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Genes Transferred by Retroviral Vectors into Normal and Mutant Myoblasts in Primary Cultures Are Expressed in Myotubes

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Retroviral vectors were used to transfer genes efficiently into rat and dog myoblasts in primary cultures under conditions which permitted the transduced myoblasts to differentiate into myotubes expressing the transferred genes. The transduced myotubes expressed normal markers of differentiation and were morphologically indistinguishable from uninfected myotubes. Retroviral vector-mediated gene transfer was also used to correct a genetic enzyme deficiency in mutant canine muscle cells.

During histogenesis, multinucleated muscle cells form by fusion of mononucleated myoblasts. This process can be reproduced in vitro, where explanted myoblasts fuse and differentiate into myotubes (12). Gene transfer studies in this system are of interest for analyzing the regulatory events associated with formation of the contractile apparatus (13). In addition, certain metabolic genetic diseases involving muscle cells are potential candidates for somatic-cell gene transfer as a therapeutic approach to correcting the specific defect (14). Retroviral vectors, which have been used to transfer genes into other cell types, appear to be useful for such studies, since the transferred genes become stably integrated into the host cell genome (6).

Myoblasts are likely to be good target cells for retroviral vector-mediated gene transfer because they undergo mitosis, an apparent requirement for retroviral vector integration (6). Retroviral vectors have been used for gene transfer into a transformed muscle cell line (9), but for many studies of muscle differentiation in vitro, whole muscle tissue is used to establish primary cultures of myoblasts (1). In these preparations, the concentrations of myoblasts affect the ability of the cells to fuse and form differentiated myotubes (1). Thus, a foreign gene must be transferred into a high percentage of primary myoblasts at the initiation of an experiment to adequately assess the effects on subsequent expression during differentiation. Retroviral vectors appear suitable for such studies, since they can infect a high percentage of target cells in culture (6).

In this study, retroviral vectors were used to transfer the bacterial neomycin phosphotransferase (*neo*) gene into primary cultures of muscle cells from rats and dogs. The infected myoblasts proliferated, fused, and developed into myotubes in the presence of G418. G418-resistant myotubes and uninfected myotubes had the same characteristic features of fully differentiated myotubes, such as multinucleation, cross striations, active contraction in tissue culture, and expression of myosin heavy chains (MHCs). A retroviral vector was also used to introduce a β-glucuronidase cDNA into canine myoblasts deficient in this enzyme. These cells also differentiated normally in the presence of G418, and the resultant myotubes expressed β-glucuronidase enzymatic activity.

Rat myoblasts were obtained from hind-limb muscles of 19-day-old fetal Sprague-Dawley rats and cultured as previously described (3). The culture technique for the canine muscle cells was modified from that used to culture the rat cells. Briefly, myoblasts were obtained postmortem from the quadriceps and triceps of young dogs, including a dog affected with β-glucuronidase-deficient mucopolysaccharidosis type VII (MPS VII) (7). The samples were minced and dissociated by gentle agitation with 4 mg of Collagenase D (Boehringer Mannheim Biochemicals) per ml in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution for 2 h at 37°C. Large debris was removed by low-speed centrifugation (20 × *g*) for 2 min. The cells remaining in suspension were pelleted by centrifugation (200 × *g*) for 5 min and washed three times with Hanks balanced salt solution. The cells were cultured in Dulbecco modified Eagle medium containing 4.5 g of glucose per liter, penicillin, streptomycin, amphotericin B, and 10% horse serum. The cells were seeded onto collagen-coated cover slips in plastic culture dishes at a density of 10^5 cells per cm^2 and incubated at 37°C in a tissue culture incubator (5% CO_2). In both rat and dog cell cultures, bipolar cells were evident in 1 to 3 days, myotube formation began in 3 to 6 days, and fully formed myotubes in which striations and spontaneous contractions were observable by light microscopy were apparent in 8 to 11 days.

The N2 retroviral vector (2) was used to transfer the bacterial *neo* gene to the cultured muscle cells. The NTK-BGEO-1 retroviral vector (15) was used to transfer the β-glucuronidase (*gus*) gene. This vector contains a rat *gus* cDNA (11) driven by the thymidine kinase promoter inserted in the sense orientation into the *Xho*I site of the N2 vector (Fig. 1) (15). Each vector was converted to amphotropic vector virus in the PA-317 packaging cell line (10). Vector virus was harvested by incubating subconfluent cultures of packaging cells in growth medium (approximately 10^6 cells per ml) for 12 h. The medium was filtered (pore size, 45 μm) and stored at −70°C until use. Each of these supernatants contained 2×10^6 CFU of vector virus per ml, as determined by G418 resistance in NIH 3T3 cells (15).

