in the biolistic device between the rupture disk and the stopping screen. Cells were shot utilizing a PDS 1000/He (Biolistic Particle Delivery System. Bio-Rad), in a partial vacuum of -18 mmHg, with a helium driving pressure of 650 PSI. Pressure on the rupture disk is increased using compressed helium until it ruptures; the resulting pressure shock wave propels the macrocarrier against the stopping screen at high velocity. The stopping screen allows only the particles to pass through to bombard the cellular monolayer placed in their trajectory. The target cells were placed at a distance of 12 cm from the helium pressure source.

Tissue culture and media

Mouse embryo primary cultures were obtained by enzymatic and mechanical dissociation of fetuses 16 days *post coitum* according to standard methods [9] and grown in Dulbecco's modified Eagle's Medium (DMEM) (Gibco, Life Technology) supplemented with 10% fetal bovine serum (FBS), Gentamycin and Amphotericin B (Gibco, Life Technology). 2.2 x 10^6 cells on a 6 cm Petri dish were shot 24 hours after plating. Six hours after the bombardment cells were trypsinized and plated on four 10 cm Petri dishes coated with polylysine and laminin (Gibco, Life Technology). Human skin fibroblasts were obtained by dissection of a human skin biopsy, obtained with informed consent, according to standard protocols [9], grown in M199 medium (Gibco, Life Technology) supplemented with 15% FBS, Gentamycin and Amphotericin B.

5 x 10^5 cells on 6 cm diameter Petri dishes were shot 24 hours after plating. 6 hours after the bombardment, cells were trypsinized and plated on three 10 cm Petri dishes. The G8 mouse myoblast cell line was grown in DMEM supplemented with 4.5 g/L glucose and 10% FBS and 6 x 10^5 cells were shot, reaching a confluency of only 60%, in order to avoid spontaneous differentiation.

Myotube formation was induced in confluent cells cultures by mitogen deprivation: each medium described above was supplemented with 5% heat inactivated horse serum (HHS) instead of FBS, creating "differentiating media".

X-gal cytochemical assay

The cells expressing β-galactosidase of E. Coli were identified following incubation with X-gal (5-bromo-4-chloro-3-indolyl-β-galac topyranoside, Boehringer Mannheim) as described [16]. Briefly, transfected cells were fixed in a solution containing 1% glutaraldehyde, and then incubated with a solution containing 0.2% X-gal. After an incubation for 2-4 hours at 37°C, the X-Gal

solution was removed and 50% glycerol was added to the dish. The blue colored cells were identified under a phase-contrast light microscope.

Immunocytochemical procedures

Cells collected on coverslips were fixed in paraformaldehyde 4% and permeabilized with 0.01% Triton X100. Antibodies used: anti-myosin heavy-chain mouse monoclonal antibody (Novocastra Laboratories) recognizing sarcomeric myosin was used as myoblast-specific marker, as described [6]; anti-dystrophin monoclonal Dys 1 (Novocastra laboratories) immunocytochemistry was performed on differentiated myotubes as described [20]; anti-lacZ monoclonal antibody (Boehringer Mannheim) was used to confirm the reporter gene presence as described (fig. 2B) [15]. Standard indirect immunofluorescence was performed with second fluorescent rabbit anti-mouse antibody (Novocastra Laboratories). The preparations were studied with Zeiss microscope with appropriate filters to detect fluorescence.

Formation of transfected myotubes

Cells were shot with microprojectiles coated with: pECE.MyoD and MRF4Z, pECE.MyoD~E47 and MRF4-Z, pECE.MyoD and pCMVlacZ (described in detail in figure 1). 5 plates of each tissue type were shot with one of the plasmid combinations. 6 hours after bombardment the bombarded plates of each combination were collected, mixed and replated in order to neutralize the differences of the single shots. A very small quantity of the bombarded cells was collected on coverslips coated with polylysin and laminin. Three days after bombardment the medium was exchanged with the differentiating one in all plates with the exception of those shot with pECEMyoD~E47 that were kept in 10% FBS. At days 6, 8 and 10 respectively a third of the plates of each condition were stained with X-gal and Blue polynucleated myotubes appeared. The efficiency of transfection was determined by X-gal staining since all cells stably transfected with the two plasmids should express lacZ under the control of the constitutive viral or muscle-specific promoter (fig. 2). The number of X-gal stained clones was compared with the cellular morphology as seen through phase-contrast microscopy assuming that cells with three or more nuclei were myotubes.

