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Limited diffusibility of gene products directed by a single nucleus in the cytoplasm of multinucleated myofibres

Tomoko Ono[a], Katsuhiko Ono[b], Kiminao Mizukawa[b], Tetsuya Ohta[a], Tomofusa Tsuchiya[a], Masaaki Tsuda[a,*]

[a]*Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-naka, Okayama 700, Japan*
[b]*Department of Anatomy, School of Medicine, Okayama University, Shikata-cho, Okayama 700, Japan*

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Abstract

Two types of β-galactosidase genes, whose products are distributed in the nucleus (Nβ-gal) or cytoplasm (Cβ-gal), were injected with fructose intramuscularly into the quadriceps of adult mice. Regionally restricted and overlapped distributions of both gene products were observed in the myofibres. These findings indicate that Nβ-gal is incorporated into the nucleus responsible for its synthesis and that Cβ-gal becomes located in the vicinity of the nucleus after its synthesis. This restricted location of Cβ-gal in myofibres remained unchanged during the development of infant mouse muscle. Thus, the gene products directed by the nucleus of myofibres seem to show limited diffusibility, suggesting a universal localization of subcellular domains in myofibres.

Key words: Gene transfer; Myofibre; Subcellular domain; β-Galactosidase; Gene therapy; Duchenne's muscular dystrophy

1. Introduction

Myofibres of skeletal muscles are multinucleated; in each myofibre hundreds of nuclei are evenly distributed throughout its length, each capable of producing mRNAs and muscle proteins which could be distributed throughout the cytoplasm of myofibre. However, in innervated adult muscle, the expression of the nicotinic acetylcholine receptor is highly restricted to the subsynaptic nuclei located near the motor endplates [1,2]. In addition, in fused heterokaryons between muscle and non-muscle cells muscle gene products are localized in the vicinity of the nuclei which are responsible for their syntheses [3]. Therefore, it is not certain whether muscle gene products synthesized in the extrasynaptic nuclei located apart from the motor endplates could freely diffuse throughout the cytoplasm of the myofibre or restrictedly in the vicinity of the corresponding nuclei. Elucidation of this point is important to understand subcellular domains in muscle fibers, which could be maintained by self-sufficient supply of gene products derived from individual nuclei within myofibres. In this study, we used direct gene transfer into the quadriceps of adult mice with several DNA mediators and observed the expression patterns of introduced reporter gene products in the myofibres.

*Corresponding author. Fax: (81) (862) 55-7456.

2. Materials and methods

2.1. Plasmid DNA injection and measurement of luciferase activity

A volume of 100 μl plasmid DNA solution (0.7 mg/ml) containing pRSVL [4], L7RHβ-gal [5] or pRSVβ-gal [6] was injected at 3 mm depth into the quadriceps of adult (7 weeks old) mice (ICR) by a microsyringe. On the indicated days, the quadriceps were removed, minced with scissors and homogenized in an Ultra-disperser (Yamato Co., Japan) with 400 μl of buffer A (0.1 M potassium phosphate (pH 7.8), 1 mM dithiothreitol (DTT), 0.1% Triton X-100) and the suspension was further homogenized by twenty strokes of a Dounce homogenizer. The homogenate was transferred to an Eppendorf tube with 200 μl of buffer A and freeze-thawed three times by cycles of cooling in liquid nitrogen for 3 min and incubation at 37°C for 2 min. The extract was centrifuged at 10,000 × *g* for 5 min and a 100 μl volume of the supernatants was used to measure luciferase activity. The muscle extracts (100 litres) were mixed with 500 μl buffer B (25 mM glycylglycine (pH 7.8), 5 mM ATP, 15 mM $MgSO_4$) and luciferase activities were measured in an LKB1251 luminometer after adding 100 μl of 1 mM luciferin. One unit of luciferase represents one count per mg protein measured with a luminometer.

