Effective restoration of dystrophin-associated proteins in vivo by adenovirus-mediated transfer of truncated dystrophin cDNAs

Katsutoshi Yuasa[a], Yuko Miyagoe[a], Kanji Yamamoto[a], Yo-ichi Nabeshima[a,b], George Dickson[c], Shin'ichi Takeda[a,*]

[a] *Department of Molecular Genetics, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187, Japan*
[b] *Institute for Molecular and Cellular Biology, Osaka University, 1-2 Yamada-oka, Osaka 565, Japan*
[c] *School of Biological Sciences, Royal Holloway College, University of London, Surrey TW20 0EX, UK*

Received 24 February 1998

Abstract A series of truncated dystrophin cDNAs (3.1–4.2 kbp) containing only three, three, two or one rod repeats with hinge 1 and 4 (named ΔDysAX2, AX11, AH3, M3, respectively) or no rod repeat retaining either hinge 1 or 4 (named ΔDysH1, H4, respectively) were constructed. These cDNAs were introduced into skeletal muscle of adult *mdx* mice using the adenovirus vector with a strong CAG promoter. ΔDysAX2, AX11, AH3 and ΔDysM3 expressed themselves successfully and recovered dystrophin-associated proteins effectively. Especially 3.7 kbp cDNA for ΔDysM3 offers the possibility of an approach utilizing newly developed virus vectors, such as an adeno-associated virus vector, toward gene therapy of Duchenne muscular dystrophy.

Key words: Dystrophin; Duchenne muscular dystrophy; Gene therapy; Adenovirus vector; Rod domain

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked, lethal disorder of skeletal muscle caused by a defect in the dystrophin gene. Progressive muscle weakness, cardiomyopathy and early death characterize the disease. Epidemiological studies have estimated its prevalence to be approximately 1 in 3500 newborn males, one-third of these cases being sporadic [1]. Currently, there is no effective treatment for the disease, but the relatively new field of gene therapy offers promising possibilities.

The native dystrophin gene measures 2.4 Mbp, while the full-length dystrophin cDNA is about 14 kbp [2]. The gene product, dystrophin, is a 427 kDa cytoskeletal protein that is situated at the inner surface of the sarcolemma [3,4]. From its position on the sarcolemma, dystrophin uses its N-terminal domain to anchor actin filaments [5] and its cysteine-rich and C-terminal domains to bind the integral and peripheral membrane proteins and glycoproteins called dystrophin-associated proteins (DAPs) [6]. It now seems clear that dystrophin stabilizes the sarcolemma against contraction stress by linking to the cytoskeleton [7]. Dystrophin's rod domain, which accounts for 76% of the molecule, mainly consists of 24 triple-helical repeats and four hinge segments [8]. Although the role of the rod domain is not fully understood, it has been shown that a large omission in this domain results in a mild form of dystrophy, Becker muscular dystrophy (BMD) [9]. 'Mini-dystrophin', isolated from a benign BMD patient, largely lacks the central region of this repetitive domain; this includes exons 17–48 (nucleotide positions 2201–7306), resulting in a total of eight rod repeats with three hinge segments [10].

As an initial step toward gene therapy of DMD, researchers have first employed the 6.3 kbp mini-dystrophin cDNA, because it is small enough to accommodate an E1-substitution-type adenovirus (Ad) vector. The mini-dystrophin is successfully expressed in dystrophic *mdx* mice, especially when the Ad construct is injected during the neonatal period [11–14]. In addition, mouse transgenic studies have shown that the phenotypes of muscular dystrophy dramatically improved in *mdx* mice carrying the mini-dystrophin gene [15,16]. It is noteworthy that these studies utilizing mini-dystrophin cDNA and clinical data from a series of BMD patients [9] show the possibility that long-period expression of dystrophin deleted for a large part of the rod domain would maintain the cytoskeleton-sarcolemma linkage and prevent the dystrophic change in dystrophin-deficient muscle.

However, when Ad is injected into adult *mdx* mice, an immunological reaction to the Ad vector prevents long-lasting expression of the dystrophin gene [14], indicating that a new generation of Ad vector or a new virus vector is required. Newly developed virus vectors, which are available in gene transfer to skeletal muscle, have a limitation in the size of the foreign gene. For example, adeno-associated virus (AAV) vector allows an insertion of up to 4.6 kbp of exogenous DNA [17]. Thus, truncation of dystrophin to a small size would make it possible to employ a wider spectrum of virus vectors.

Based on these observations, we designed a series of truncated dystrophins with consecutive deletions in the rod domain, and examined their effects on the expression of dystrophin and DAPs at the sarcolemma after Ad-mediated gene transfer into skeletal muscles of *mdx* mice. Here we report that truncated dystrophins bearing one to three rod repeats and two hinge segments can effectively accumulate at the sarcolemma and recover DAPs, and 3.7 kbp cDNA for a truncated dystrophin, which has only one rod repeat, can be a good candidate to be inserted into AAV vectors.

*Corresponding author. Fax: (81) (423) 46-3864.
E-mail: takeda@ncnaxp.ncnp.go.jp

Abbreviations: Ad, adenovirus; AAV, adeno-associated virus; BMD, Becker muscular dystrophy; DAP, dystrophin-associated protein; DMD, Duchenne muscular dystrophy; DMEM, Dulbecco's modified Eagle's medium; moi, multiplicity of infection; pfu, plaque-forming units; PBS, phosphate-buffered saline; TA, tibialis anterior; PCR, polymerase chain reaction