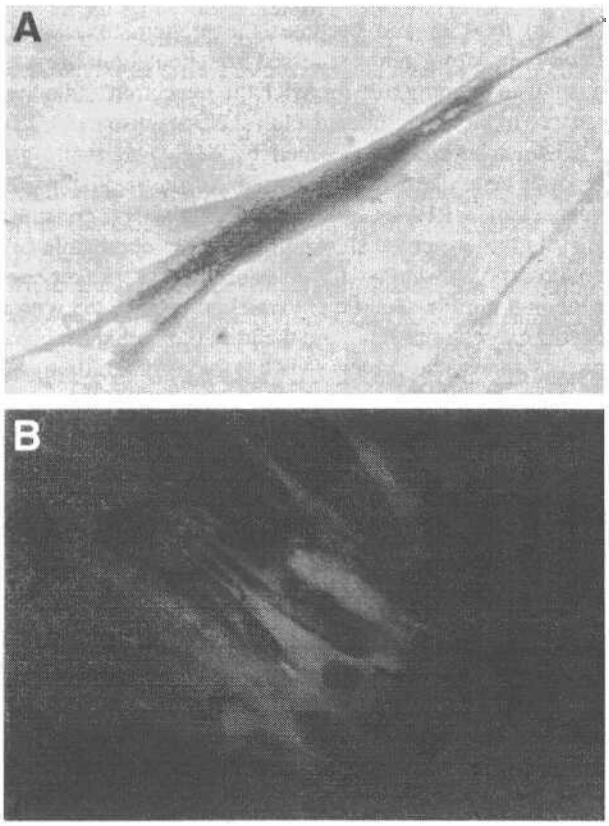

Figure 2. Myoblast derived from the transformation of human fibroblasts with MyoD and MRFZ showing to be positive for lacZ expression with the X-gal cytochemical assay (blue color) (A) and myoblasts positives for FITC immunoflurescence with anti-lacZ ***antibodies*** *(B), 500 X magnification.*

Results

Cells of mouse primary cultures were obtained from fetuses 16 days *post coitum*. These cells are mainly derived from chorionic villi and the presence of myoblasts is normally very low. Nevertheless we tested them for immunohistochemical reaction with anti-myosin heavy chain and anti-dystrophin antibodies, which are specific for myoblasts and myotube respectively [16-20]. The result was completely negative (data not shown) as it was negative for human fibroblasts (data not shown).

According to the literature [18-21] and to our preliminary experiments, human fibroblasts and mouse embryo-derived cells begin myotube formation after three days from MyoD transfection, and the phenomenon was triggered by changing the medium to the differentiating one, for this reason we began our analysis on the fourth day after transfection.The results summarized in figure 3 show that for human fibroblasts the peak of efficiency of this co-transfection is 8 days after the bombardment, at this time the efficiency per shot and the absolute number of myotubes were maximal for each of the three plasmid combinations. This result was confirmed also in the cells derived from mouse primary cultures but with a different time span: the maximum efficiency was registered to be after 10 days from the bombardment in each of the plasmids combinations tested. This experiment was repeated three times yelding very similar results and their average is plotted in figure 3. A small quantity of the transfected cells were collected on coverslips and exposed to antibodies against lacZ as a further confirmation of the technique for the detection of exogenous gene expression (fig. 2B). Non bombarded cells from both types tested were stained with X-gal and no spontaneous blue cells were found.

Figure 4 shows the induced myogenesis on small quantities of the bombarded cells collected on coverslips. Fluorescein immunohistochemical reactivity after exposure to

