Molecular Biology of Muscular Dystrophy

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Proceeding of International Workshop betwenn Japan and France

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Preface

This book represents the proceeding of international workshop between Japan and France entitled "Molecular Biology of Muscular Dystrophy" held in Tokyo, 17-18 January, 1996. The antecedent of the workshop dates back to 1995, when Robert G.Whalen and Hideo Sugita organized a small workshop in Paris. Progress in the field of muscular dystrophy has clearly been facilitated by the international cooperation, exchange of ideas and generous sharing of biological reagents. As such, we would like to acknowledge our indebtedness to Drs. Whalen and Sugita for setting these conferences in motion. All of us look forward to the next meeting tentatively scheduled for France in the Autumn of 1997.

Progress in the field of muscular dystrophy over the past several years has been impressive, and the contribution of Japan and France was becoming bigger and bigger. Especially, the cloning of the sarcoglycan genes and the subsequent identification of the mutations in some of the patients of limb-girdle muscular dystrophy has been done by Japanese and French researchers. This finding was high-lighted in this meeting. After identification of the causative gene of muscular dystrophy, most important step is to verify underlying mechanism of muscle necrosis, nevertheless the cause of muscle necrosis was deficiency of dystrophin, sarcoglycan, or laminin molecule.

One more important subject in this field is to develop gene therapy for muscular dystrophy. In life-threatened disease such as Duchenne muscular dystrophy, there is no more hopeful treatment than gene therapy. In this meeting, some of the challenging approach using cell-mediated transfer as well as using direct gene transfer, has been proposed. Progress next few year should tell the advantage and disadvantage of gene therapy.

We gratefully acknowledge Science and Technology Agency(STA) and Japan International Science and Technology Exchange Center(JISTEC) for their sponsorship of this meeting.

Eijiro OZAWA
Organizer

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IV. TOWARD GENE THERAPY INTO MUSCULAR DYSTROPHY

The localization of dystrophin and its associated proteins on the sarcolemma of mammalian striated muscle cells

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Abstract

In confocal laser microscopy combined with indirect immuno-fluorescence with a monoclonal anti-dystrophin antibody, dystrophin was seen to be localized along the sarcolemma in a nonuniform and even discontinuous pattern in normal guinea pig skeletal and cardiac muscle cells. Sections cut tangential to the sarcolemma showed a lattice-like image of longitudinal and transverse striations. The transverse striations were often regularly spaced resembling the sarcomere pattern. An intense immuno-fluorescence staining was seen at the myotendinous junctions in skeletal muscle cells and at the intercalated discs in cardiac muscle cells. This staining pattern suggests that dystrophin may also be localized in the adherens junctions where myofibrils attach. When muscle cells were stained with both anti-dystrophin and anti-β-dystroglycan, the staining pattern was completely identical suggesting that both proteins are colocalized along the sarcolemma. In contrast, the staining of vinculin was not well superimposed on that of dystrophin.

To clarify the exact relationship between the sarcolemma and dystrophin and other related proteins, crude sarcolemmal vesicles were prepared from rabbit and rat skeletal muscles according to the methods described by Ohlendieck et al.(1991). When such crude sarcolemmal vesicles were examined by negative staining and thin section electron microscopy, they were found to be heterogeneous in population varying in size and shape. Among them, large vesicles of irregular shape contained fine filamentous structures and vesicular fragments. Such filamentous structures formed networks which were closely associated with the inner surface of the vesicle membrane. Interestingly, the

filamentous networks were restricted to the membrane domains where particulate structures projected from the outer surface. Gold-labeled wheat germ agglutinin (WGA) selectively bound to such large vesicles. With some other findings, the large vesicles were considered to be of sarcolemmal origin. When the crude sarcolemmal vesicles were mounted on a grid and immunologically stained with anti-dystrophin antibody after brief Triton treatment, immunogold particles were found only on the filamentous networks beneath the vesicle membranes. Replica electron microscopy showed characteristic granular structures on most of large vesicles. They seemed to correspond to the particulate structures projecting from the outer surface of vesicles as seen in thin sections. Exogenous laminin could be reconstituted on the sarcolemmal vesicles in close association with such particulate structures, suggesting that they may represent dystroglycan complexes.

