5' Azacytidine Enhances Exogenous Gene Expression in Skeletal Muscle

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Abstract

DNA methylation is one of the mechanism for regulating gene expression. Transfected cells in tissue culture are a mosaic of expression of the exogenous gene. 5' azacytidine (Aza C), inhibiting nuclear DNA methyltransferase, causes an increase of expression of *E.coli* a-galactosidase (LacZ) in transfected CHO cells.

Murine muscle cell lines G8 and C2C12 were transfected by biolistic technique with plasmids carrying a reporter gene pCMVlacZ and pY3 that confers resistance to Hygromycin B. Resistant clones have been treated with different concentrations of 5' azacytidine and analyzed by X-gal stain and Southern Blotting of DNA, after various time intervals up to one month. Hygromycin resistant cells were transplanted in the tibialis anterior muscle of living mice. Aza C was injected subcutaneously above the treated muscle. Histochemistry with X-gal was performed after different treatment periods, in order to evaluate the reporter gene expression timings.

The myogenic cell lines, after treatment with 5' azacytidine *in vitro*, express the exogenous gene at different levels, with four fold enhancement in G8 cells, after 15 days from transfection. The enhancement of expression is directly derived from the inhibition of methylation as shown by DNA restriction analysis. Mouse muscular samples treated subcutaneously with 5' azacytidine showed an enhancement of lacZ expression *in vivo* up to 20 days after transfection.

5' azacytidine enhances the level and the time span of exogenous gene expression in muscle cells facilitating gene therapy studies.

Key words: 5' azacytidine, myogenic cell, methylation, gene expression, *in vivo* treatment.

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All cell lines containing an exogenous gene after permanent transformation are a mosaic of expression of the introduced gene, that diminishes with time; in fact most commonly used reporter genes allow an appropriate level of expression only up to two weeks from transfection [15].

5' azacytidine is an analog of cytidine with a nitrogen atom replacing the carbon at the 5' position of the pyrimidine ring; because of this substitution the nitrogen atom cannot be methylated and incorporation of Aza C into DNA results in hypomethylation of the DNA [9, 6, 13, 10]. DNA methylation is one of the mechanism for regulating gene expression; the methylation pattern of specific genes changes during development and hypomethylation correlates with activation of some genes [14]. The degree of methylation of a gene reflects the state of that gene's transcriptional activity. This last property is very useful for the prolongation of the expression timing of exogenous genes [23, 25].

Aza C is also incorporated into RNA causing at high concentration alteration in RNA synthesis and processing, resulting in inhibition of protein synthesis [2, 19, 3, 20, 14].

In CHO cells it was shown that the main mechanism involved in the block of exogenous genes expression was methylation, and the inhibition of this phenomenon with Aza C enhances their expression up to 1 month after transfection [15].

In the present work we studied the effects of 5 azacytidine on the expression of a reporter gene in permanently transformed myogenic cell lines in tissue cultures and after transplantation *in vivo* in tibialis anterior muscle of mice.

For this study we utilized *E. coli* b-galactosidase (LacZ), that has been used extensively as a reporter gene in a variety of systems, including bacterial, yeast, mammalian and insect cells. 5' azacytidine, inhibiting DNA methyltransferase, an house-keeping enzyme in mammalian cells, causes an increase of expression of b-galactosidase, both in quantity and duration.

Materials and Methods

Cell cultures and transfection

Murine muscle cell lines G8 and C2C12 were purchased from the American Type Culture Collection (Rockville, Maryland). Myoblasts were mantained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with fetal horse serum, at 37°C in a humified atmosphere of 5% CO_2 in air.

Transfection was performed on 60 mm diameter dishes at cellular confluence, 24 hours after plating.

The plasmids utilized were a reporter gene pCMVlacZ, where a cytomegalovirus promoter drives the expression of *E. coli* b-galactosidase, and pY3 that confers resistance to the toxin Hygromycin B [1].

Cells were transfected by the biolistic technique. Briefly, M10 tungsten microprojectiles with a diameter ranging from 0.7 to 1 :m were coated with plasmid DNA by a precipitation with 1.25 M $CaCl_2$ and 0.02 M free base Spermidine according to the method described [22]. DNA coated particles were loaded onto a Kapton membrane ("macrocarrier") in an ethanol suspension and allowed to dry. The macrocarriers were mounted in the biolistic device between the rupture disk and the stopping screen.

Cell dishes, at 90-95% confluence, 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 (Pounds per Square Inch). Pressure on the rupture disk was increased using compressed helium until its rupture; the resulting pressure shock wave propelled the macrocarrier against the stopping screen at high velocity. The stopping screen allowed only the DNA coated particles to pass through to hit the cellular monolayer placed in their trajectory.

