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Adenovirus as an expression vector in muscle cells *in vivo*

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ABSTRACT **Attempting gene transfer in muscle raises difficult problems: the nuclei of mature muscle fibers do not undergo division, thus excluding strategies involving replicative integration of exogenous DNA. As adenovirus has been reported to be an efficient vector for the transfer of an enzyme encoding gene in mice, we decided to explore its potential for muscle cells. Advantages of adenovirus vectors are their independence of host cell replication, broad host range, and potential capacity for large foreign DNA inserts. We constructed a recombinant adenovirus containing the β-galactosidase reporter gene under the control of muscle-specific regulatory sequences. This recombinant virus was able to direct expression of the β-galactosidase in myotubes *in vitro*. We report its *in vivo* expression in mouse muscles up to 75 days after infection. The efficiency and stability of expression we obtained compare very favorably with other strategies proposed for gene or myoblast transfer in muscle *in vivo*.**

The eventual correction of muscle diseases, and especially Duchenne muscular dystrophy (DMD), raises very difficult problems. As much as 50% of DMD cases are sporadic (1) and thus cannot be prevented by current genetic counseling and prenatal diagnosis approaches; this frequency is even higher in mitochondrial myopathies (2). It is thus necessary to actively explore therapeutic strategies. The main problems to overcome are the large number of muscles to be treated and the nondividing nature of mature muscle cells. Moreover, the size of the sequences coding for dystrophin [11,056 base pairs (bp)] is incompatible with most vectors used for gene transfer; in addition, dystrophin is an intracellular protein thought to have a structural role in the muscle cells, whereas most of the current gene therapy approaches concern circulating proteins such as α_1-antitrypsin (3), or diseases due to an enzymatic defect, where a very partial restoration of the activity might be sufficient, such as adenosine deaminase (4).

Two strategies have been proposed to date: myoblast transplantation (5) and direct DNA injection (6). We decided to explore a third route using adenovirus as a vector for gene expression in muscle cells. Such a vector has already been used to correct postnatally an ornithine transcarbamoylase deficiency in mouse (7, 8) and more recently to direct the expression of α_1-antitrypsin in the rat lung epithelium (9).

The advantages of adenovirus include (*i*) a large host range, thus allowing the use of the animal models available for DMD (the *mdx* mouse and the *CXMD* dog), (*ii*) a low pathogenicity in man, (*iii*) a capacity for foreign DNA in principle compatible with the size of the dystrophin mRNA [the properties of adenovirus should allow the insertion of >30 kilobases (kb) of exogenous DNA, provided that a helper virus is used to propagate the recombinant virus], and (*iv*) the possibility to obtain high titers of virus, which is important for *in vivo* applications.

Our results suggest that adenovirus can be used to direct efficient gene expression in myotubes derived from rodent myogenic cell lines as well as in mouse muscle.

MATERIALS AND METHODS

Cell Lines, Cell Transfection, and Virus. Monolayer cultures of 293 cells (10) were propagated in minimum essential medium (MEM) supplemented with 10% fetal calf serum. The C2.7 (11) and the L6 myoblast cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) with 20% fetal calf serum. Fusion of C2.7 myoblasts was induced when cells were confluent by lowering the serum to 2%. The induction medium used for the L6 cell line was DMEM with 1% FCS and 10 μg of insulin per ml.

Transfections were performed using the calcium phosphate method (12). Adenovirus stocks were prepared by infection of 293 cells. Cells and media were harvested 25–30 hr after infection, and virus was released by three cycles of freezing and thawing. The dl 324 mutant of adenovirus type 5 contains a deletion of E3 (78.5–84.7 map units) and of E1 (nucleotides 1334–3639).

Animals. Mice were F_1 individuals of the C57B6 × SJL strain. Injections (10–20 μl of the viral suspension) were performed in the thigh of the animals either newborn, 1 week old, or 4 weeks old, at a single site.