Muscle cells were seeded in culture at 10^5 cells per cm^2. The next day the cells were infected by incubating them at 37°C for 2 to 4 h in medium containing 10^6 CFU of vector virus and 8 μg of Polybrene per ml. The vector-containing medium was removed, culture medium was added, and the cells were incubated at 37°C. The next day, and every 2 days thereafter for 8 to 11 days, the culture medium was changed to medium containing 0.8 mg of G418, a toxic analog of neomycin to which cells containing the *neo* gene are resis-

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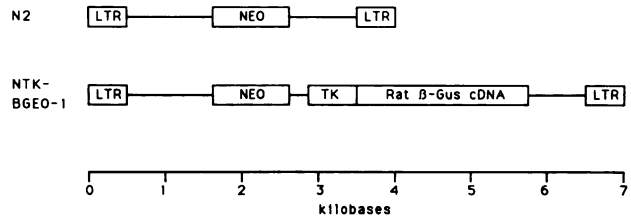

FIG. 1. Structures of retroviral vectors used for gene transfer to muscle cells. The NTK-BGEO-1 vector (15) was constructed from the N2 vector (2). LTR, Long terminated repeat; β-Gus, β-glucuronidase; TK, thymidine kinase promoter.

tant, per ml. The cells in uninfected control cultures from each species failed to proliferate or fuse and eventually died when cultured in this concentration of G418 (data not shown).

The rat and the dog muscle cells that were exposed to the N2 vector virus proliferated, fused, and developed into multinucleated myotubes in the presence of G418 (Fig. 2B and Fig. 3B). This indicated that the cells were infected with the vector and were expressing the *neo* gene. Equal numbers of cells were seeded in infected and uninfected cultures. In the infected cultures, the number of cells surviving in the presence of G418 was greater than 95% of the number of cells in infected control cultures grown in normal medium, indicating that most of the cells exposed to the vector viruses had become infected. Myoblast proliferation and differentiation in the infected cultures exposed to G418 was 1 to 2 days behind that in uninfected cultures, a phenomenon which also occurs with fibroblasts. Infected myoblasts were also maintained in a proliferative state by passing them at low density (approximately 5×10^4 cells per cm^2) in high-serum (20%) medium. The ability of these cells to differentiate into myotubes was maintained through at least 20 doublings in the presence of G418.

After myotubes began to form, striations became visible through phase-contrast light microscopy. This appearance, which is characteristic of fully differentiated myotubes, results from the expression and assembly of myofibrillar proteins. These myotubes spontaneously contracted and relaxed in the culture dish, indicating that the contractile apparatus was functional. The cultures were fixed, and the presence of embryonic MHCs (E-MHCs) was demonstrated immunohistochemically by using the E-MHC-specific 2B6 monoclonal antibody (5). In vitro, myosin is expressed only after myoblasts become postmitotic (12). In the mature myotube, myosin is incorporated into cross-striated myofibrils. The pattern of E-MHC expression in the vector virus-infected rat cells (Fig. 2D) was identical to the pattern seen in the uninfected control cells (Fig. 2C). The fibroblasts in the cultures did not express E-MHC. The 2B6 anti-rat antibody also detected expression of MHCs in both the infected and uninfected canine myotubes (not shown).