PII S0014-5793(98)00251-8

2. Materials and methods

2.1. Construction of truncated dystrophin cDNAs

We constructed a series of truncated dystrophin cDNAs with varying sizes of the rod domain (Fig. 1A). A 6.3 kbp *Not*I/*Sal*I fragment from human mini-dystrophin cDNA [18] was cloned into the *Not*I/*Sal*I sites of pBluescriptII(SK$^+$) (Stratagene), generating pBSBMD. Four plasmids including cDNA of truncated dystrophin (ΔDys), named AX2, AX11, AH3, and M3, were generated as follows. The sequences of the primers and other oligonucleotides used in the construction of the cDNAs are shown in Table 1. The PCR product which was amplified with pBSBMD as the template and primers F1/R1 or F2/R2 was digested with *Afl*II/*Xho*I and inserted into the *Afl*II/*Xho*I sites of pBSBMD, generating pBSΔDysAX2 and pBSΔDysAX11, respectively. The PCR product amplified with pBSBMD as the template and primers F4/R4 was digested with *Mun*I/*Hin*dIII and inserted into the *Mun*I/*Hin*dIII sites of pBSBMD, generating pBSΔDysM3. A fragment that was formed by annealing oligonucleotides, F3/R3, was used to link between the *Afl*II and *Hin*dIII sites of pBSBMD, generating pBSΔDysAH3.

Two other plasmids including cDNA for ΔDysH1 and ΔDysH4 were generated from pBSΔDysM3 (Fig. 1A). pBSΔDysM3 was digested with *Apa*I, then blunted and self-ligated to remove one of the *Eco*O109I sites. The PCR product amplified using pBSΔDysM3 as the template and primers F5/R5 was digested with *Eco*T22I/*Eco*O109I and inserted into the *Eco*T22I/*Eco*O109I sites of the resulting plasmid, generating pBSΔDysH1. To generate pBSΔDysH4, two PCR reactions were independently accomplished using pBSΔDysM3

