Volume 296, number 2, 128–134 FEBS 10573 January 1992

Retroviral-mediated transfer of a dystrophin minigene into *mdx* mouse myoblasts in vitro

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Received 6 November 1991

We have demonstrated expression of a 6.3 kb Becker muscular dystrophy (BMD) human dystrophin cDNA following retroviral-mediated transduction of cultured myoblasts from the dystrophin-deficient *mdx* mouse. The truncated dystrophin protein was localised to the sarcolemma of differentiated myotubes by antibodies against the C-terminus of the molecule, and produced an identical immunostaining pattern to that observed in control myotubes expressing normal endogenous dystrophin. These results indicate that retroviral-mediated gene transfer may be useful for experimental in vivo studies on the complementation of dystrophin gene mutations.

Dystrophin; Recombinant retrovirus; Gene transfer, *mdx* mouse; Skeletal muscle

1. INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is a recessive, X-linked condition caused by nonsense mutations or complete deletion of the gene for dystrophin, a large (mol. wt. 427 kDa) cytoskeletal protein most abundant in skeletal muscle [1]. It affects 1 in 3500 males leading to progressive muscular weakness and death usually in the third decade [2].

The *mdx* mouse is an animal model of DMD, lacking dystrophin expression in all but a very small percentage of myofibres [1,3] due to a point mutation in the mouse homologue of the DMD gene [4]. Although severe dystrophic changes are observed in the muscles of the diaphragm [5], the *mdx* mouse does not show the progressive degeneration of limb muscles characteristic of the human disease. Instead, a high level of myofibre regeneration in the limbs seems to effectively restore muscle integrity and function [6]. Nevertheless, *mdx* mouse skeletal muscle provides an excellent model for the early myopathic phase of DMD.

Recently, retroviral vectors have been widely used for the efficient, stable introduction of foreign genes into eukaryotic cells in vitro and in vivo [7–11]. The 14 kb full-length cDNA of the DMD gene [12], however, exceeds the 10–12 kb packaging limit of retroviruses. Therefore, a 6.3 kb dystrophin minigene cDNA derived from a patient with very mild Becker Muscular Dystrophy (BMD) [13] has been incorporated into the MoMuLV-based retroviral vector, pBabe Neo [14]. This minigene expresses a truncated protein lacking over 40% of the central rod domain of the normal dystrophin molecule, yet the patient suffers very little functional loss. Transduction of the retroviral minigene construct into *mdx* mouse myoblasts in vitro lead to the expression of a truncated dystrophin protein at the sarcolemma of differentiated myotubes.

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2. MATERIALS AND METHODS

2.1 *Construction of the retroviral plasmid, pBN1*

The 6.3 kb dystrophin cDNA [15] was inserted into the *Sal*I site of the retroviral vector pBabe Neo to yield an 11.5 kb construct, pBN1 (Fig. 1). Constitutive transcription of the cDNA insert is driven from the Moloney murine leukaemia virus promoter in the 5′ LTR and polyadenylated from the 3′ LTR of the provirus. An internal SV40 early promoter drives transcription of the *neo* gene which confers resistance to the aminoglycoside G418 in mammalian cells and kanamycin sulphate in bacteria. The vector can be packaged into infectious retrovirus particles in combination with the packaging cell line, ΩE [14].

2.2. *Transfections, cell lines and determination of viral titres*

Transient transfections of COS cells and the ecotropic helper-free packaging cell line, ΩE were performed with plasmids pBabeNeo and pBN1 by DNA/calcium phosphate precipitation [16]. Supernatants containing transiently-produced recombinant retrovirus from transfected ΩE cells were used to infect ΩE cells treated with 30 ng/ml tunicamycin (Sigma) as previously described [17] in order to achieve stable integration of the proviral DNA into the genome. Drug resistant colonies were selected in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum and 500 μg/ml G418 (Geneticin, Gibco). Stable producer cell lines manufacturing retrovirus at the highest titre were selected following infection-assay of NIH-3T3 cells. The volume of media in 100 mm dishes of subconfluent producers was reduced to 5 ml, harvested 72 h later and filtered through 0.45 μm filters. Serial dilutions were made in 2 ml of medium (DMEM; 10% FCS) containing 8 μg/ml polybrene (Sigma) and applied to 7.5×10^5 NIH3T3 cells on 100 mm dishes for 4 h. The following day, cells were split at a ratio of 1:20 in medium containing 500 μg/ml G418 and incubated until drug resistant colonies were clearly visible.