Dystrophin: localization and regulatory regions for expression

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Introduction

Although the gene that, when defective, results in Duchenne muscular dystrophy was isolated and called dystrophin gene[1-2)], mechanism of muscle degradation in this condition is still obscure, because of the unknown physiological or mechanical function of the gene transcript. To determine the localization and functional significance of dystrophin, we studied various tissues from almost entire body of control and mdx mice, and control rats, using polyclonal antibodies against dystrophin. We also studied human muscle biopsy specimens.

It is known that the muscle type promoter is involved in expression of the dystrophin gene in skeletal, cardiac and smooth muscle as well as in glial cells. It is conceivable that the muscle type dystrophin promoter contains the different regulatory regions for skeletal, cardiac and smooth muscle in vivo, but the detail is not well known. With this background we started to investigate the mechanism of developmental and tissue-specific regulation of the muscle type dystrophin gene, especially promoter region in mice, using transgenic mice.

Materials and methods

Immunohistochemical study

All the tissues from mice and rats, including the cerebrum, cerebellum, spinal cord, eye, tongue, nose, heart, lung, kidney, digestive organs, liver, spleen, muscle, skin and blood vessels were obtained immediately after anesthetic death. Indirect immunofluorescence and ABC method were used to stain cryostat sections on gelatinized coverslips. We used several kinds of dystrophin antibodies(Fig. 1).

Transgenic mice

We generated transgenic mice carrying the 900bp genomic fragment from the muscle type dystrophin promoter region, fused to the coding region of the bacterial *lacZ* gene(Fig. 2). Inserts from pBlue dys promoter-*lacZ* were excised by Xhol-BamHl digestion, purified with Gene Clean(Bio 101), and micro-injectedinto 400 fertilized mouse eggs collected after mating(Fig. 3).

Results

Immunohistochemical study

We observed a immunohistochemical dystrophin reaction in synaptic regions such as neuromuscular junctions, equatorial region of intrafusal fibers, myo-tendinous junctions, outer plexiform layer of the retina, the taste buds, papillary layer of the dermis, tactile nerve endings in the skin and neurons in the brain, as well as on the surface membrane of skeletal, cardiac and smooth muscle fibers. Then we analyzed mRNAs from the retina of mice with the use of the reverse transcription and polymerase chain reaction (RT-PCR) method. The 5' sequences, corresponding to the first exon, of dystrophin transcripts (DT) in the retina was mainly the brain type(Fig. 4), whereas in the 3' region of DT that corresponds to the C-terminal domain of dystrophin, some additional RT-PCR products were detected.

Base sequences in three of them showed homology to these for previously reported human brain type dystrophin isoforms(Fig. 5).

Transgenic mice

Nineteen independent transgenic founder mice were identified, in six of which the expression of *lacZ* was detected in the right heart. Fourteen of the nineteen transgenic lines produced offspring. Various tissues of transgenic adult mice were stained with X-gal to detect *lacZ* expression. Six of the fourteen transgenic mouse lines showed *lacZ* expression only in the right heart(Fig. 6). The reporter gene expression was detected first in the presumptive right atria and ventricular myocardium of the embryos at 8.5 days post coitum.

We compared the promoter sequence to that of the previously reported the human sequence of 850bp region upstream from the cap site[3] and reported the mouse sequence of 400bp upstream from the cap site[4]. The homology between the human and mouse 140bp regions upstream from the initiation codon reaches the 93%. Upstream from this point, the mouse and human sequences look more divergent (66.8% homology). Non-specific region, ATA and GC box, and muscle specific regions, CArG box, are conserved in human and mouse.

Discussion

We observe rather strong immunohistochemical reactivity in synaptic regions[5-9] as well as on the skeletal, cardiac and smooth muscle fiber membrane. It is well known that these synaptic regions, which showed immunohistochemical dystrophin reaction, contain abundant synapses and/or non-myelinated nerve endings, as a common histological characteristics. With this background we consider that the localization of dystrophin in the synaptic regions suggests its physiological function in the conduction system rather than a mechanical one. In 1993, Pillers et al.[10] reported abnormal electroretinogram in Duchenne and Becker muscular dystrophy patients, that is markedly reduced amplitude of the b-wave in the dark-adapted state. The results suggested that the dystrophin has some function to keep normal function in the retina. Recently, D'Sauza et al.[11] finally reported a novel isoform of dystrophin (Dp260) present in the mouse retina and that is required for normal function in the retina. So, we still don't know the full length brain type dystrophin has function or not.