30 cell dishes (60 mm diameter) of each cell line were shot for each experimental procedure, each plate was trypsinized after 5 hours from the bombardment and cells were dished to 4 plates (100 mm diameter).

After 10 days of selection with Hygromycin B (40 mg/ml), resistent clones (permanent transfection efficiency 1.3 $x10^{-3}$) were trypsinized and replated. Cells were then exposed to different concentrations of 5' azacytidine (5-10-25 mM) and analyzed after 1-2-4-5-10-15-20 days of treatment. The whole procedure was repeated twice for each cell line.

Histochemistry

Tranfected cells were rinsed twice in PBS, fixed in O.25% glutaraldehyde and then incubated in a O.5 mg/ml solution of X gal (5-bromo-4-chloro-3-indolyl-b-galactopyranoside) for 3 hours. The blue cells were identified and counted by phase-contrast light microscopy.

Southern Blot analysis of DNA methylation

Hpa II restriction enzyme that does not recognize the methylated sites but recognizes the unmethylated sites on the DNA sequence, and Msp I that recognize both the methylated and unmethylated sites were utilized for the detection of methylation pattern. The Hpa II or Msp I digests of the plasmid DNA or of the genomic DNA extracted from treated cells were electrophoresed, blotted and probed, according to the method described by Maniatis et al [16], with a ^{32}P labelled probe obtained by excision and purification of the CMV promoter of pCMVlacZ. The whole procedure was repeated twice.

Cell transplantation

The right Tibialis Anterior (TA) muscles of fourty eight C57B6L mice were injected with 40 ml of 0.75% bupivacaine (BVC) 24 hours prior to the transplant. Mice were immunosuppressed with daily intraperitoneal injections of Cyclosporine A at the dosage of 10 mg/Kg, from the day before the transplant to the day of sacrifice. 1 x 10^6 transformed G8 cells, in 100 ml PBS were injected in each TA muscle. Mice were anesthetized with an intraperitoneal injection of Ketamine (10 mg/Kg). Mice were sacrificed 5-10 and 15 days after the transplant of selected myoblasts and TA muscles were taken and frozen in liquid nitrogen cooled isopentane.

Treatment of mice with 5' azacytidine

Animals were divided into seven groups, defined by different concentrations of 5' azacytidine (Sigma); these were 0, 0.3 mg/g/day, 0.6 mg/g/day, 1.2 mg/g/day, 1.5 mg/g/day, 2.4 mg/g/day and 4.8 mg/g/day. Mice in each group were daily injected subcutaneously just above the TA muscle. The total volume injected was 100 ml.

Histological analysis

All tissues were frozen in liquid nitrogen cooled isopentane and sectioned in 12 mm thick transverse serial sections. Six cross sectional areas of the muscles taken in proximity of the intramuscular injection were examined, each comprising three 12 mm sections for histochemical detection of b-gal activity and three 10 mm sections for hematoxylin and eosin.

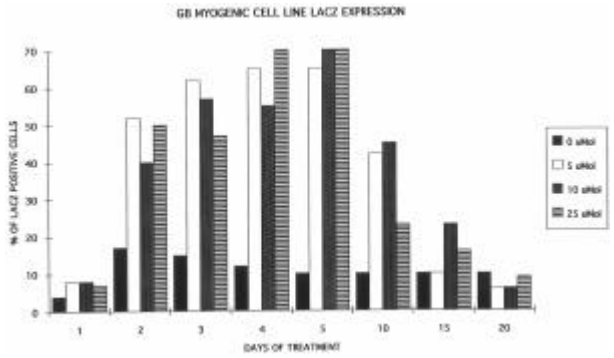

Figure 1. Percentage of LacZ positive G8 cells (myogenic cell line from mouse) as a function of 5' azacytidine concentration and days of the treatment.

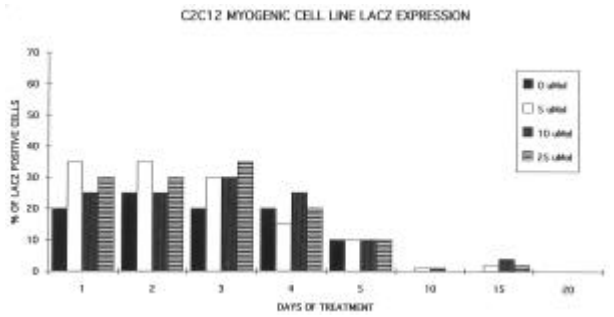

Figure 2. Percentage of LacZ positive C2C12 cells (myogenic cell line from mouse) as a function of 5'azacytidine concentration and days of the treatment.