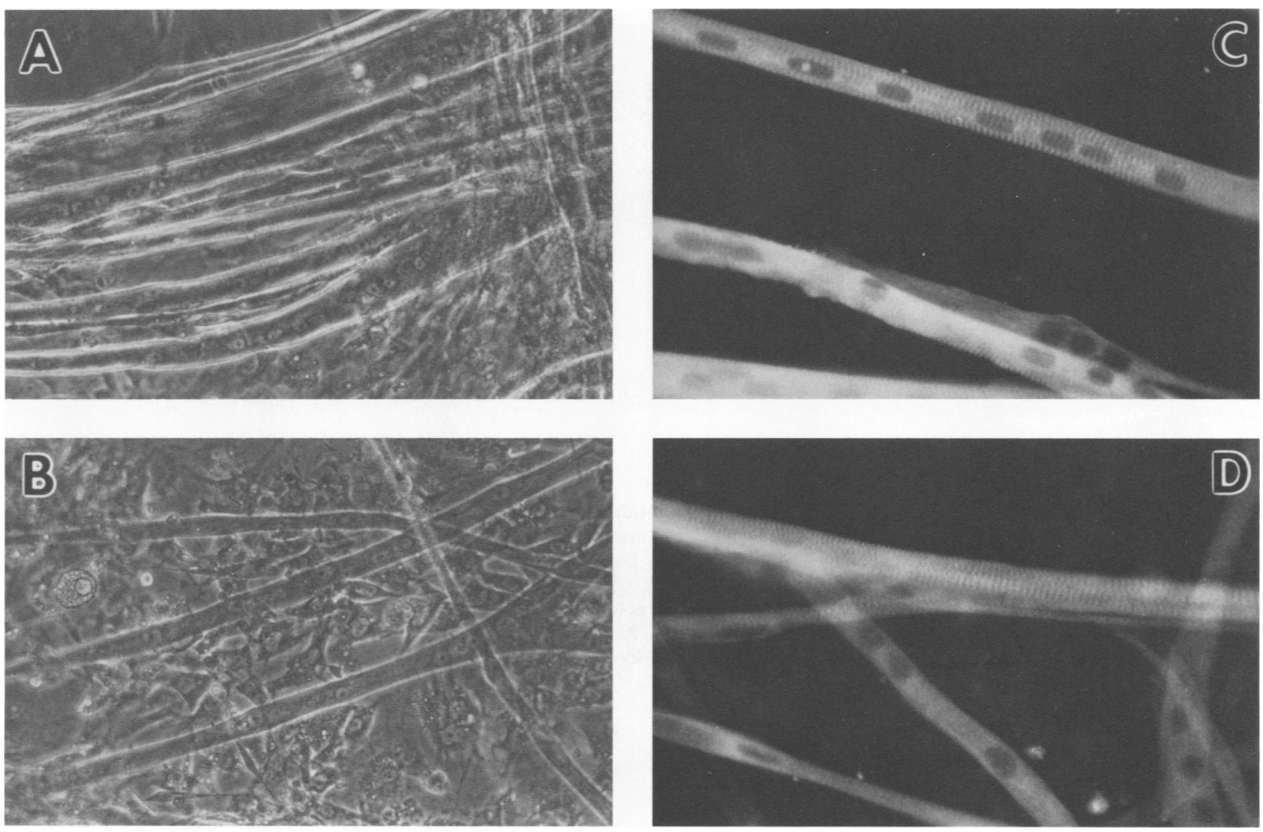

FIG. 2. Expression of retroviral vector-transferred *neo* gene in primary cultures of rat muscle cells. (A and B) Phase-contrast photographs of the cultured cells, showing multinucleated myotubes which developed from explanted myoblasts. (C and D) Cultures were fixed and stained by fluorescent immunohistochemistry with the 2B6 monoclonal antibody to E-MHC (5). Note the cross striations. (A and C) Uninfected cells cultured in normal medium; (B and D) cells that differentiated from myoblasts infected with N2 vector virus and were cultured in medium containing G418. Magnification, ×250 (all panels).