Construction of Plasmid pMβgal. Plasmid pMβgal is derived from plasmid pCH110 (13). It contains the first 454 nucleotides of adenovirus 5, followed by an enhancer fragment of the mouse myosin light chain 1/3 locus. This fragment was obtained by screening a plasmid containing the 3′ end of the mouse $MLC_{1/3F}$ gene with a rat probe (14). An 800-bp fragment that was isolated and sequenced was found to have >90% homology with the rat sequence and displayed at least a 30-fold enhancement, which was myotube specific, with the homologous promoter and a heterologous thymidine kinase promoter (S.T. and C. Biben, unpublished results). A fragment equivalent to nucleotides 387–680 of the rat sequence was amplified by PCR before cloning into the plasmid pMβgal. A mouse skeletal α-actin gene promoter, nucleotides −682 to +127 of the published sequence (15), was added between the enhancer and the β-galactosidase sequences. The poly(A) signal is from simian virus 40 (*Hpa* I–*Bam*HI fragment), and, finally, nucleotides 3328–6241 of adenovirus 5 (including sequences coding for peptide IX) were added downstream of the poly(A) signal.

Assay for β-Galactosidase Activity. β-Galactosidase activity in cell lines was assayed as described using histochemical methods (16) or a colorimetric assay (17). For the *in vivo*

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Abbreviations: DMD, Duchenne muscular dystrophy; ITR, inverted terminal repeat.

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experiments, muscle samples were fixed and β-galactosidase activity was revealed as described (16).

RESULTS

Construction of a Recombinant Adenovirus. A recombinant adenovirus was designed to direct the muscle-specific expression of β-galactosidase. It was constructed via homologous recombination *in vivo* between the dl 324 mutant of adenovirus 5 and the plasmid pMβgal. The dl 324 mutant is deleted for two segments of the viral genome (≈5 kb total) that contain the E1 and the E3 regions. E3 is not necessary for viral growth, and the absence of E1 can be transcomplemented by the use of 293 cells, which harbor the E1 region of adenovirus 2. About 7 kb of exogenous DNA may be inserted without interfering with encapsidation of the recombinant genome.

Plasmid pMβgal contains sequentially the 5′ inverted terminal repeat (ITR), which is necessary for replication, and adjacent packaging sequences of adenovirus 5, the β-galactosidase gene of *Escherichia coli* under the control of a mouse α-actin gene promoter fragment reinforced by a muscle-specific enhancer sequence from the mouse $MLC_{1/3F}$ gene (myosin light chain), a poly(A) signal from simian virus 40, and sequences coding for polypeptide IX of adenovirus 5 with 3′ flanking sequences. The presence of polypeptide IX is necessary for encapsidation of a DNA with a size up to 104% that of the wild-type viral genome (18); it is absent from the dl 324 mutant. Initial experiments had shown that a recombinant adenovirus containing only the α-actin promoter fragment as a muscle-specific regulatory sequence was not able to direct efficient expression in myotubes in culture.

The linearized plasmid was cotransfected together with the large *Cla* I fragment of dl 324 DNA in 293 cells, which contains all of the viral genome except for 914 bp at the left end. Restriction analysis of the DNA content of the plaques obtained allowed us to isolate the recombinant adenovirus (Fig. 1); 8 of the first 10 plaques analyzed corresponded to the expected recombinant virus.

Expression of β-Galactosidase in Myogenic Cell Lines. We have tested the expression of β-galactosidase in two rodent myogenic cell lines infected with the recombinant virus. The mouse C2.7 myoblast line (11) can be induced to fuse into myotubes when confluent, by lowering the serum level in the culture medium. The cells were infected for 24 hr at various times before or after induction of fusion. Very little β-galactosidase activity was observed before fusion (however, occasional blue-stained mononuclear cells were observed by histochemical staining), whereas strong expression was detected when virus was added at the time of induction or up to 48 hr after it (further time points could not be obtained as the myotubes tend to detach from the plate, independent of virus infection) (Fig. 2). In similar experiments, high expression was also observed in the rat L6 myoblast line infected at 0, 24, or 48 hr after induction of fusion. As in both culture systems, a majority of myotubes is present 48 hr after the beginning of fusion, our experiments suggest that nondividing myotubes can be infected efficiently. In contrast, no β-galactosidase activity was detected in infected 3T3 mouse fibroblasts, confirming the muscle specificity of the regulatory sequences used.