Quantification of lac-Z staining

The cryostat sections were transferred to gelatin coated glass slides. The sections were then fixed by dipping the slides in a cold (4°C) solution of 0.25% glutaraldehyde in PBS (pH 7.4) for 15 minutes, rinsed twice for 5 minutes in PBS, and stained overnight at 37°C with X-gal at a concentration of 400 mg/ml in 5 mM $K_3Fe(CN)$, 5 mM $K_4Fe(CN)$, 1 mM $MgCl_2$ in PBS. The slides were rinsed using PBS and examined microscopically for the presence of b-gal-labeled ("blue") myofibers.

Results

The two myogenic cell lines, after 10 days of selection for Hygromycin B resistance and several days of treatment with 5' azacytidine, expressed permanently the exogenous gene at different levels, with a more striking effect on G8 cells. The best results for each treated cell line were: a seven fold increase of LacZ positive cells in comparison with not treated cells, in G8 murine myogenic cell line after 5 days of treatment with 10 and 25 mM 5'azacytidine (Figure 1); a 1.5 fold increase of

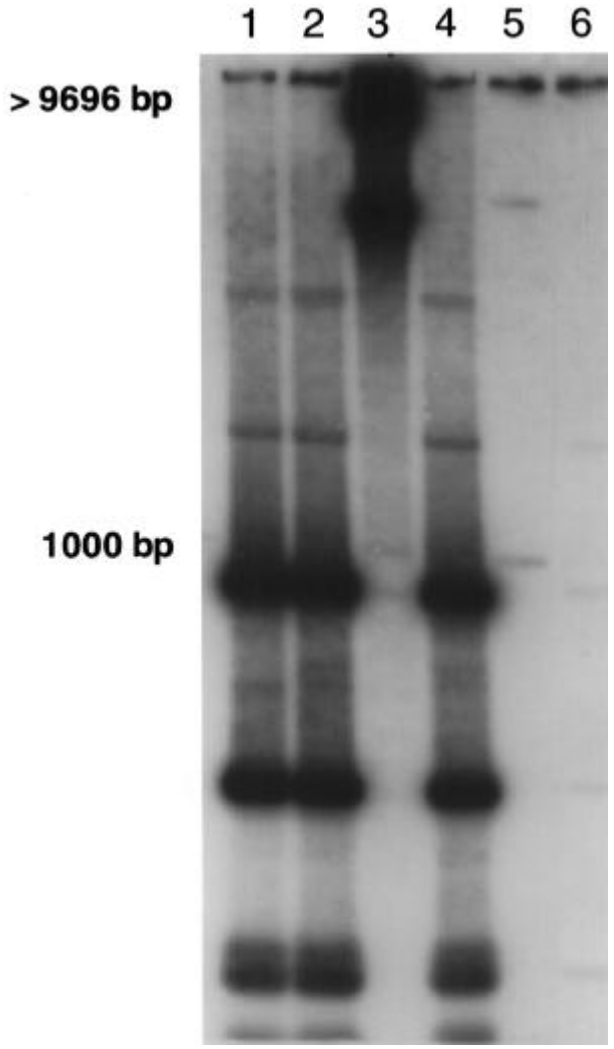

Figure 3. Southern Blot analysis of DNA methylation: Hpa II restriction enzyme that does not recognize the methylated sites but recognizes the unmethylated sites on the DNA sequence, and Msp I that recognizes both the methylated and unmethylated sites are utilized for the detection of methylation pattern. The Hpa II or Msp I digests of the plasmid DNA (lane 1-4) and of the genomic DNA (lane 5-6) extracted from treated cells were electrophoresed, blotted and probed with a ^{32}P-labelled probe obtained by excision and purification of the CMV promoter of pCMVlacZ. LANE: 1) naked pCMVlacZ digested with Hpa II; 2) naked pCMV-lacZ digested with Msp I; 3) in vitro methylated pCMV-lacZ digested with Hpa II; 4) in vitro methylated pCMV-lacZ digested with Msp I; 5) DNA extracted from G8 cells not treated with Aza-C digested with Hpa II; 6) DNA extracted from G8 cells treated for 96 hours with 5 mM Aza-C digested with Hpa II.

LacZ positive cells in C2C12 murine myogenic cell line after 3 days of treatment with 25 mM AzaC (Figure 2).