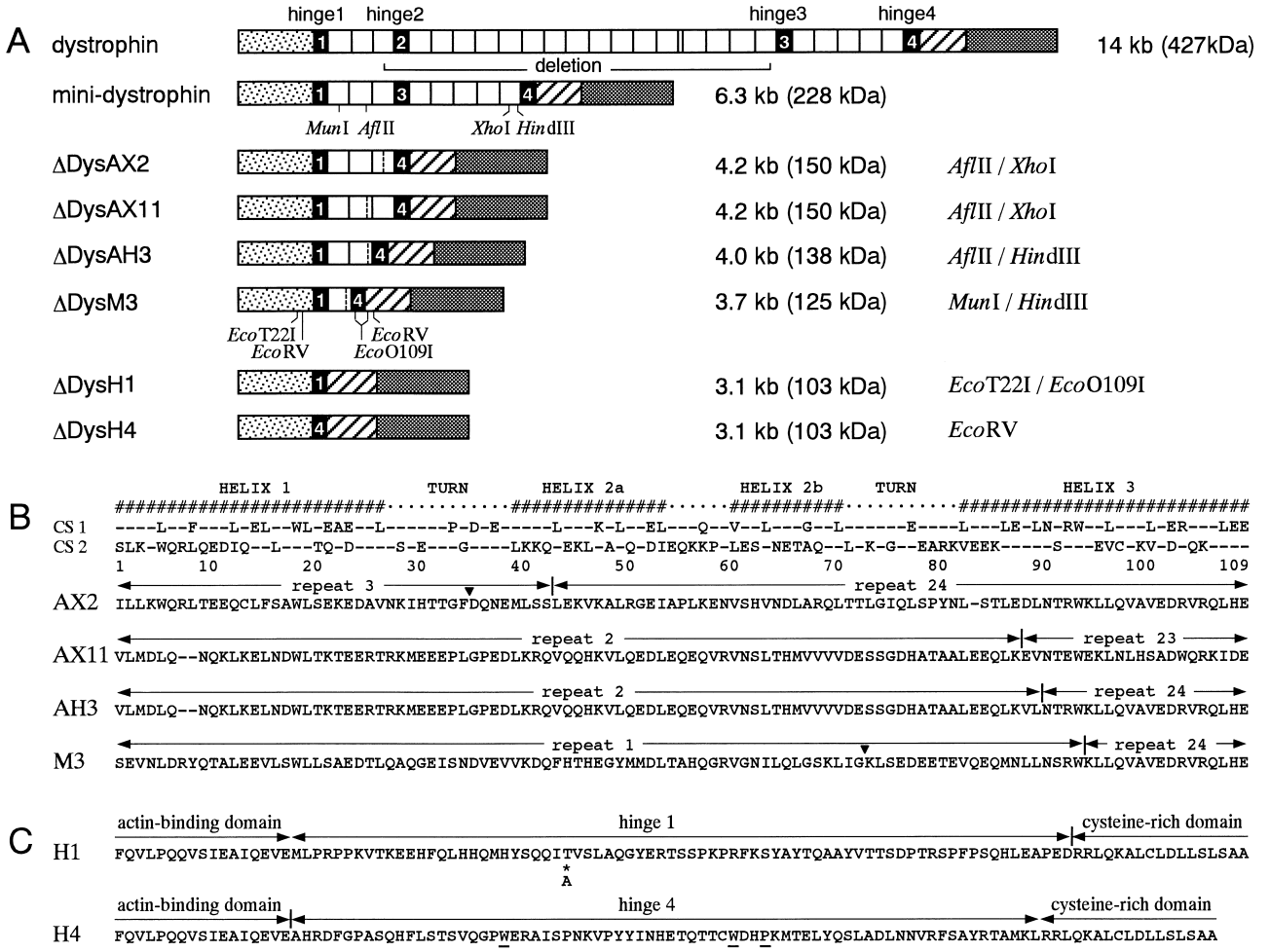

Fig. 1. Construction of truncated dystrophin cDNAs with varying numbers of rod repeats. A: Diagram of human full-length dystrophin, mini-dystrophin [10,18], and newly generated truncated dystrophin cDNAs. To construct ΔDysAX2, ΔDysAX11, ΔDysAH3, and ΔDysM3, the central rod domain in the mini-dystrophin cDNA was digested with the restriction enzymes indicated on the right of each construct. Both of the resulting ends were linked together by a PCR-amplified fragment or synthetic DNA fragment to reconstruct the rod repeat structure (for details, see Section 2). To construct ΔDysH1 and ΔDysH4, ΔDysM3 was digested with the restriction enzymes indicated and both ends were ligated using PCR-amplified fragments. The dotted line indicates the junction. The sizes of the cDNAs and the deduced molecular weights of truncated dystrophins are indicated. The actin-binding domain is illustrated by dotted boxes, the rod domain by open boxes (each repeat depicted by a box), the cysteine-rich domain by hatched boxes and the C-terminal domain by shaded boxes. Black boxes indicate hinges. The hinges are numbered according to the description by Koenig and Kunkel [8]. B: Amino acid sequences of the reconstructed rod repeat in ΔDysAX2 (AX2), ΔDysAX11 (AX11), ΔDysAH3 (AH3) and ΔDysM3 (M3). Vertical bars indicate the positions of junctions. Triangles and dashes indicate the positions of gaps and deletions to optimize the alignment of rod repeats [8]. CS 1 and CS 2 show consensus sequences from 24 repeats in dystrophin [8]. CS 1 indicates amino acids found in at least eight repeats and CS 2 shows amino acids appearing in 5, 6, or 7 repeats among the 24. C: Amino acid sequences of ΔDysH1 (H1) and ΔDysH4 (H4) at the area of ligation. In ΔDysH1, hinge 1 is directly connected to the cysteine-rich domain. In ΔDysH4, the actin-binding domain is connected to hinge 4. Tyrosine (T), marked with an asterisk, in hinge 1 was found to be substituted to alanine (A) in a family with X-linked dilated cardiomyopathy in North America [33]. The dashed line below hinge 4 indicates the WW domain; the most conserved amino acids in the WW domain are underlined [31].

as the template and primers F5/R6 or F6/R7. A mixture of two resulting PCR products was used as the template for the second PCR reaction with primers F5/R7. The second PCR product was digested with *Eco*RV and was inserted between two *Eco*RV sites of pBSΔDysM3. The amino acid sequence of the linked regions is shown in Fig. 1B,C.

2.2. Generation of recombinant adenoviruses expressing truncated dystrophin

E1-substitution-type recombinant Ad vectors expressing each truncated dystrophin were generated by the COS-TPC method [19]. Briefly, each truncated dystrophin cDNA, ΔDysAX2, AX11, AH3, M3, H1 or H4, was inserted into the CAG expression unit, which has proven to have strong expression in vitro [20] and in vivo [21], in the cassette cosmid pAxCAwt [22]. Each recombinant Ad was generated by homologous recombination in 293 cells between the resulting cosmid and the DNA-terminal protein complex of Ad5 dlx [19,23]. The obtained recombinant Ad vectors, designated AxCAΔDys, were propagated, purified and titrated as described [24]. Each AxCAΔDys was stored in phosphate-buffered saline (PBS), containing 10% glycerol, at −80°C.

2.3. Gene transfer into cultured myoblasts and Western blot analysis

A subclone of C2C12 myoblasts [25] (approximately 1.0×10^5) was plated and incubated for 1 day on 6 cm collagen-coated dishes and in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (v/v) fetal calf serum. C2C12 myoblasts were then infected with AxCAΔDys at a multiplicity of infection (moi) of 100 plaque-forming units (pfu)/cell and growth medium was replaced by differentiation medium consisting of DMEM and 5% (v/v) horse serum. After 3 days, cells were harvested and suspended in SDS-PAGE lysis buffer (15% SDS, 70 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 10 mM EDTA). 20 μg/lane of cell lysate was then separated on 5% SDS-PAGE and electro-transferred to a PVDF membrane (Immobilon, Millipore). The blot was incubated with the anti-dystrophin monoclonal antibody, DYS2 (Novocastra), at 1/100 dilution. This antibody recognizes the final 17 amino acids of human dystrophin. Immune complexes on the blot were detected with horseradish peroxidase-conjugated rabbit anti-mouse IgG1 (Zymed) and the ECL Western blotting detection reagents (Amersham).

2.4. Immunohistochemistry of adenovirus-injected muscles

Prior to in vivo injection, each AxCAΔDys stock solution was run through a CHROMA SPIN column (Clontech) saturated with PBS in order to eliminate glycerol. 50 μl purified AxCAΔDys solution was then directly injected using a 27-gauge needle into the left tibialis anterior (TA) muscle of *mdx* mice of 12–16 weeks of age. One week after infection, the muscle was isolated and then frozen in liquid nitrogen-cooled isopentane. Cross-sections (6 μm) from injected and non-injected *mdx* muscles and control C57BL/10 muscle were prepared on the same slide, dried and fixed in acetone for 10 min. Immunohistochemical procedures were carried out using the following antibodies: a rabbit polyclonal antibody against the C-terminal 25 amino acids of dystrophin (anti-C, generous gift of Dr. Y. Nonomura), a rabbit polyclonal antibody against amino acids 2360–2409 of dystrophin, corresponding to a part of the 19th rod repeat (P23a, obtained from Dr. M. Yoshida) [26], a sheep polyclonal antibody against β-dystroglycan [27], a rabbit polyclonal antibody against α-sarcoglycan (obtained from Dr. Y. Wakayama) [27], and a rabbit polyclonal antibody (generous gift of Dr. S. Kameya) against amino acids 191–206 of α1-syntrophin [28]. The primary antibody was detected with either FITC-conjugated goat anti-rabbit IgG (Tago Immunologicals) or rabbit anti-sheep IgG (Organon Teknika). The signals obtained from the same slide were recorded photographically under the same condition using the laser scanning confocal imaging system MRC-1000 (Bio-Rad).