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2.3. *Retroviral infection of primary cultures*

Muscle tissue was dissected from the hind limbs of 3–6-week-old control C57/B10 and dystrophin-deficient *mdx* mice and dissociated for primary culture essentially as described [18]. Dissociated cells were preplated on uncoated plastic tissue culture dishes for 30 min to remove fibroblasts prior to plating onto glass coverslips or 35 mm tissue culture dishes coated with 25 μg/ml poly-L-lysine and 8 μg/ml laminin (Sigma) [19]. Three days later, coverslips and 35 mm dishes were transferred to 16 mm wells or 100 mm tissue culture dishes previously plated with control or minigene producer cell lines (containing integrated pBabeNeo or pBN1 proviruses, respectively), and grown in growth medium (DMEM; 20% FCS) containing 2 μg/ml polybrene. At onset of differentiation to myotubes, the media was changed to DMEM containing 5% (v/v) horse serum.

2.4. *Immunocytochemistry and Western blot analysis*

For immunostaining analyses, cells grown on coverslips were fixed in methanol (–20°C) and incubated individually or simultaneously with a mouse monoclonal antibody MANDYS 1 [20,21] against a central rod domain epitope of dystrophin and rabbit antibodies, G6 and G8, raised, respectively, to N- and C-terminal synthetic peptide antigens of the molecule. Subsequent incubations were carried out with fluorescein-conjugated anti-mouse and biotinylated anti-rabbit antibodies, prior to tertiary labelling of the biotinylated antibody with streptavidin-conjugated Texas Red (Amersham). Stained cells were viewed on a Zeiss microscope equipped with phase contrast and epi-fluorescence optics.

3. RESULTS

3.1. *Expression of the Becker-type dystrophin cDNA in COS cells*

Retroviral-mediated gene transfer is currently the most effective way of stably integrating DNA into mammalian genomes. Therefore a human dystrophin minigene was cloned into the retroviral vector pBabe Neo to achieve stable gene transfer in cultured *mdx* mouse skeletal muscle cells. pBabe Neo contains an SV40 origin of replication, enabling high level expression of plasmid-borne cDNAs in transfected COS cells [22]. Thus before proceeding to isolate recombinant retroviral producer cell lines, the integrity of the Becker minigene construct, pBN1 (Fig. 1) was confirmed by detection of recombinant dystrophin expression in COS cells transfected with pBN1 plasmid DNA. Incubation of methanol fixed COS cells with antibodies against the N- and C-terminal epitopes of the dystrophin molecule resulted in strong fluorescent labelling of the truncated dystrophin in pBN1, but not pBabe Neo-transfected cells (Fig. 2A,B).

3.2. *Production of stable, high titre producer cell lines*

Production of a high titre of recombinant retrovirus is necessary to achieve high efficiency gene transfer. Therefore, 24 stable ΩE producer cell lines were screened for infectivity against NIH 3T3 cells. Retrovirus particles released by these producer cells into the media were referred to as RV.Babe Neo and RV.BN1. Average counts of G418 resistant colonies of NIH 3T3 cells following infection with retroviral supernatants were used to calcutate retroviral titres according to the formula of Cepko [23], expressed as colony-forming units (cfu) per ml. The RV.BN 1 producer cell line of highest titre yielded an average of 4.1×10^4 cfu/ml. Retroviral titres were lower than reported elsewhere [7,24], perhaps due to less efficient packaging of such a large amount (10.5 kb) of proviral RNA from pBN1, which is close to the packaging size limit of retroviral capsids.