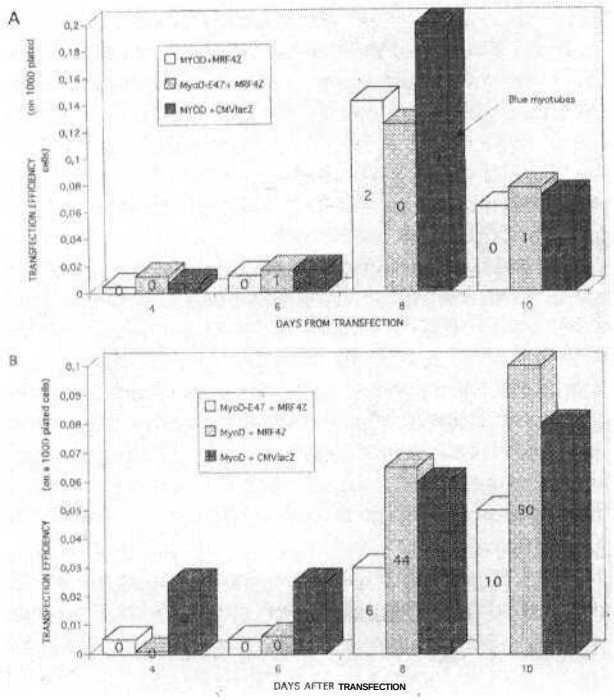

Figure 3. The results of the experiments of MyoD driven expression of lacZ are showed as plotted averages of transfection efficiency in relation to time in human fibroblasts (A) and mouse embryo primary cultures (B). The numbers expressed on top of each column represent an average of myotubes counted in each condition.

anti-myosin heavy chain antibody, specific for myoblasts [6], of these transformants (figure 4A) is evident if compared to the reactivity of G8 mouse myoblasts (figure 4B). The collected cells and G8 myoblasts were exposed to anti-dystrophin antibody (figure 4C, D), specific for myotubes [20] confirming the MyoD forced myogenesis.

pMRF4Z was shot alone in human fibroblasts, mouse primary cultures and G8 cell line. No blue stain was detected even after the induction of differentiation in human fibroblasts and mouse primary cultures. Only in G8 cell line the blue stain was present but very faint as reported [15].

Discussion

Due to the time consuming procedures involved in the isolation process of myoblasts and the need of a large number of them for most transfection methods, the testing of an exogenous gene in a muscle-specific cellular environment could take more than a month. The use of myogenic cell lines could solve this problem, but so far there are no human myogenic cell lines available, and even murine myogenic cell lines do not offer a physiologic environment, in fact when transplanted *in vivo* they are tumorigenic [24]. Physiologic human myoblasts proliferate in culture for a definite number of doublings, 25-30, before they senesce meanwhile mouse myoblasts senesce even earlier, after about 12 to 15 doublings [8]. In order to test the expression of an exogenous gene in a culture of muscular tissue several delicate and time consuming procedures have to be performed: the dissection of the biopsy specimen, emission of cells from the specimen, cloning, expansion of clones, antibody testing of clones [9], transfection of the myogenic clones, growth and differentiation of transfected cells. Satellite cells can also be isolated from muscles of boys affected by Duchenne Muscular Dystrophy (DMD) [21] (a progressive, X-linked disease with an incidence of about 1 in 3000, caused by mutations in the 2.5 Mb gene that codes for dystrophin, which result in the absence of a functional protein product [10]). These cells have already spent a great deal of their proliferative capability by participating in repeated cycles of regeneration [3], thus they tend to senesce after relatively few doublings *in-vitro*. For this reason to test the expression of an exogenous gene in DMD myoblasts is practically impossible. Our present results demonstrate that these problems can be circumvented if non-muscle cells, i.e., fibroblasts, amniocytes or chorionic-villus cells (which are not encumbered by near exhaustion of their proliferating potential and are easily obtained in significant quantities without time-consuming cloning procedures), are permanently converted into myoblasts by transfection with a Myo D construct.

MRF4 appears consistently at day 16 p.c. in all fetal skeletal muscles and becomes the predominant MyoD family regulatory gene expressed troughout adult life suggesting an ongoing role for MRF4 in execution and maintenance of the differentiated adult skeletal muscle [20, 15]. MRF4 expression is activated by MyoD as well as a reporter gene like lacZ downstream of the regulatory sequences of MRF4 as utilyzing MRF4Z DNA construct [15]. In this paper we showed how easily obtainable cell cultures (notably human fibroblasts) can express a reporter gene in a muscle-specific pattern if co-transfected with a MyoD expression vector and the promoter/enhancer complex of a muscular protein (MRF4) that is naturally trans-