The enhancement of expression was directly derived from the inhibition of methylation as shown by the DNA restriction analysis (Figure 3). However, each cell line showed a mosaic of expression (Figure 4A, G8 cell line; 4B, C2C12 cell line), also in the treated samples.

Signs of cellular degeneration appeared in the plates after 10 days of treatment with different concentrations of 5'azacytidine; in particular C2C12 cells appeared to be more sensitive to the toxicity of the treatment rather than to the benefits of it on the expression of the exogenous gene.

It appeared too toxic the contemporary exposure of the cells to Hygromycin B and AzaC, in fact no Hygromycin resistant clones resulted after selection.

A mosaic of LacZ expression was observed in the tibialis anterior muscles of C57B6L mice after different period of daily treatment with distinct doses of 5' azacytidine.

At low doses (beetwen 0.6 and 1,5 mg/g/day), after 5 days of AzaC treatment a few number of b-gal positive fibers in muscle areas injected with trasformed G8 cells were found. Control muscles, injected with the same amount of G8 cells also showed some cellular clusters.

After10 days of treatment with AzaC, b-galactosidase expression in TA muscle was still evident, meanwhile no reaction was observed in the control group.

Animals treated with 2.4 and 4.8 mg/g/day 5' azacytidine showed after 5 and 10 days of treatment a greater enhancement of LacZ expression in comparison with those treated with different drug concentrations (Figure 5 A, B). In these conditions very few signs of drug toxicity were present. After 15 days treatment the b-galactosidase expression in TA muscle was diminished, more than half in comparison with the samples taken after 10 days of treatment. The blue fibers in the treated animals were polygonal with nuclear centralization (figure 5 A, B), meanwhile in the control group, where no blue fibers were detectable, the treated muscle showed only fully mature fibers.

Discussion

The DNA where 5'azacytidine substitutes for cytidine, remains hypomethylated; this mechanism could be responsible for maintenance of some genes in the active state and the induction of cell differentiation [5, 19, 11, 7].

The demethylation of genes such as MyoD1 is proba-

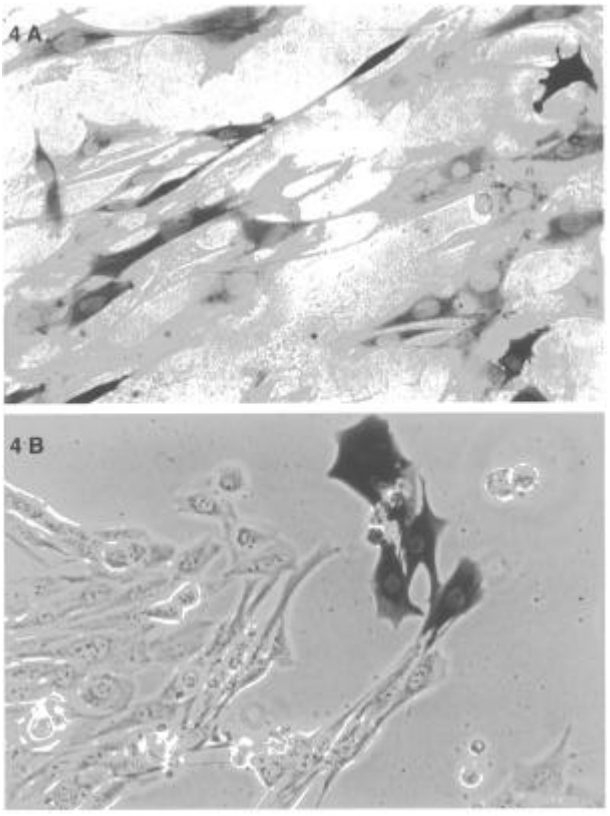

Figure 4A. X-gal stain on murine myogenic cell line G8 after 5 days of treatment with 10 mM 5' azacytidine (brigh light, 40X magnification). Figure 4B. X-gal stain murine myogenic cell line C2C12 after 3 days of treatment with 25 mM 5' azacytidine (phase contrast, 40X magnification).