But, anyway it is not likely that dystrophin plays a critical role in the conduction system, because there has been no report of apparent retinal abnormalities in DMD patients. Dystrophin may play a delicate role, acting as

only a trigger propagating contraction within the cell. Dysfunctioning of the conduction system due to a defect in dystrophin is still an important factor in the muscle degradation in DMD. As for the vascular system, no smooth muscle cells receive nerve endings, which are usually several microns away from the cells. This suggests that the vascular system may be one of the most vulnerable tissues to a defect in dystrophin although it play a minor role in the conduction system due to anatomical characteristics[12]. I would like to emphasize that we should not ignore the localization and function of dystrophin on the smooth muscle layer, especially on the vascular system. As for the transgenic mice, by using the E.coli *lac Z* reporter gene instead of the CAT assay, it has been possible to follow visually the temporal and spatial expression of dystrophin both during embryonic and postnatal mouse development. The *lacZ* gene has advantage of visualizing expression in specific organs.

We found that the 900bp promoter region of mouse muscle type dystrophin was active only in the right heart, i.e. not in skeletal smooth or left heart muscle. On the other hand, Muntoni et al.[13] reported family cases with only severe cardiomyopathy, i.e. no clinical muscle symptoms, due to possible deletion the muscle type dystrophin promoter, but the cardiac-specific region of the dystrophin promoter has not been identified to date. Our data in this study may be convenient to explain the cases reported by Muntoni et al.

These results suggest that several delicately divided elements for dystrophin expression exist in muscle tissues, such as left heart, skeletal and smooth muscle cell.

Fig. 1 **Antidystrophin antibodies**

Affinity-purified, region-specific dystrophin antibodies used in this study. kD=kDa

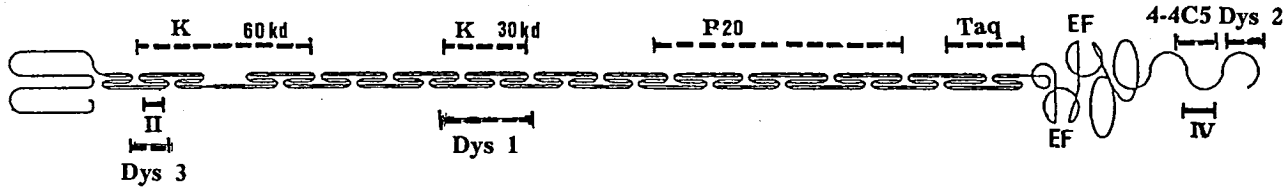
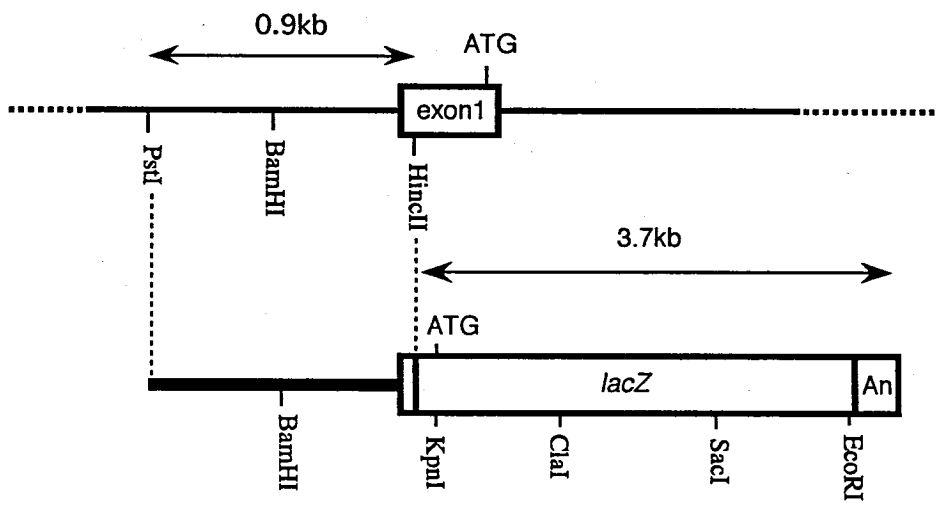


Fig. 2

Mouse Dystrophin Promoter-*lacZ* Constract



Structure of dystrophin muscle type promoter lacZ transgene.
900bp PstI-HincII fragment derived from Mouse dystrophin promoter region was fused to the coding region of the E.coli, lacZ gene.