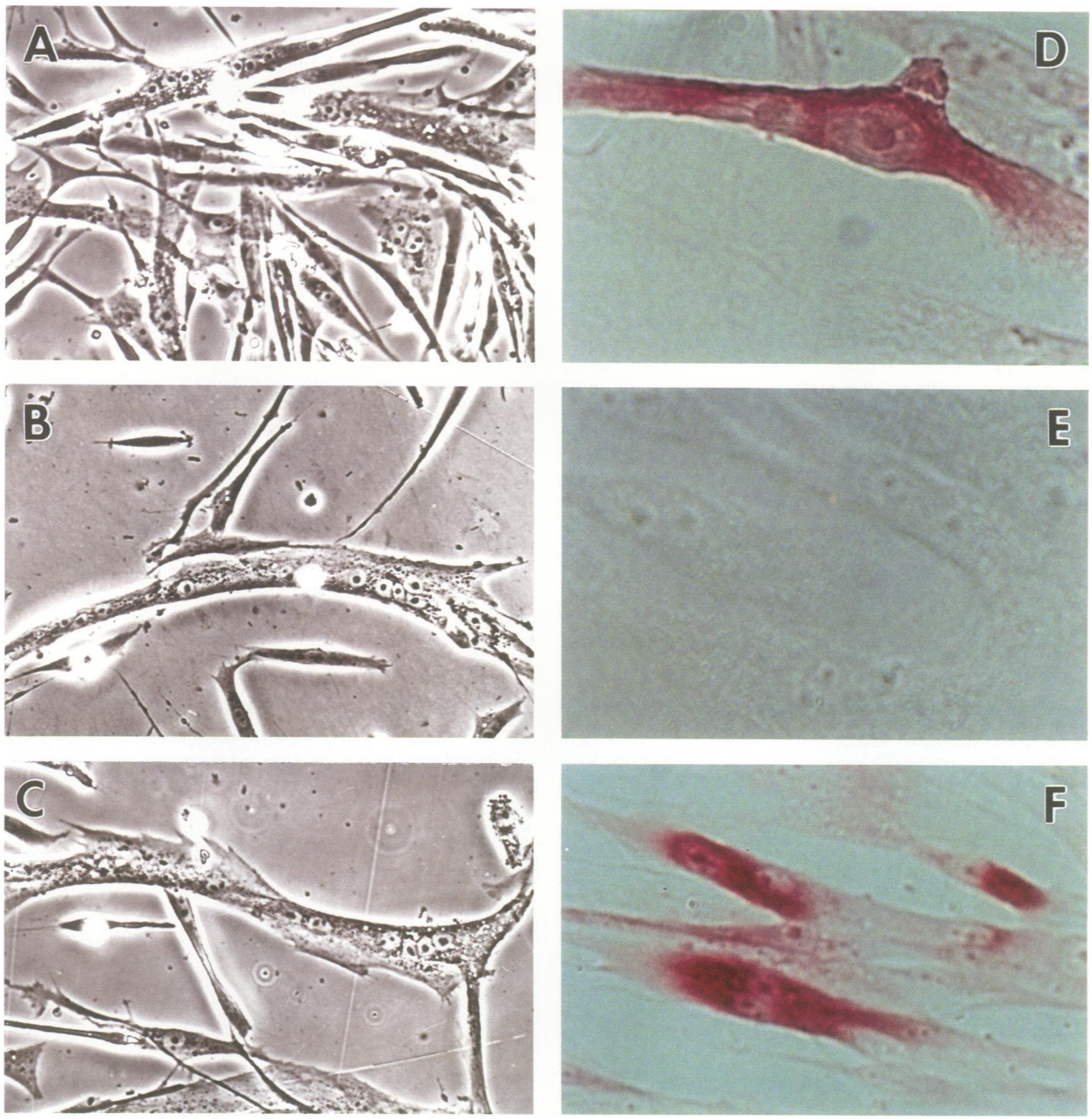

FIG. 3. Correction of β-glucuronidase deficiency in canine myotubes by retroviral vector transfer of a rat *gus* cDNA into MPS VII myoblasts. (A through C) Phase-contrast photographs of the cultured cells, showing multinucleated myotubes which developed from explanted myoblasts. (D through F) Cells were fixed and stained for β-glucuronidase activity by using hexazotized pararosaniline to detect cleavage of the naphthol-AS-BI-β-D-glucuronide substrate (4, 8, 15). (A) Uninfected MPS VII muscle cells; (D) uninfected normal muscle cells; (B, C, E, and F) MPS VII muscle cells that developed from vector-infected myoblasts grown in medium containing G418; (B and E) cells infected with N2 vector virus; (C and F) cells infected with NTK-BGEO-1 vector virus. Positive staining for β-glucuronidase activity is red (D and F) and can be seen in all of the cells, whereas the negatives are completely unstained (E). Magnifications, ×250 (A through C and F) and ×350 (D and E).

The canine MPS VII myoblasts were used as a model system to determine if vector-mediated gene transfer into myoblasts could be used to correct a genetic deficiency in differentiated myotubes. The MPS VII myoblasts were infected with the N2 vector, containing only the *neo* gene, or with the NTK-BGEO-1 vector, which has been shown to express normal levels of β-glucuronidase activity in other canine and human MPS VII cells (15). The vector-infected myoblasts proliferated, fused, and differentiated into myotubes in the presence of G418 (Fig. 3B and C), indicating that vector transfer had occurred. By enzyme activity determinations, β-glucuronidase was expressed in the cultures infected with the NTK-BGEO-1 vector (data not shown). However, the cultures contained a mixture of fibroblasts, myoblasts, bipolar cells, and myotubes. Therefore, to determine if β-glucuronidase was expressed in the differentiated myotubes, histochemical staining for β-glucuronidase activity was performed as previously described (4, 8, 15). Normal

canine myotubes stained positive (Fig. 3D), and uninfected MPS VII myotubes grown in normal medium stained negative (not shown). In the infected MPS VII myotubes grown in the presence of G418, the cells infected with N2 stained negative (Fig. 3E) and the cells infected with the NTK-BGEO-1 vector stained positive for β-glucuronidase activity (Fig. 3F). This demonstrated that, after vector-mediated transfer into myoblasts, the *gus* minigene was expressed in the mutant cells.

Our experiments indicated that retroviral vectors can be used to efficiently introduce genes into primary myoblasts. The stably transduced myoblasts can fuse and differentiate into apparently normal myotubes which express the vector-transferred genes. These results indicate that retroviral vector-mediated transfer of muscle structural protein genes, including genes with specific mutations, may be a feasible approach for studying the functional role of specific domains in contractile proteins. In view of this, we went on to show that a metabolic genetic defect could be corrected by vector transfer of a minigene into myoblasts. The product of the transferred gene was present after the infected myoblasts fused and differentiated into myotubes. Thus, it may be possible to use this method to genetically correct myoblasts in vitro and transfer them to muscles in vivo as an approach to the treatment of inherited metabolic myopathies (14). It should be noted that the size of foreign sequences that can be transferred by retroviral vectors is limited to a few kilobases at most (6). Thus, only diseases involving small genes, such as encode many enzymes, would be candidates for this approach.

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