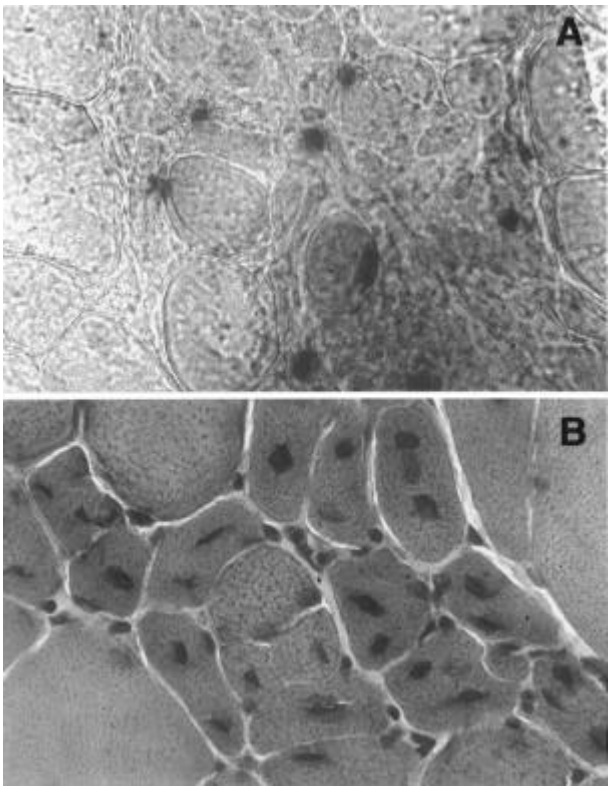

Figure 5. Photomicrographs of TA muscle after X-gal staining for LacZ expression (in blue) in the section: muscle treated with 2.4 mg/g/day 5' azacytidine for 10 days, after injection of transformed cells (5A: X gal staining, 250 X; 5B: Hematoxylin-Eosin100X magnification).

bly involved in the process of differentiation into fusing myotubes of not myogenic cell lines [4]; on the other hand the control of methylation of MyoD genes is a mechanism that ensures the inheritable quiescence of the gene in immortalized cultured cells [21, 24].

The increased number of G8 cells expressing b-galactosidase after treatment with 5 and 10 mM 5'azacytidine for 5 days (in addition to 8 days selection with Hygromycin) in comparison with G8 cells not treated with the drug, confirmed in myogenic cell lines, data previously reported in CHO cells, about an enhancement of expression of exogenous genes induced by demethylation [15]. Partial pCMV-LacZ integration in several Hygromycin resistant subclones may account for the observed variability in reporter gene expression in G8 cells. This hypothesis is also supported by the presence of a shorter pCMV-LacZ hybridizing band in Southern blot analysis of G8 transfected cells.

Our experiment showed a lower enhancement of the reporter gene in C2C12 cell line after treatment which is probably due to a different sensitivity to the drug in different cell lines [17]. No differences in cell morphology were observed in contrast to data reported by Parrow and coworkers [18].

The prolongation of the exogenous genes expression *in vitro* could be useful for facilitating muscle specific studies of gene expression, although it must be kept in mind that the AzaC effect varies between different cell types and probably between different species [12].

Jaenisch et al demonstrated that the drug 5'azacytidine injected into MOV 7 and MOV 10 mouse activates silent retroviral genomes. MOV 7 and MOV 10 mouse carry a Moloney murine leukemia provirus; the proviral genomes are highly methylated and are not expressed in animals. A single injection of the drug in newborn mouse induced transcription of the silent proviruses [8].

Keeping this in mind, we performed our *in vivo* study, with the aim of opening the way to different scenarios of enhancement and prolongation of exogenous genes expression driven by viral promoters.

We observed an enhancement of expression of b-gal in TA muscle treated with 2.4 and 4.8 mg/g/day 5'azacytidine for 10 days in comparison with the untreated ones. The most important information that we found was that it is possible to obtain a prolongation of exogenous gene expression up to one month *in vitro* and *in vivo*. Since the transplantation method used, the single intramuscular injection, has a low efficiency per se, it became important in our experiment to find blue fibers after certain lapse of time in AzaC treated mice, in comparison with transplanted mice not treated that appeared negative.

At concentrations of the drug above 4.8 mg/g/day, for 15 days of treatment, tissue was completely disorganized, and it was no longer possible to distinguish between myonuclei and infiltrating cells. As a consequence, the yield of the implantation in this condition was particularly low.

Treatment with concentration of 2.4 mg/g/day showed less degenerative changes, edema and stasis and did not interfere with fusion of myoblasts with regenerating fibers. Subcutaneous administration of 5'azacytidine in this range caused a prolongation of the expression of the reporter gene in muscle tissue up to 15 days of treatment (25 days after cell transfection), in comparison with untreated muscles, that appear completely negative. The treatment of the tissue confirmed, but did not reproduce at the same level, the results obtained in cells culture.

This observation could be explained by cellular scattering in the tissues after transplantation and by an incomplete immunosuppression.

Our results show that the employment of this molecule could help solving the problem of the exogenous genes shut-off that poses a serious limit to the development of gene therapy for muscular diseases both with viral and physical transfection methods.

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