3. Results

3.1. Generation of recombinant adenoviruses encoding truncated dystrophin

We designed a series of six truncated dystrophins with consecutive deletions within the rod domain, as shown in Fig. 1. Using the six dystrophin cDNAs, we successfully generated six recombinant adenoviruses. While all constructs retain the N-terminal actin-binding, cysteine-rich, and C-terminal domains, the designed ΔDys, AX2, AX11, AH3 or M3 contain three, three, two and one rod repeat, respectively, as well as both hinges 1 and 4; and then, the ΔDysH1 and ΔDysH4 have no rod repeats and either hinge 1 or 4, respectively. In cDNAs for the former four ΔDys, we reconstructed a putative triple-helical structure [8] at the fused position of the rod domain (Fig. 1B). We digested the genome of each propagated AxCAΔDys with restriction enzymes and confirmed the size of inserted cDNA (data not shown).

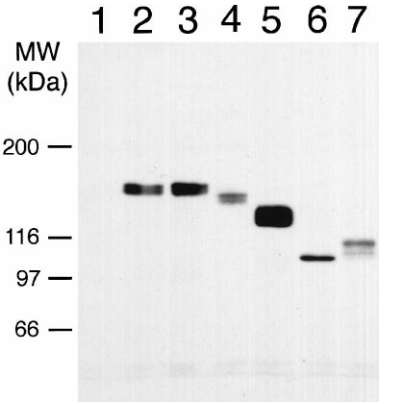

Fig. 2. Ad vector-mediated transfer of truncated dystrophin cDNAs with varying numbers of rod repeats into the murine myogenic cell line. C2C12 cells were infected with each recombinant AxCAΔDys at a moi of 100, after which they were induced to differentiate by changing the culture medium. Three days after infection, the cells were harvested. The total cell extract (20 μg/lane) was separated by SDS-PAGE (5% acrylamide), transferred to PVDF membrane and incubated with monoclonal antibody DYS2, which acts against the last 17 amino acids of dystrophin. Lane 1: non-infected C2C12 cells; 2: AxCAΔDysAX2; 3: AxCAΔDysAX11; 4: AxCAΔDysAH3; 5: AxCAΔDysM3; 6: AxCAΔDysH1; 7: AxCAΔDysH4. All AxCAΔDys expressed truncated dystrophin of the expected size, except AxCAΔDysH4; the product of AxCAΔDysH4 migrated more slowly than estimated (lane 7). MW: molecular weight.

3.2. Adenovirus vector-mediated expression of truncated dystrophins in a myogenic cell line

To verify the correct transcription and translation of truncated dystrophins after the propagation of their corresponding AxCAΔDys, we infected C2C12 myoblasts with each AxCAΔDys and performed Western blot analysis. Each ΔDys revealed its expected size (Fig. 2, lanes 2–6), but the band for ΔDysH4 appeared at a higher position than expected (103 kDa) (Fig. 2, lane 7). Endogenous dystrophin was not clearly detected in C2C12 cells because the cells were not fully differentiated into mature myofibers (Fig. 2, lane 1). When the quantity of each truncated dystrophin was compared, ΔDysM3 appeared in the largest amount. These results indicate that recombinant AxCAΔDys effectively infect cultured muscle cells and express truncated dystrophin under the regulation of the CAG promoter [20]. They also indicate that ΔDysM3 protein is most stably expressed in vitro.

3.3. Adenovirus vector-mediated expression of truncated dystrophins in the skeletal muscle of mdx mice

To determine whether truncated dystrophins are stably expressed in muscle fibers in vivo, we injected recombinant Ax-