For safety reasons, it is important to ensure that infected target cells are unable to generate replication-competent recombinant retrovirus which could act as 'helper' virus and lead to uncontrolled infections [25]. The ΩE cell line adopts the strategy developed by Markowitz et al. [26] where the genes *gagpol* and *env*, vital for viral replication and infection, were introduced on separate plasmids to prevent recombination events

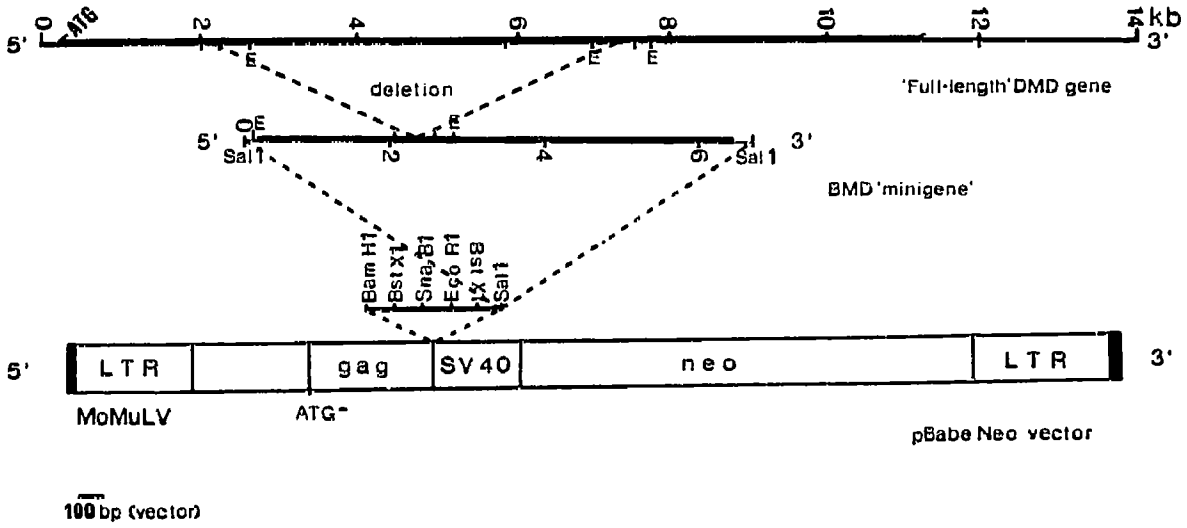

Fig. 1. The retroviral plasmid, pBN1, contains a 6.3 kb Becker-type dystrophin cDNA inserted into the *Sal*I site of the retroviral vector pBabe Neo. This minigene lacks 5.1 kb from the central region of the 14 kb normal human dystrophin cDNA.