2.2. β-Galactosidase staining

The entire quadriceps were removed on indicated days after the DNA injection, fixed with 2% formalin/0.2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) for 3 h and stored overnight in 20% sucrose solution in 0.1 M PBS. Serial longitudinal sections (140 μm) of frozen quadriceps were prepared by microtome with an electro-freezer. For detection of β-gal-positive cells, all the sections were treated with staining solution (84 mM phosphate buffer (pH 7.2), 1 mM $MgCl_2$, 3 mM potassium ferrocyanide, 3 mM pottasium ferricyanide, 0.1% Triton X-100, 0.02 % Nonidet P40 and 0.05% 4-Cl,5-Br,3-indolyl-β-galactoside (X-gal)] for 12–18 h in a dark-room, and sections containing positive cells were placed on gelatin-coated slides. After being embedded, the sections were photographed and the lengths of the β-gal-positive regions were measured. The lengths of the β-gal stained regions did not change during treatment of the sections with staining solution for at least 18 h (data not shown).

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2.3. Microscopic observation

After detection of β-gal positive cells by light microscopy, sections were post-fixed in 1% osmium tetroxide for 1 h, dehydrated in a graded ethanol series, and then embedded in Epon 812 (Oken, Japan). Ultrathin sections were cut and observed under a transmission electron microscope (JEOL, 1200EX) without electron staining.

3. Results and discussion

3.1. Expression of luciferase gene injected with several mediators

Direct injection of plasmid DNA into mouse skeletal muscles with a sucrose solution is effective for expressing reporter genes in myofibres for as long as 60 days [7]. Here we examined the ability of other reagents, glucose, fructose, sodium chloride (NaCl) and lipopolyamine, to act as delivery mediators and the time course of luciferase gene expression after injection of DNA into quadriceps of adult mice. As shown in Fig. 1, luciferase activity due to injection of a pRSVL plasmid DNA containing the firefly luciferase reporter gene [4] was detected with all these mediators as well as with sucrose for at least 60 days after injection at almost the same expression levels. The dose response effect of injected DNA was almost saturated at 70 μg DNA with 100 μl of each mediator solution (data not shown). Water and potassium chloride were not effective as DNA mediators (data not shown). Thus, glucose, fructose, NaCl and lipopolyamine as well as sucrose can be used as delivery mediators for the introduction of plasmid DNA into mouse skeletal muscles.

3.2. Distribution of β-galactosidase in myofibres

To determine the distribution of introduced gene products in myofibres, we used two types of DNA constructs: one was L7RHβ-gal [5] containing the *E. coli* β-galactosidase gene linked with SV40 enhancer-promoter and nuclear location-signal sequences of SV40 large T-antigen and the other was pRSVβ-gal [6] containing the gene linked with Rouse sarcoma virus (RSV) enhancer-promoter. As shown in Fig. 2A and C, histochemical staining for β-galactosidase activity in muscle longitudinal sections showed a nucleus-concentrated distribution of the L7RHβ-gal product (Nβ-gal) and a cytoplasmic distribution of the pRSVβ-gal product (Cβ-gal). In both cases, the positively stained regions were restricted in the myofibres, whose lengths varied from about 50 μm to 1 mm but mostly ranged between 200 and 500 μm (data not shown). As shown in Fig. 2A, the nuclei stained with Nβ-gal were evenly stained throughout the positively stained regions. In a few cases, however, only one stained nucleus located alone or apart from a group of stained nuclei was observed (Fig. 2B), suggesting that Nβ-gal directed by a single nucleus could not be transferred to the nearby nuclei.