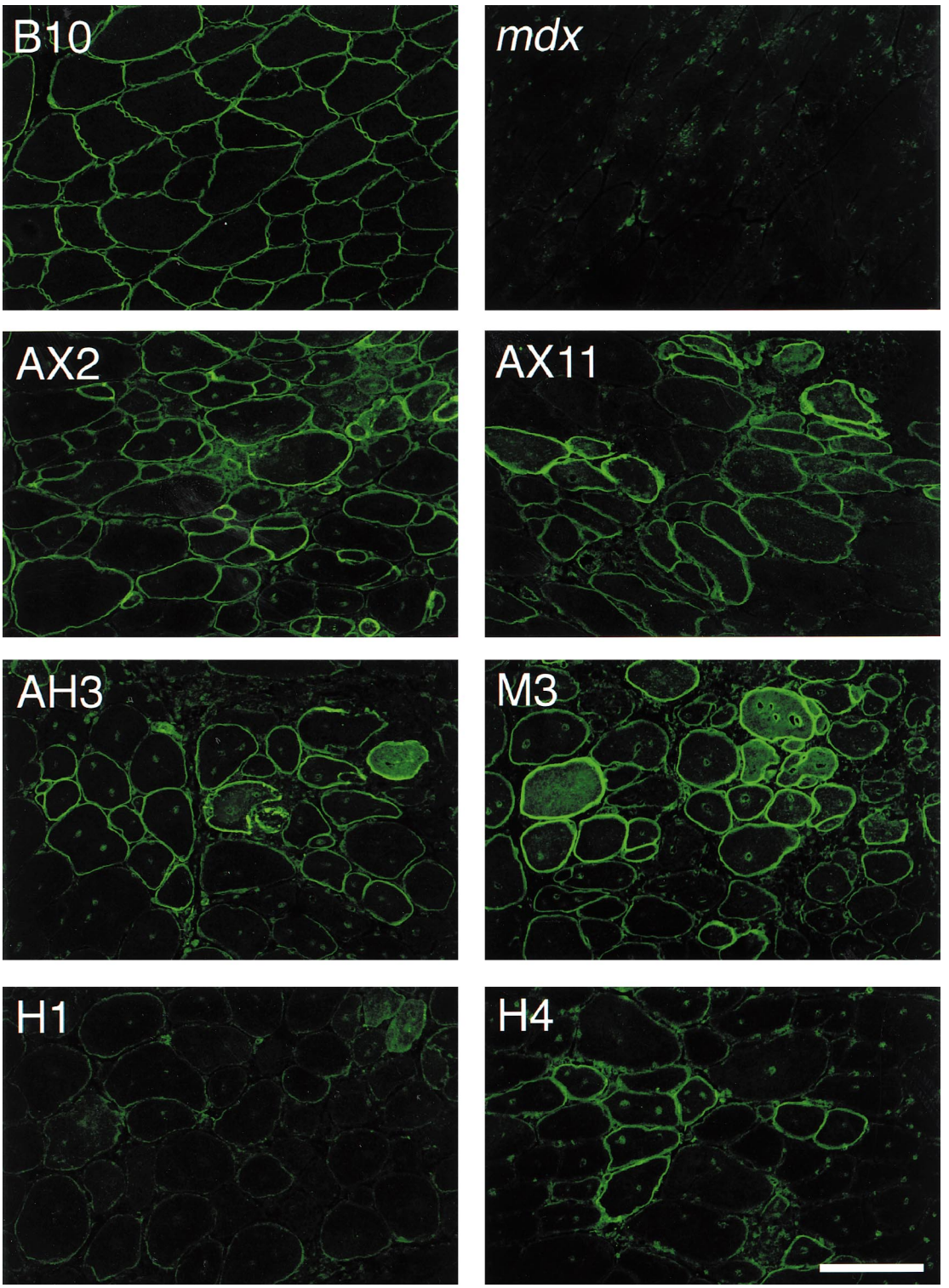

Fig. 3. Ad vector-mediated transfer of truncated dystrophin cDNA into the skeletal muscle of dystrophic *mdx* mice. Recombinant Ad was directly injected into the TA muscle of adult *mdx* mice. The dose of vector injected is listed in Table 2. Seven days after injection, mice were killed and frozen sections of the TA muscle were immunostained for dystrophin using a rabbit polyclonal antibody, anti-C, which recognizes the carboxy-terminus of dystrophin. B10: adult normal C57BL/10 mouse; *mdx*: non-injected *mdx* mouse; AX2, AX11, AH3, M3, H1, and H4: AxCAΔDys-injected *mdx* mice. Bar = 100 μm.

CAΔDys directly into the TA muscle of adult *mdx* mice and performed immunohistochemical analyses (Fig. 3). In the AxCAΔDys-injected *mdx* muscles, recombinant Ads evoked a strong infiltration of mononuclear cells and necrosis of muscle fibers (data not shown), as previously reported [14]. Dystrophin-positive fibers had a tendency to appear in a cluster at the periphery of the damaged area. This is where all truncated dystrophins, except ΔDysH1, are expressed at the sarcolemma; they often did so in greater intensity than endogenous dystrophin in control C57BL/10 mice when both were stained

Table 1
DNA sequences of synthetic oligonucleotides used for construction of truncated dystrophin cDNAs

Primer	Primer sequence (5′-3′)	Sequence position
F1	GCCGGCGAACAACTTAAGGTATTG	1799–1816
2	GCCGGCCTTAAGGAGGTCAATACTGAG	8936–8950
3	TTAAGGTATTGAACACCAGATGGA	1806–1816, 9269–9281
4	GCCGGCCAATTGGGAAGTAAGCTG	1409–1426
5	GGAACATGCATTCAACATCGAA	796–817
6	CAGGAAGTGGAAGCCCACAGGGACTTTGGTCCAG	953–964, 9329–9350
R1	GCCGGCCTCGAGACTTGATAACATTTC	2005–1991
2	GGCGCCTTGACTTTCTCGAGGTGATC	9144–9125
3	AGCTTCCATCTGGTGTTCAATACC	9285–9269, 1816–1810
4	GCCGGCAAGCTTCCATCTTGAATTTAG	1501–1486
5	CGGCAGGGCCTTCTGCAGTCTTCGGTCTTCAGGAGCTTCC	9564–9545, 1189–1174
6	GTCCCTGTGGGCTTCCACTTCCTGGATGGC TTC	9340–9329, 964–944
7	ATCTGCAGGATATCCATGG	9657–9639

Oligonucleotides F3 and R3 were used for direct annealing to form a DNA fragment. The other oligonucleotides were used as primers for the PCR reaction. Underlines correspond to the sequence of human dystrophin cDNA, GenBank accession number M18533.

on the same slide. The proportion of dystrophin-positive fibers is considerably higher than that of revertant fibers in *mdx* skeletal muscle. In addition, using antibody P23a [26], we also confirmed that dystrophin-positive fibers were not revertant fibers (data not shown). Though the intensity of dystrophin immunostaining in AxCAΔDys-injected muscles varied from fiber to fiber, strong immunofluorescence was consistently observed in AxCAΔDysM3-injected muscles. By contrast, the signal of the dystrophin-positive fibers at the sarcolemma was faint and patchy in AxCAΔDysH1-injected muscle.

To evaluate the effectiveness of each ΔDys in *mdx* mouse skeletal muscle, we selected, in each AxCAΔDys-injected *mdx* muscle, more than three areas where dystrophin-positive fibers formed clusters. Three independent researchers evaluated the numbers of truncated dystrophin-expressing fibers and the intensity of dystrophin immunofluorescence and the results are summarized in Table 2. The results indicate that truncated dystrophins containing some rod repeats and both hinge 1 and 4 localized effectively at the sarcolemma. The loss of hinge 4, as demonstrated by ΔDysH1, results in a great reduction of the dystrophin's localization at the sarcolemma.

3.4. Recovery of dystrophin-associated proteins at the sarcolemma

To evaluate dystrophin's function as a key molecule for the formation of the dystrophin-DAP complex, we examined the expression of DAPs at the sarcolemma of *mdx* skeletal muscles after AxCAΔDys injection (Fig. 4). In comparison with the reduced expression of DAPs in *mdx* skeletal muscle [29], sarcolemmal expression of DAPs was markedly retrieved in the dystrophin-positive fibers of AxCAΔDys-injected muscles, except AxCAΔDysH1-injected muscles. Very interestingly, the intensity of immunofluorescence for DAPs was homogeneous irrespective of the levels of dystrophin expression. In AxCAΔDysH1-injected *mdx* muscle, however, immunofluorescence of DAPs along sarcolemma was difficult to detect. The signals, especially for β-dystroglycan and α-sarcoglycan in ΔDysH1-injected muscle, were extremely low in the majority of dystrophin-positive fibers. These results show that truncated dystrophins, except for ΔDysH1, can effectively restore DAPs at the sarcolemma.