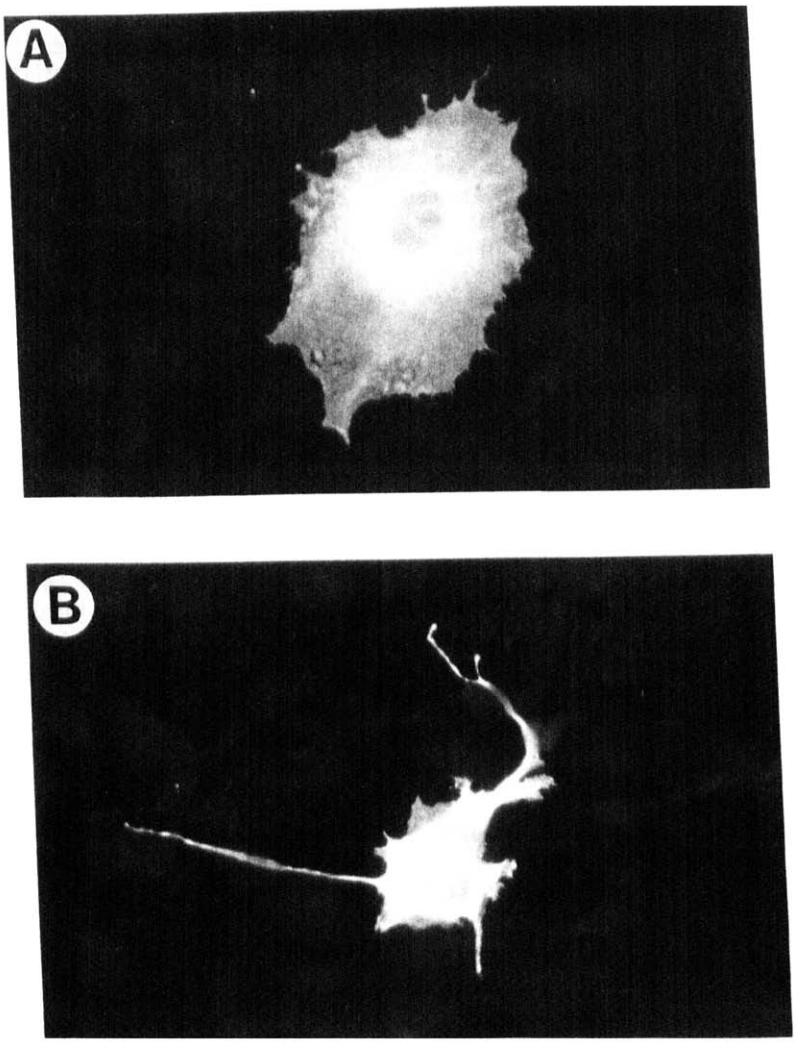

Fig. 2. Immunostaining of pBN1-transfected COS cells with antibodies against (A) the N-terminal and (B) C-terminal domains of the dystrophin molecule. Magnification, ×600.

leading to helper virus production. In order to test for helper activity, NIH 3T3 cells were incubated with the conditioned media from previously-infected NIH 3T3 cells in the presence of 8 μg/ml polybrene. No G418-resistant cells were subsequently detected, validating the 'helper-free' status of the producer cell lines.

3.3. *Expression of the Becker-type dystrophin cDNA in transduced* mdx *myotubes*

Recently, Hoffman et al. [3] have reported the very occasional (<1.0%) occurrence of *mdx* mouse myofibres which appear to express the full-length dystrophin protein. Therefore, dual antibody labelling of infected cultures was employed to distinguish myotubes expressing the dystrophin minigene from 'revertant' or 'suppressor mutant' fibres. Staining patterns observed using a monoclonal antibody (MANDYS 1) against part of the dystrophin central rod domain absent from the Becker-type protein [13] was compared with concurrent staining of the same samples with an antibody (G8) against the C-terminal region, common to both full- length and truncated molecules.

10–15 days after infection of primary myoblast cultures with the recombinant retrovirus, RV.BN1, intense Texas-Red immunostaining with the C-terminal dystrophin antibody was observed at the sarcolemma of all C57/ B10 and 5–10% of *mdx* myotubes (Fig. 3.) No C-terminal labelling was observed in *mdx* cultures exposed to control retrovirus RV.Babe Neo alone. In contrast, the rod domain antibody showed no fluorescein labelling of *mdx* myotubes exposed to either RV.BN1 or RV.BabeNeo, but only at the sarcolemma of C57/ B10 normal mouse myotubes. No 'revertant' *mdx* myotubes were observed. At high power magnification of C-terminal labelled *mdx* myotubes, a veined pattern and sarcolemmal localisation of the truncated dystrophin could be seen that was identical to the staining pattern observed at the surface of normal dystrophin-positive

Fig. 3. Expression of recombinant human dystrophin in transduced *mdx* myotubes. Myotube cultures from (A–C) C57/B10 normal mouse, (D–F) *mdx* mouse transduced with the control retrovirus, RV.Babe Neo, and (G–I) *mdx* mouse transduced with the dystrophin minigene retrovirus, RV.BN1, were subjected to double immunostaining with antibodies to central rod domain (B,E,H; MANDYS 1) and C-terminal (C,F,I; G8) epitopes. Magnification ×200.