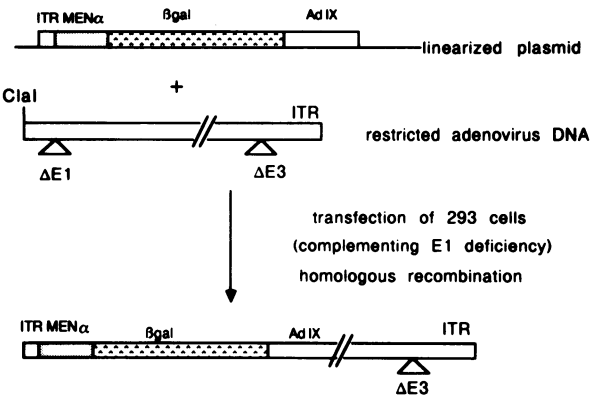

FIG. 1. Isolation of a recombinant adenovirus. MEN, enhancer of the mouse $MCL_{1/3F}$ gene; α, mouse skeletal α-actin promoter; βgal, β-galactosidase gene of *E. coli*; AdIX, sequences coding for peptide IX of adenovirus, with 3′ flanking region (nucleotides 3328–6241 of adenovirus 5); ITR, ITR and packaging sequence (nucleotides 1–454 of adenovirus 5).

Expression of β-Galactosidase in Muscle of Infected Mice. To test whether the adenovirus construct is able to direct β-galactosidase expression in muscle, we injected 10^8 plaque-forming units into the thigh of either newborn, 1-week-old, or

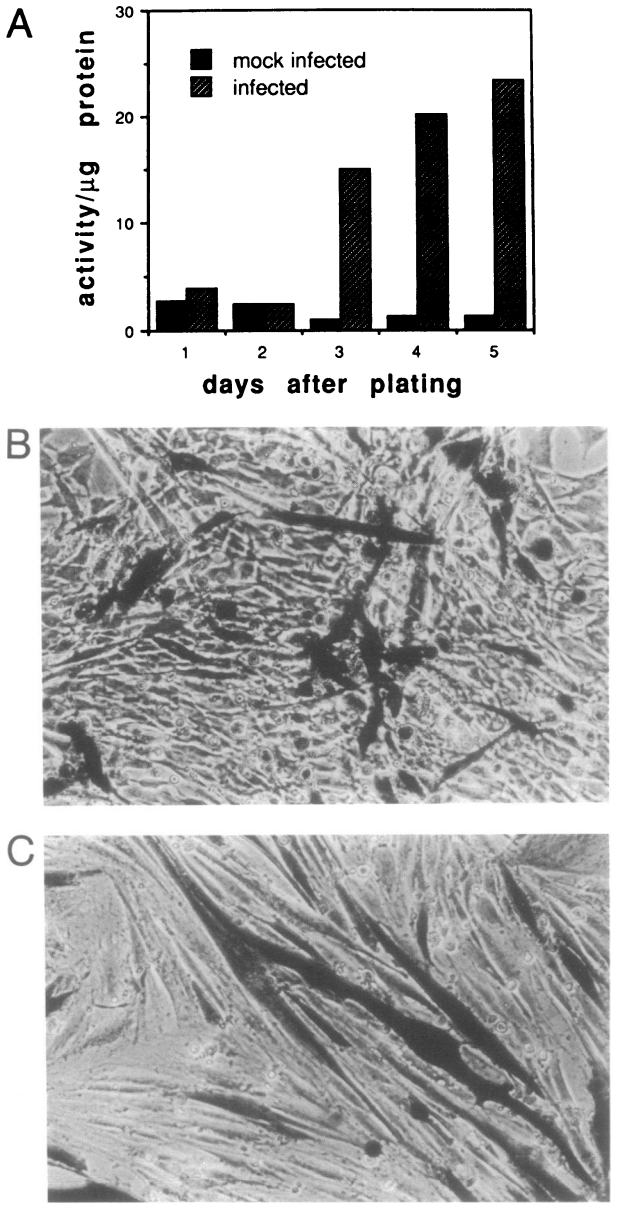

FIG. 2. Expression of β-galactosidase in myogenic cell lines. (*A*) Cells were infected at different stages and β-galactosidase was assayed by a colorimetric method. The fusion medium was added after day 2. (*B* and *C*) C2-7 myotubes (*B*) and L6 myotubes (*C*) infected 2 days after addition of the fusion medium.

Table 1. Expression of β-galactosidase in muscle of infected mice

Age at infection	Time of assay after injection, days	Number of mice			
		0	+	++	+++
1 day	5				3
	7		1		2
	10–15	1	1		5
	20–25	1	2		
	40	1			
	60–65	3	3	1	2
	75		3	2	4
1 week	25	1	1		
	48			1	
	53	2		2	
4 weeks	7				4
	14				4
	40	4			
	52	3		1	

+, 1–10 positive fibers; ++, 11–30 positive fibers; +++, >31 positive fibers.