This nucleus-concentrated distribution of Nβ-gal was further supported by transmission electron microscopic observations (Fig. 3). The precipitates formed by Nβ-gal activity were concentrated in the nucleus and its surrounding region (Fig. 3A and B). Two adjacent nuclei were evenly stained and no significant precipitate formed by Nβ-gal was observed between them (Fig. 3C). In addition, when a nucleus was poorly stained with β-galactosi-

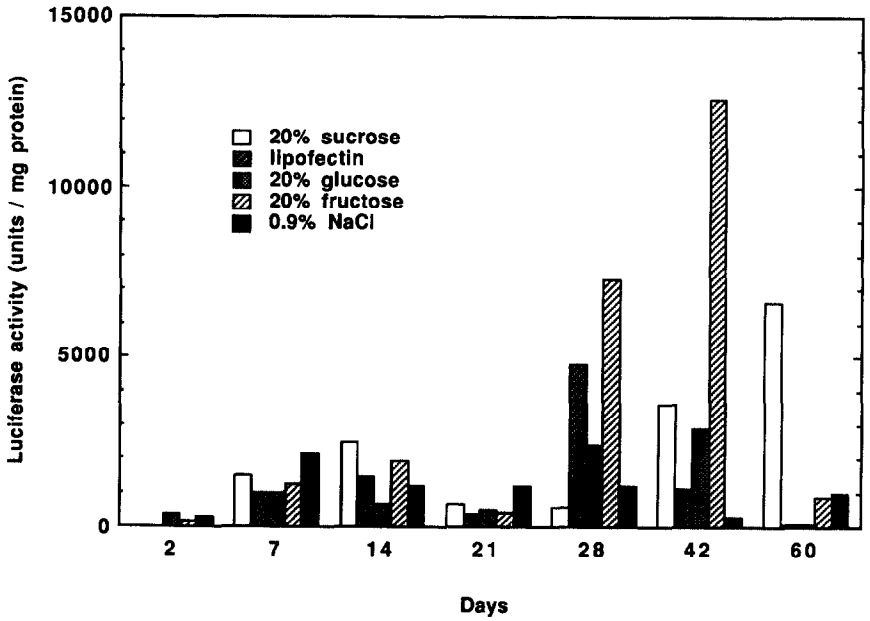

Fig. 1. Time course of change in luciferase activity after intramuscular injection of pRSVL plasmid DNA. Luciferase activity was measured on the indicated days after intramuscular injection of pRSV DNA with 20% sucrose, 20% fructose, 20% glucose, 0.9% sodium chloride or lipofectin (BRL) solution.

dase, much precipitate was visible in the vicinity of the nuclear membrane (Fig. 3D). In every case (Fig. 3A, C and D), the precipitates in the cytoplasmic region surrounding the nucleus were distributed with a gradient toward the nucleus. By contrast, the precipitates formed by Cβ-gal were distributed all over the myofibrils in the cytoplasmic region (Fig. 3E). These findings indicate that L7RHβ-gal plasmid DNAs were incorporated into each positively stained nucleus and the Nβ-gal synthesized was transported into the nucleus responsible for its synthesis before it diffused into the cytoplasm.

Co-injection of L7RHβ-gal and pRSVβ-gal DNA constructs showed three types of staining patterns which were detected in the nuclei, cytoplasmic regions or both. In the regions detected with Nβ-gal and Cβ-gal, the stained signals of nuclei and cytoplasmic regions mostly overlapped (Fig. 2D and E). Sometimes we observed heterogenously stained cytoplasmic regions with uneven locations of stained nuclei (Fig. 2E), probably due to unequal incorporation of either plasmid DNA into the nuclei in the β-gal positive regions. These findings suggest that the co-injected DNA constructs could be separately or concomitantly incorporated into the nuclei and Cβ-gal did not diffuse freely to the cytoplasm but became located in the vicinity of the nucleus responsible for its synthesis. However, the continuous staining of Cβ-gal positive regions (Fig. 2C) suggests that the distributions of Cβ-gal derived from neighboring nuclei could overlap.