4. Discussion

We examined the expression of recombinant Ad constructs of truncated dystrophins in both cultured skeletal muscle cells and adult *mdx* mouse skeletal muscle in vivo. The use of a combination of a widely infectious Ad vector and the strong promoter in skeletal muscle, CAG [20,21], enabled us to compare the expression of recombinant truncated dystrophin cDNAs.

Surprisingly, ΔDysM3, which has only one rod repeat, showed the highest expression in vitro. When AxCAΔDys were injected into adult *mdx* skeletal muscle, ΔDysM3 was effectively expressed as well as ΔDysAX2, AX11, and AH3. Though the precise protein structure of ΔDysM3 is currently not known, we speculate that the ΔDysM3 molecule might be stable in the skeletal muscle of adult mice as well as in vitro. Clemens et al. [30] have generated three truncated dystrophin constructs with an in-frame deletion (3.0, 4.4 or 5.7 kbp dele-

Table 2
Quantitative analysis of Ad vector-mediated transfer of truncated dystrophin cDNAs into *mdx* skeletal muscle

Recombinant adenovirus	Dose of virus ($\times 10^8$ pfu/muscle)	Dystrophin-positive fibers[a] mean (range)	Intensity of immunofluorescence at sarcolemma[b]	*n*
AxΔDysAX2	8.6	32% (22–39)	++	4
AxΔDysAX11	2.2	27% (11–56)	++	4
AxΔDysAH3	14	33% (15–45)	++	4
AxΔDysM3	16	33% (22–51)	+++	8
AxΔDysH1	6.0	12% (3–22)	+	3
AxΔDysH4	13	21% (16–31)	++	3

Three regions were selected from each injected muscle and evaluated blind by three independent observers.
[a]The percentage of dystrophin-positive fibers in the selected regions.
[b]Intensity of sarcolemmal labeling of dystrophin was evaluated from 0 to +++.