4-week-old mice. Very strong histochemical staining (>30 positive fibers) was detected in the muscle around the injection site up to 2 weeks after infection, even in mice that were infected at 4 weeks (Table 1). In the latter case, staining was present over a distance of at least 8–9 mm, and most of the positive fibers appeared stained over a length of 1.5–2 mm (Fig. 3). Of the 37 injected muscles (35 mice) that were analyzed at >20 days after infection, 16 showed low (1–10 positive fibers) or moderate staining (11–30 positive fibers), and 6 showed strong staining at 60–75 days (Table 1, Fig. 3). No staining was observed in nonmuscle tissues surrounding the injection site. These data were obtained by staining the muscles as a whole. The internal fibers may not have been accessible to the reagents because of the tight structure of the muscle, thus leading to an underestimation of the number of stained fibers. This was confirmed by incising some infected muscles before staining: in addition to external fibers, some internal fibers appeared positive.

The status of viral DNA sequences was followed by Southern blotting of injected muscle DNA. Most or all of the viral DNA was nonintegrated, since we only detected the expected fragments containing the inverted terminal repeat sequences, up to the last tested time point of 33 days (data not shown). The amount of viral DNA detected appeared rather stable, if the increase in muscle mass is taken into account.

DISCUSSION

Correction of muscle diseases by gene transfer represents a very difficult problem because of the very large mass of tissue involved. Two approaches have recently been proposed toward this long-term goal: myoblast transplantation (5) and direct introduction of plasmid DNA into mature muscle, by injection or by particle bombardment (6, 19). Although retroviruses have been used for gene transfer in muscle cells in culture (20), they cannot be used on noncycling cells and thus on myofibers. Their use would thus also involve myoblast transplantation (for instance, for gene correction using autologous cells). We have decided to explore the potential of adenovirus vectors for gene transfer in muscle, as host cell proliferation is not required for expression of the viral proteins. Such vectors have been used previously for efficient transfer of the ornithine transcarbamoylase minigene into the liver of the *spf-ash* mice, leading to phenotypic correction (7, 8), and more recently to target expression of α_1-antitrypsin in the lung epithelium (9).

We constructed a recombinant adenovirus that allows muscle-specific expression of a reporter gene, β-galactosidase. It was tested successfully on two rodent myogenic cell lines and was then used *in vivo* by injection into muscle of mice.

Very efficient expression was observed in short-term experiments (up to 15 days), with intense staining in many fibers over distances of at least 8–9 mm. This appears much better than that observed for similar experiments carried out by direct injection of plasmid, where staining was reported over a depth of 1.2 mm at 7 days after injection (6), or by particle bombardment (19). Results were more variable in long-term experiments; however, moderate to high expression was