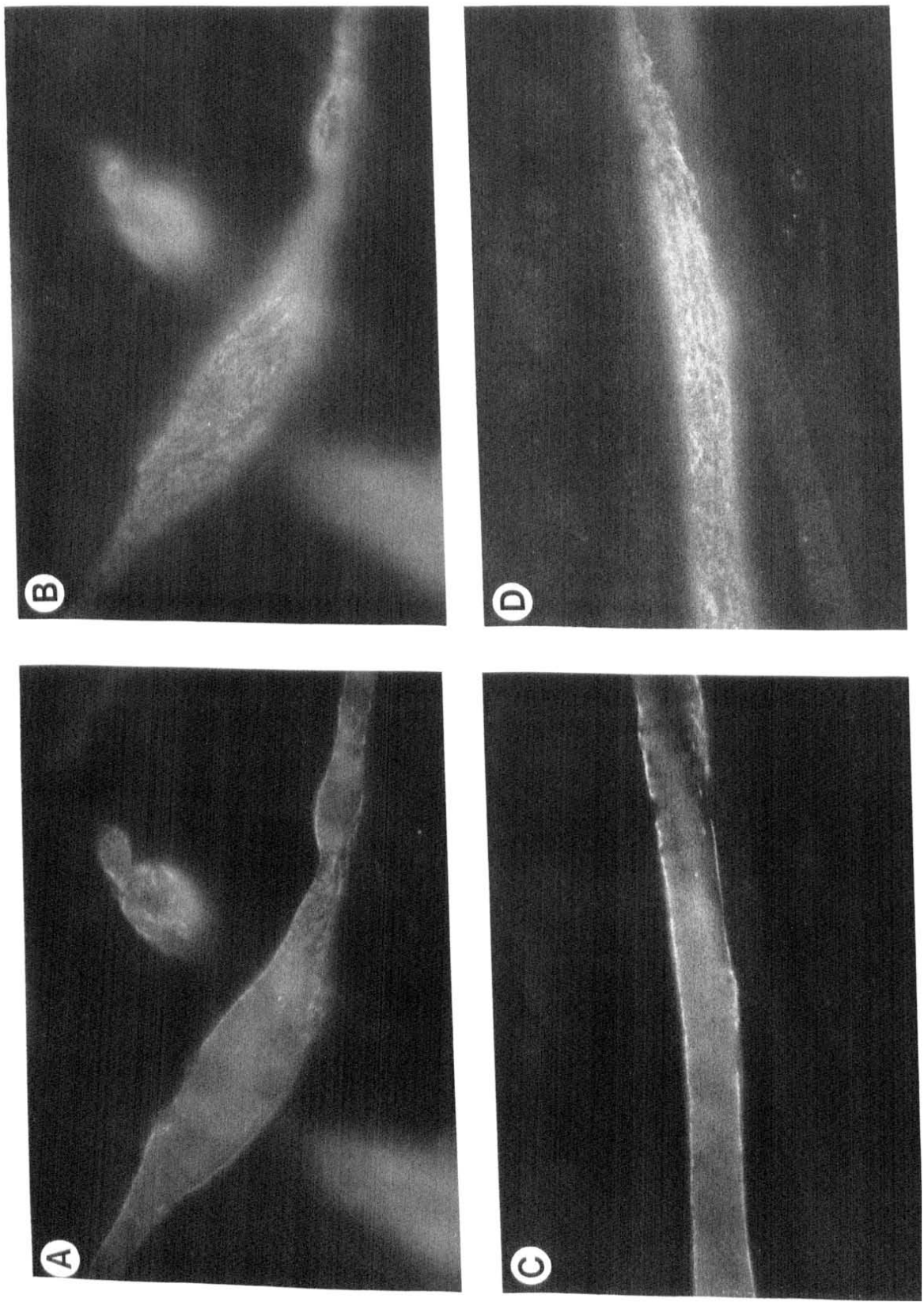

Fig. 4. Immunolocalisation of endogenous dystrophin at the sarcolemma of normal mouse myotubes (A,B) compared with expression of the Becker-type dystrophin in transduced *mdx* mouse myotubes immunostained with the C-terminal dystrophin antibody, G8. Note the very similar immunostaining patterns viewed through the mid-plane (A,C) and at the surface (B,D) of the myotubes. Magnification, ×480.

myotubes [27] (Fig. 4). These results clearly demonstrate expression of the Becker-type dystrophin in transduced *mdx* myotubes.