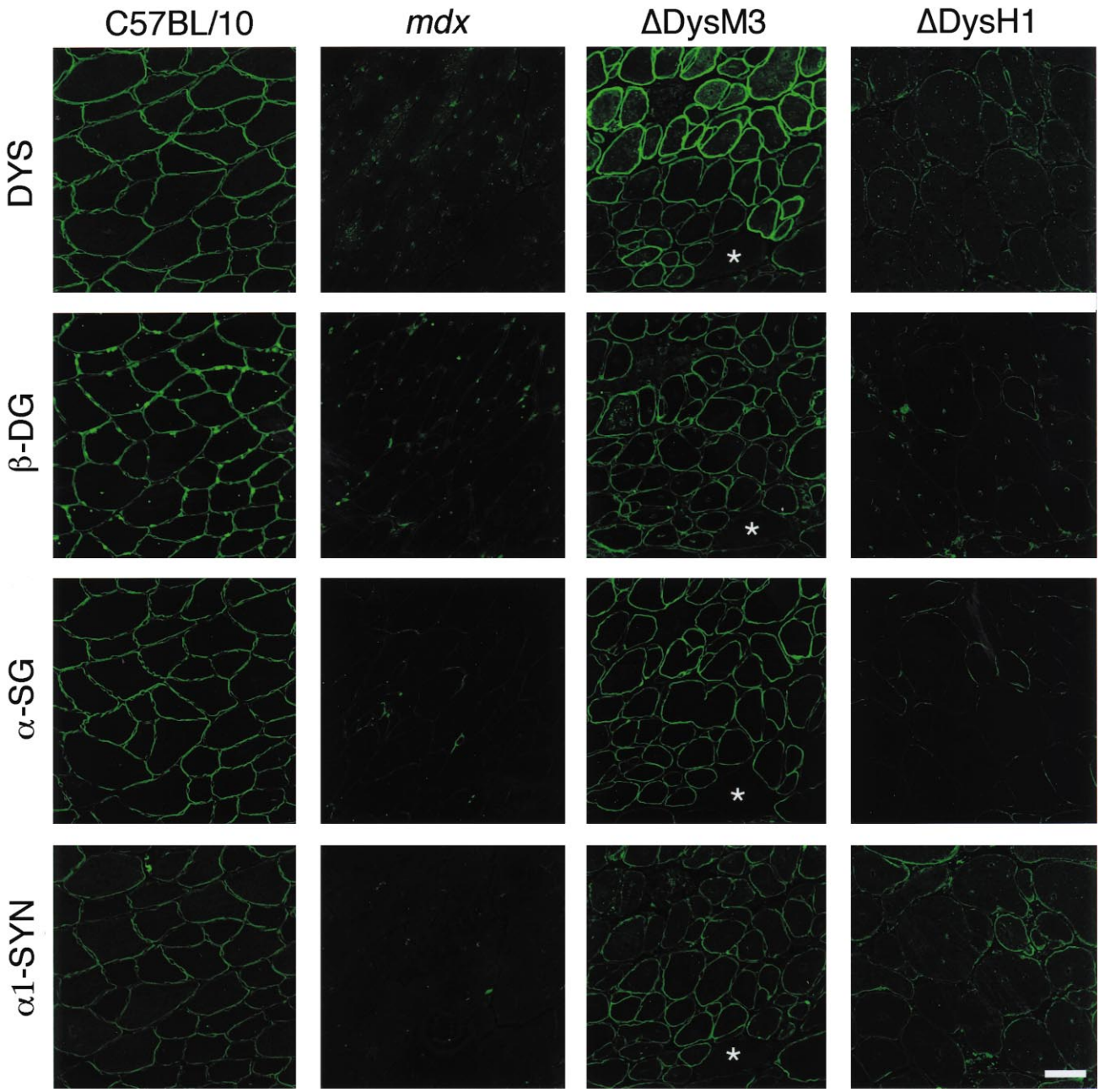

Fig. 4. Restoration of dystrophin-associated proteins at the sarcolemma in AxCAΔDysM3-injected *mdx* skeletal muscle, but not in AxCAΔ DysH1-injected *mdx* mice. Injections and immunostaining were performed as described in Section 2. The muscle fibers successfully expressing dystrophin in AxCAΔDysM3-injected *mdx* mice were stained intensely with antibodies against β-dystroglycan, α-sarcoglycan, and α1-syntrophin. The dystrophin-negative fibers, marked with an asterisk, were negative for DAPs. In AxCAΔDysH1-injected *mdx* muscle, the signal for dystrophin was markedly weak. In such dystrophin-positive fibers, DAPs, especially β-dystroglycan and α-sarcoglycan, were not detected at the sarcolemma. Bar = 50 μm.

tion) of the rod domain, where 15, 10 or six rod repeats remained in the final constructs. These constructs demonstrated that the amount of dystrophin produced in cultured myogenic cells is not exclusively determined by the size of the deletion. Together with our findings, we can conclude that the stability of the truncated dystrophin is independent of the number of rod repeats. These results are consistent with the observation that the length of the deletion in the dystrophin gene in BMD patients correlates with neither the amount of dystrophin produced nor the severity of the disease [9].

Although AxCAΔDysH1 and AxCAΔDysH4 were injected in high viral doses, their expression was considerably lower than that of the other constructs. This is likely because both ΔDysH1 and ΔDysH4 completely lack the rod repeats. In addition, it must be noted that the expression of ΔDysH1 was extremely low. ΔDysH1 lacks hinge 4, an area containing a WW domain [31] which seems to connect the dystrophin molecule to the sarcolemmal membrane by binding to the XPPXY motif of β-dystroglycan [32]. We suggest that ΔDysH1 is less stable because of its reduced binding ability to β-dystroglycan. ΔDysH4 does not have hinge 1. Although its missing rod repeat may largely explain its lower stability,

the lack of hinge 1 could be another contributing factor. Ortiz-Lopez et al. [33] identified a missense mutation in the hinge 1 region of a family of X-linked dilated cardiomyopathy found in North America, and presumed that the mutation results in a configurational change in the dystrophin molecule. Together with our results of retarded migration of ΔDysH4 in Western blot, hinge 1 might be critical for conformation of dystrophin molecule.

As predicted from transgenic studies of mini-dystrophin cDNA [15], small truncated dystrophins can support the accumulation of DAPs at the sarcolemma in *mdx* skeletal muscle if C-terminal parts of the dystrophin molecule are intact. Our results demonstrate that a truncated dystrophin that maintains both hinge 4 and the cysteine-rich domain can effectively accumulate DAPs at the sarcolemma. Our results also indicate that high level expression of ΔDys is not necessarily required for the full recovery of DAPs, as suggested by previous mini-dystrophin research [15].

It is, however, also important to note that the recovery of DAPs at the sarcolemma is not sufficient for the restoration of dystrophin function. For example, transgene experiments with Dp71 cDNA, which encodes one of the dystrophin isoforms lacking the actin-binding and rod domains, showed complete recovery of DAPs, but the dystrophic phenotype rather deteriorated [34,35]. Furthermore, Chamberlain and colleagues introduced *mdx* mice with a series of truncated dystrophin cDNAs as transgenes. They ascertained that a dystrophin construct which contained hinges 1 and 4 but has no rod repeats is stably expressed at the sarcolemma, but could not improve the dystrophic phenotype (personal communication). Thus, long-term expression of our truncated dystrophins is required to prove their function in vivo. The Ad vectors we used evoke strong immune reactions in adult mice and prevent long-lasting expression of the exogenous gene. Therefore, we injected AxCAΔDysM3 into neonatal *mdx* mice. Four weeks after the injection, degeneration of muscle fibers decreased and centrally nucleated fibers were almost absent in the Ad-injected regions (unpublished data). Thus, at present, ΔDysM3, containing only one rod repeat and intact N-terminal and C-terminal domains, is the smallest molecule required to alleviate the dystrophic phenotype. To confirm that this truncated dystrophin does indeed prevent dystrophic change, we are now generating transgenic mice of ΔDysM3 cDNA.

In this report we showed that the truncated dystrophins, ΔDysAX2, AX11, AH3, and M3, reveal a high efficiency of expression in adult *mdx* skeletal muscle. New vectors which evoke fewer immune reactions and allow long-standing expression of the transferred gene are under active consideration in the gene therapy of DMD. The AAV vector, which is one of them, has several attractive features including targeted integrating capacity, but only allows the insertion of up to 4.6 kbp of exogenous DNA fragment [17]. Accordingly, even the 6.3 kbp mini-dystrophin cDNA is too long to be inserted into AAV vectors. Our truncated dystrophin cDNAs, especially the 3.7 kbp cDNA for ΔDysM3, can be a good candidate to be inserted into AAV vectors.

Acknowledgements: We thank Dr. Y. Nonomura for a polyclonal antibody against the C-terminus of dystrophin, Dr. Y. Wakayama for polyclonal antibodies against β-dystroglycan and α-sarcoglycan, Dr. S. Kameya for a polyclonal antibody against α1-syntrophin, and Dr. M. Yoshida for a polyclonal antibody against the 19th rod repeat of dystrophin. The authors thank Dr. J.S. Chamberlain for providing information prior to publication and Mr. D.L. North for his review of the manuscript. This work was partly supported by grants for Nervous and Mental Disorders from the Ministry of Health and Welfare.