3.3. Time-course of distribution of Cβ-gal

Next, we examined the time course of distribution of Cβ-gal after injection of pRSVβ-gal into adult mouse quadriceps. As shown in Fig. 4A and B, the lengths of the Cβ-gal-positive regions detected 2 days and 3 weeks after injection mostly ranged between 200–500 μm. When pRSV β-gal was injected into 2-week-old mice (Fig. 4C), no tendency for extension of the distribution of Cβ-gal-positive regions was observed even 4 weeks after the injection. These findings indicate that the distribution of the products of the introduced gene directed by individual nuclei did not change with the time after injection or development of the muscle.

Thus, the diffusibility of gene products of each nucleus in myofibres seems to be limited. The nuclear proteins of myofibres may, in principle, be self-sufficiently supplied by the nucleus responsible for their syntheses, while cytoplasmic proteins derived from a single nucleus of a myofibre diffuse to a limited extent in the cytoplasm and their distributions overlap those of the products directed by neighboring nuclei. This limited diffusibility of gene products in myofibres seems to correspond to the nuclear domain observed with fused heterokaryons between

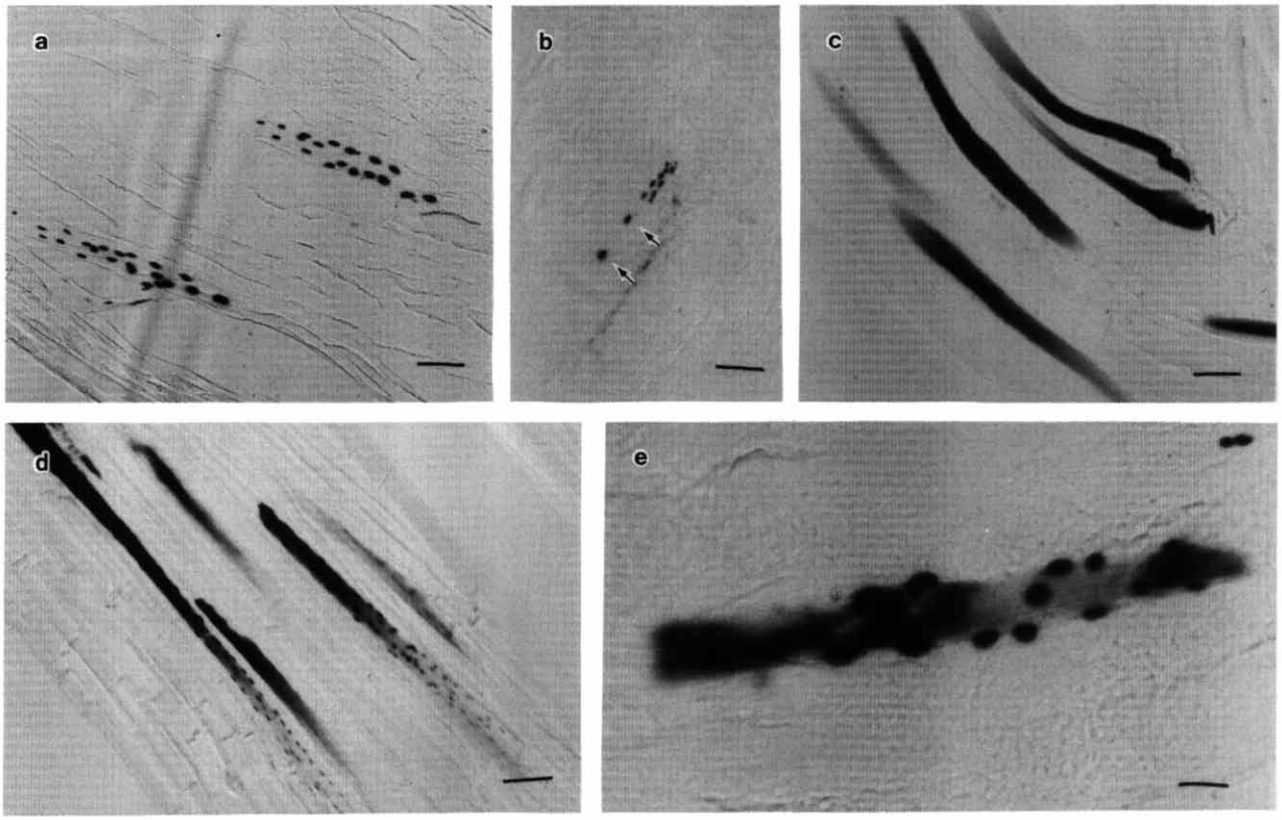

Fig. 2. Detection of *E. coli* β-galactosidase activities in muscle cells by in situ cytochemical staining. A dose of 70 μg of L7RHβ-gal (A and B), 70 μg of pRSVβ-gal (C) or 70 μg of L7RHβ-gal plus 70 g of pRSVβ-gal (D and E) plasmid DNA was injected into the quadriceps with 20% fructose solution. The entire quadriceps were removed 7 days later and subjected to in situ cytochemical analysis. Arrows (B) indicate the nuclei located apart from a group of stained nuclei. Bars = 100 (A–D); and 25 (E) μm.