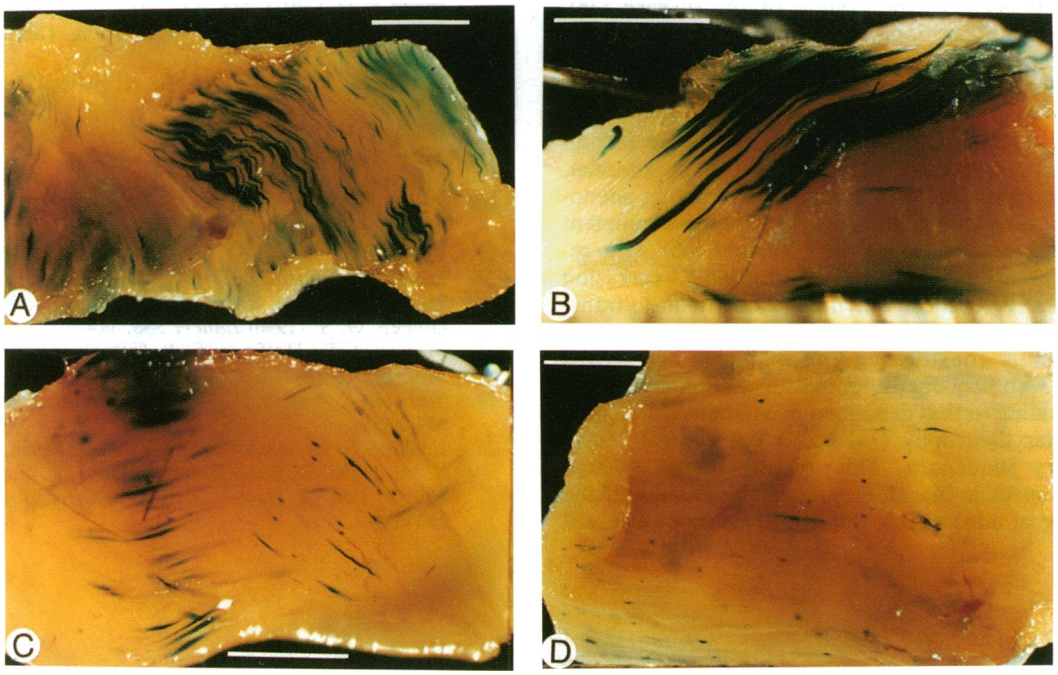

FIG. 3. Expression of β-galactosidase in muscle of infected mice. (*A* and *B*) Muscle of 4-week-old mice analyzed 14 days after injection. (*C* and *D*) Muscle of newborn mice analyzed 75 days after injection. (Bars = 2 mm.)

observed in 13 of 27 mice analyzed 48–75 days after injection, with positive fibers dispersed up to 1 cm. This can be related to the stable persistence of unintegrated adenovirus DNA, which was observed for at least 33 days in our experiments and for 3 months in various organs of rabbits injected intravenously with a different adenovirus recombinant (21). Stable muscle expression of reporter genes up to 60 days has also been reported by Wolff *et al.* (6) following direct injection of plasmid DNA. It is difficult to compare our results with myoblast transplantation, where the presence of donor proteins was ascertained up to 99 days in the *mdx* mouse (5) and 92 days in a single DMD patient (who was also under parallel cyclosporin treatment) (22). In the most successful experiments in *mdx* mouse, 10–30% of fibers were dystrophin positive. However, it was reported that the success rate was higher in nude mice, even when compared with major histocompatibility complex-compatible mice made tolerant at birth. In addition, the migratory power of injected myoblasts was reported not to exceed a few millimeters (ref. 5 and references therein).

One additional advantagous feature of such an adenovirus construct is the possibility to reach several muscles with a single injection. An intravenous route has previously been shown to be efficient for obtaining expression in liver (8) and has been tested for other organs (L.D.P., I. Makeh, M. Perricaudet, and P. Briand, unpublished). We have tested other routes of injection in preliminary experiments and obtained positive fibers in the thigh and abdominal wall of mice injected via an intraperitoneal route.

The performance of the adenovirus vector for obtaining muscle-specific expression appears superior to those reported for DNA injection or particle bombardment (with respect to the size of the area where expression occurs and/or time length of expression). Up to now, the myoblast transplantation strategy has been favored (5, 23), and some assays on Duchenne patients have even been attempted (22). However, this strategy would require a large number of closely spaced injections, each with several million myoblasts. Obtaining such amounts of cells for autologous transplantation (following transfer of a normal gene construct) would appear difficult for treatment of DMD, as it was reported that the replicative life-span of myoblasts already is much decreased early in the course of the disease (24). Heterologous transplantation would very likely require an immunosuppressive treatment. We feel that these problems justify the exploration of alternative strategies, although we recognize the very important issues raised by *in vivo* infection with live virus, even though the virus is replication incompetent and does not appear to have transforming potential. Moreover, recombination with a wild-type infecting adenovirus is very unlikely to generate a replication-competent recombinant. Meanwhile, it should be noted that such recombinant adenoviruses may be useful for gene expression studies in mature myotubes, which appear difficult to transfect, and in muscle *in vivo*.

We are presently constructing a recombinant adenovirus carrying the 6.3-kb sequences coding for a minimal dystrophin, corresponding to that described in a patient with a mild Becker muscular dystrophy (25) under the control of the same regulatory sequences. This dystrophin has a large portion of the spectrin-like domain deleted. Thanks to the wide host range of adenovirus, this construct may be tested in the two available animal models for DMD, the *mdx* mouse and the *CXMD* dog. Although the size of the complete dystrophin coding sequence is incompatible with the present adenovirus vector, it should be possible to integrate it into a recombinant virus using a helper virus, since the only cis-acting sequences necessary for replication and encapsidation are <1 kb (18).

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