4. DISCUSSION

A number of methods have been used to achieve dystrophin expression in skeletal and cardiac muscles of the dystrophin-deficient *mdx* mouse, including transfer of dystrophin-positive myoblasts into regenerating skeletal muscle [28], direct injection of dystrophin cDNAs [15] and injection of fertilised mouse ova with linearised DNA to produce transgenic animals (Wells et al., unpublished). However, at present the preferred method of somatic gene transfer into mammalian cells is by means of retroviral vectors due to their high efficiency and stable integration of proviral DNA [24]. Despite the limited packaging size of retroviruses which precludes incorporation of the 14 kb full length DMD cDNA, we have shown that retroviral mediated transfer of a dystrophin minigene is possible and results in the correct localisation of the gene product at the sarcolemma of skeletal muscle myotubes.

This truncated dystrophin molecule is at least semi-functional in vivo, as the BMD patient bearing the deleted gene remains ambulatory at 62 years of age. Therefore, expression of the minigene in *mdx* myofibres may correct some of the cellular defects of dystrophic muscle, such as membrane instability [29] and raised intracellular calcium levels [30]. Indeed, Acsadi et al. [15] have demonstrated that expression of an identical cDNA following its direct injection into *mdx* muscle in vivo results in a decreased number of centrally nucleated fibres, indicating a reduction in muscle damage and regeneration. This has been extended by Wells et al. (unpublished) in transgenic mice expressing the Becker dystrophin, who also observed a significant reduction in the number of centrally nucleated fibres relative to non-transgenic *mdx* control animals.

It is thought that the presence of N- and especially C-terminal domains in Becker dystrophin molecules enables relatively normal interactions with components of the cytoskeleton, such as actin [31] and membrane-bound glycoproteins [32]. This may result in less severe membrane damage relative to dystrophin-deficient myofibres, and a consequently milder phenotype.

Differentiated myotubes are mitotically inactive, favouring stable expression of introduced genes. In this study expression of the Becker minigene in *mdx* myotubes was still detectable 3–4 weeks post-infection (data not shown) and at comparable levels to normal myofibres (Fig. 4.). Since dystrophin transcripts are present at fairly low levels in normal skeletal muscle [33], expression of a recombinant dystrophin from a strong retroviral promoter in one or two nuclei may be sufficient to prevent muscle necrosis when introduced into the post-natal animal.

Recently, Salminen et al. [34] have used a retrovirus to introduce a human multi-drug reporter gene cDNA into cultured rat myocytes which were later implanted into the skeletal muscle of immunosuppressed adult rats. Myoblasts from biopsies of dystrophin-deficient animals could be transduced with recombinant dystrophin retroviruses in vitro, expanded and replaced into the skeletal muscle of the donor without requiring immunosuppression. Fusion of these recombinant myoblasts with dystrophic muscle may result in dystrophin expression in numerous skeletal muscle fibres.

The results reported here clearly demonstrate that a functional dystrophin minigene can be packaged in infectious retrovirus particles and used to stably transduce primary *mdx* myoblasts in vitro. The integrated dystrophin cDNA is expressed at high levels from the MoMuLV retroviral promoter leading to correct localisation of the gene product at the sarcolemma of mature myotubes. Furthermore, as retroviruses only infect actively dividing cells [35] the high mitotic activity of regenerating dystrophic muscle may make it a highly suitable target for direct retroviral-mediated transfer of dystrophin genes in vivo.

Acknowledgements: This work was supported by grants from the Muscular Dystrophy Group of Great Britain and the Medical Research Council. We would also like to thank Dr. Dominic Wells for providing the *mdx* mice and Dr. Hartmut Land for providing the retroviral vector pBabe Neo with its complementary packaging cell line ΩE.

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