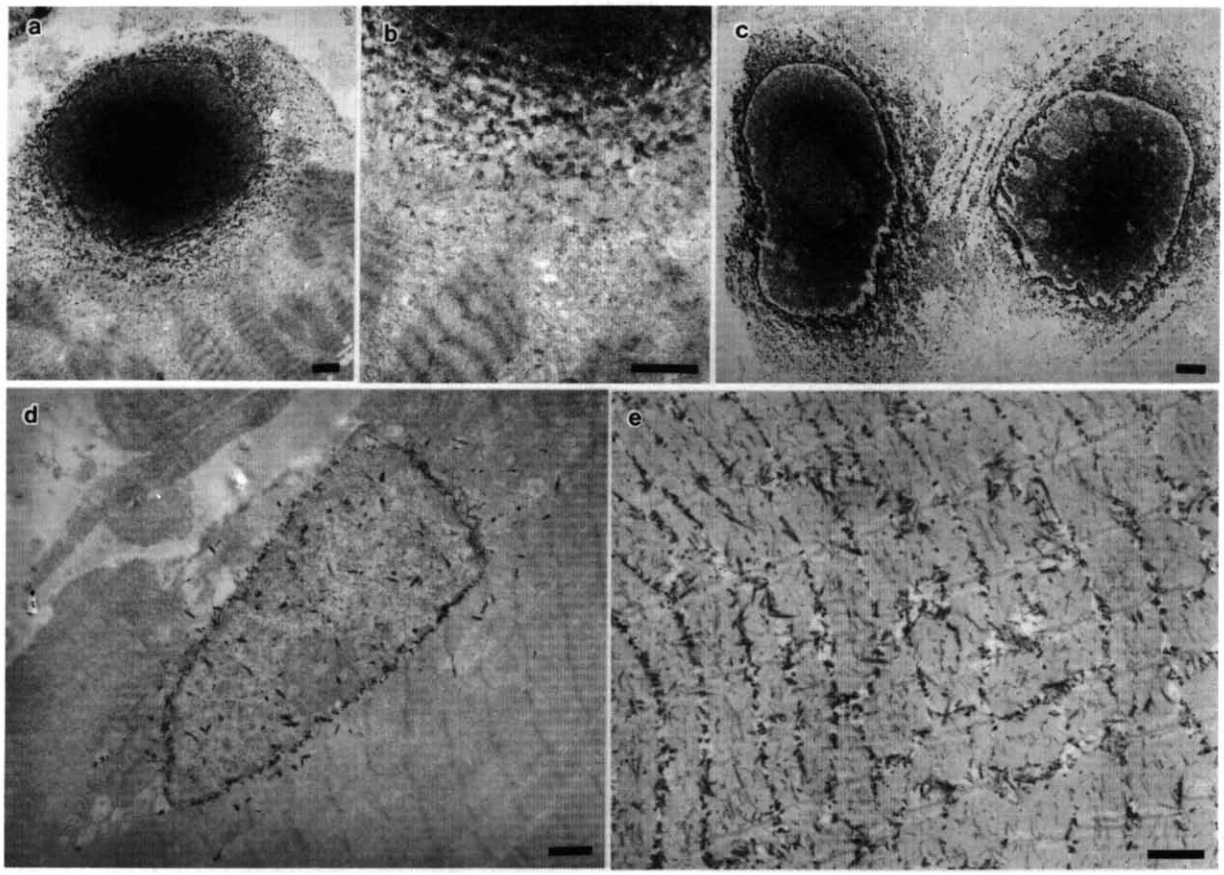

Fig. 3. Electron microscopic appearance of β-gal-positive nuclei and cytoplasm of myofibres. L7RHβ-gal (A–D) or pRSVβ-gal (E) plasmid DNA was injected into the quadriceps of adult mice and the entire quadriceps were removed 7 days later. Bars = 1 μm.

muscle and non-muscle cells in culture [3], where organella, membrane and muscle structural proteins remained localized in the vicinity of the nucleus responsible for their syntheses. Thus, some subcellular domain centered by a nucleus may be maintained even in multinucleated myofibres of skeletal muscles. This finding must be taken into consideration in developing a therapeutic method for Duchenne's muscular