References

[1] Emery, A.E.H. (1993) Duchenne Muscular Dystrophy, 2nd edn., Oxford University Press, Oxford.
[2] Koenig, M., Hoffman, E.P., Bertelson, C.J., Monaco, A.P., Feener, C. and Kunkel, L.M. (1987) Cell 50, 509–517.
[3] Zubrzycka-Gaarn, E.E., Bulman, D.E., Karpati, G., Burghes, A.H.M., Belfall, B., Klamut, H.J., Talbot, J., Hodges, R.S., Ray, P.N. and Worton, R.G. (1988) Nature 333, 466–469.
[4] Arahata, K., Ishiura, S., Ishiguro, T., Tsukahara, T., Suhara, Y., Eguchi, C., Ishihara, T., Nonaka, I., Ozawa, E. and Sugita, H. (1988) Nature 333, 861–863.
[5] Hemmings, L., Kuhlman, P.A. and Critchley, D.R. (1992) J. Cell Biol. 116, 1369–1380.
[6] Suzuki, A., Yoshida, M., Hayashi, K., Mizuno, Y., Hagiwara, Y. and Ozawa, E. (1994) Eur. J. Biochem. 220, 283–292.
[7] Campbell, K.P. (1993) Cell 80, 675–679.
[8] Koenig, M. and Kunkel, L.M. (1990) J. Biol. Chem. 265, 4560–4566.
[9] Beggs, A.H., Hoffman, E.P., Snyder, J.R., Arahata, K., Specht, L., Shapiro, F., Angelini, C., Sugita, H. and Kunkel, L.M. (1991) Am. J. Hum. Genet. 49, 54–67.
[10] England, S.B., Nicholson, L.V.B., Johnson, M.A., Forrest, S.M., Love, D.R., Zubrzycka-Gaarn, E.E., Bulman, D.E., Harris, J.B. and Davies, K.E. (1990) Nature 343, 180–182.
[11] Ragot, T., Vincent, N., Chafey, P., Vigne, E., Gilgenkrantz, H., Couton, D., Cartaud, J., Briand, P., Kaplan, J.-C., Perricaudet, M. and Kahn, A. (1993) Nature 361, 647–650.
[12] Vincent, N., Ragot, T., Gilgenkrantz, H., Couton, D., Chafey, P., Grégoire, A., Briand, P., Kaplan, J.-C., Kahn, A. and Perricaudet, M. (1993) Nature Genet. 5, 130–134.
[13] Deconinck, N., Ragot, T., Maréchal, G., Perricaudet, M. and Gillis, J.M. (1996) Proc. Natl. Acad. Sci. USA 93, 3570–3574.
[14] Acsadi, G., Lochmüller, H., Jani, A., Huard, J., Massie, B., Prescott, S., Simoneau, M., Petrof, B.J. and Karpati, G. (1996) Hum. Gene Ther. 7, 129–140.
[15] Wells, D.J., Wells, K.E., Asante, E.A., Turner, G., Sunada, Y., Campbell, K.P., Walsh, F.S. and Dickson, G. (1995) Hum. Mol. Genet. 4, 1245–1250.
[16] Phelps, S.F., Hauser, M.A., Cole, N.M., Rafael, J.A., Hinkle, R.T., Faulkner, J.A. and Chamberlain, J.S. (1995) Hum. Mol. Genet. 4, 1251–1258.
[17] Dong, J.-Y., Fan, P.-D. and Frizzell, R.A. (1996) Hum. Gene Ther. 7, 2105–2112.
[18] Acsadi, G., Dickson, G., Love, D.R., Jani, A., Walsh, F.S., Gurusinghe, A., Wolff, T.A. and Davies, K.E. (1991) Nature 352, 815–818.
[19] Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C. and Saito, I. (1996) Proc. Natl. Acad. Sci. USA 93, 1320–1324.
[20] Niwa, H., Yamamura, K. and Miyazaki, J. (1991) Gene 108, 193–200.
[21] Araki, K., Araki, M., Miyazaki, J. and Vassalli, P. (1995) Proc. Natl. Acad. Sci. USA 92, 160–164.
[22] Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S. and Saito, I. (1995) Nucleic Acids Res. 23, 3816–3821.
[23] Saito, I., Oya, Y., Yamamoto, K., Yuasa, T. and Shimojo, H. (1985) J. Virol. 54, 711–719.
[24] Kanegae, Y., Makimura, M. and Saito, I. (1994) Jpn. J. Med. Sci. Biol. 47, 157–166.
[25] Yoshida, S., Fujisawa-Sehara, A., Taki, T., Arai, K. and Nabeshima, Y. (1996) J. Cell Biol. 132, 181–193.
[26] Yoshida, M. and Ozawa, E. (1990) J. Biochem. 108, 748–752.
[27] Wakayama, Y., Inoue, M., Murahashi, M., Shibuya, S., Jimi, T., Kojima, H. and Oniki, H. (1996) Ann. Neurol. 39, 217–223.
[28] Peters, M.F., Kramarcy, N.R., Sealock, R. and Froehner, S.C. (1994) NeuroReport 5, 1577–1580.
[29] Ohlendieck, K. and Campbell, K.P. (1991) J. Cell Biol. 115, 1685–1694.

[30] Clemens, P.R., Krause, T.L., Chan, S., Korb, K.E., Graham, F.L. and Caskey, C.T. (1995) Hum. Gene Ther. 6, 1477–1485.
[31] Sudol, M., Bork, P., Einbond, A., Kastury, K., Druck, T., Negrini, M., Huebner, K. and Lehman, D. (1995) J. Biol. Chem. 270, 14733–14741.
[32] Einbond, A. and Sudol, M. (1996) FEBS Lett. 384, 1–8.
[33] Ortiz-Lopez, R., Li, H., Su, J., Goytia, V. and Towbin, J.A. (1997) Circulation 95, 2434–2440.
[34] Cox, G.A., Sunada, Y., Campbell, K.P. and Chamberlain, J.S. (1994) Nature Genet. 8, 333–339.
[35] Greenberg, D.S., Sunada, Y., Campbell, K.P., Yaffe, D. and Nudel, U. (1994) Nature Genet. 8, 340–344.