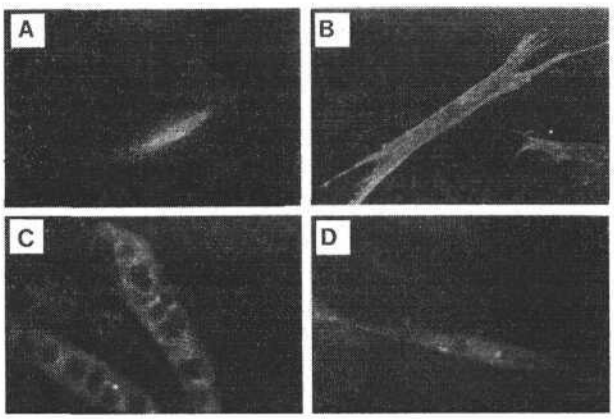

Figure 4. FITC immunofluorescence reactivity with anti-myosin heavy chain antibodies (A, B) on single myoblast cells and anti-dystrophin antibodies (C, D) on multinucleated myotubes. Human fibroblasts transfected with MyoD are A and C; G8 mouse myoblasts are B and D.

activated by MyoD. We utilized for this purpose the Biolistic gene gun because both DNA vectors can be precipitated on the same microprojectiles giving a high yield of co-transfected cells [1].

The dynamic study of MyoD transactivation of MRF4Z construct showed reproducible results (fig. 3) demonstrating that the peak expression of the reporter gene is 8 days after co-transfection in human fibroblasts and 10 days in mouse embryo cultures. A possible explanation for this is that mouse embryo cultures are farther away in the differentiation program from the full myogenic phenotype than human fibroblasts, that are mesenchimal cells so the intracellular environment is very close to the myogenic phenotype; this theory is sustained also by the facts that the DNA sequences used here are murine and that mouse embryo cultures have a faster metabolism, with a doubling time of 5 hours against the 22 hours of human fibroblasts.

In each cell type the peak efficiency is consistent for each of the plasmid combination (fig. 3), this, together with the fact that cells transfected with CMVlacZ alone have a much higher efficiency of lacZ expression [11] and that cells transfected with MRF4Z alone have no lacZ expression, confirms that the expression pattern of these experiments depends uniquely on the action of MyoD. Moreover, in both cell types the pattern of expression of MyoD or of MyoD~E47 transactivation is similar as day of peak expression, but it differs as absolute number of blue myotubes. Since in our experimental design bombarded cells are trypzinized and pooled for each plasmid combination, myotube formation depends on the fact that three transfected cells randomly adhere to the plate close to each other. The cells transfected with MyoD are kept for three days before switching the medium to the mitogen deprived differentiating medium in the differentiation-inhibiting, FBS-supplemented medium. In the FBS supplemented medium, cells can double a few times before differentiation and then they already adhere close enough to each other to form myotubes. MyoD~E47 construct is an artificial dimer of these myogenic factors (myoD and E47) and it is not sensitive to the inhibition of the differentiation induced by growth factors present in FBS [2], meanwhile MyoD alone is inhibited by these factors. On the other hand, the fact that both constructs show a peak of transactivation efficiency in the same day, depending upon the cell type, is further evidence in support of the speed of inhibition and disinhibition phenomena [13].

MyoD~E47 utilization in this setting is disadvantageous if compared to MyoD alone, because of the lower efficiency. Similarly the most evident disadvantage of the described system is the MyoD-dependent arrest of proliferation [23], which limits the absolute quantity of recovered transfected cells; unless, like in our case, a large number of cells are transfected. A possible solution to this proliferation inhibition could be the utilization of inducible promoters driving the expression of MyoD constructs. For example the mouse metallothioneine promoter could be used: in fact the presence of Zinc Sulphate in the medium activates the promoter [11] and MyoD expression could be blocked until transfected cells proliferate consistently, then MyoD expression could be restored and cells expressing an exogenous gene under the control of MRF4 regulatory sequences could be transplanted in animals or patients.

Our approach is based on the regulatory sequence of MRF4; it must be noted that the proposed system would work with the regulatory sequences of other muscle-specific genes whose expression is activated by MyoD, as for example muscle-specific creatine kinase, desmin, myosin light chain, etc. [23].

In our study we demonstrated that myogenic cells with a muscle-specific expression of an exogenous gene can be obtained speedily from non-muscular tissues. Myoblast-mediated gene delivery may be particularly advantageous for certain kinds of gene therapy, because, when a suspension of viable and fusion-competent myoblasts are injected into a muscle, some of the injected cells will fuse with the host fibers forming mosaic fibers [14] complementing them with the exogenous protein.

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