dystrophy (DMD) by transfer of the normal dystrophin gene into diseased muscle [8,9], because the diffusibility of muscle gene products throughout the cytoplasm of myofibres is in principle necessary for gene therapy of DMD.

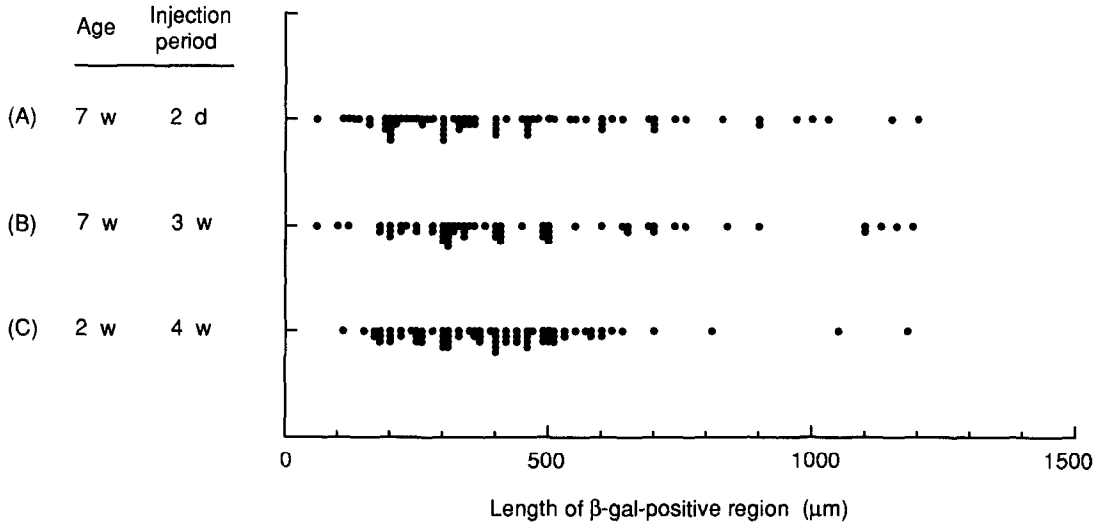

Fig. 4. Distribution of lengths of Cβ-gal-positive regions. pRSVβ-gal plasmid DNA was injected into the quadriceps of 7- (A and B) or 2- (C) week-old mice. The entire quadriceps was removed 2 days (A), 3 weeks (B) or 4 weeks (C) later and stained histochemically. After photography of β-gal-positive sections, the lengths of the Cβ-gal-positive regions were measured and plotted.

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