Fig. 3

Scheme of the procedure for producing transgenic mice

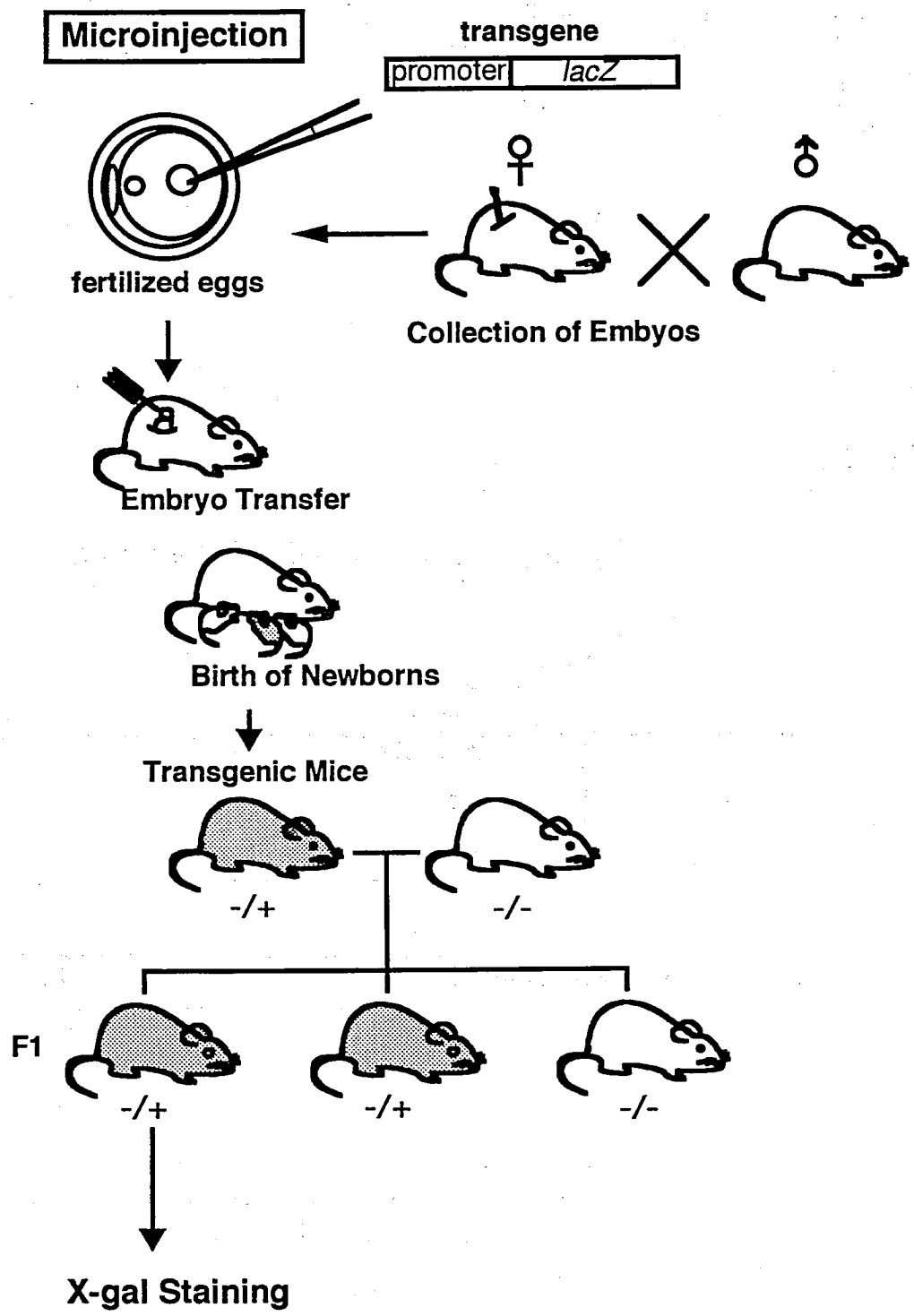
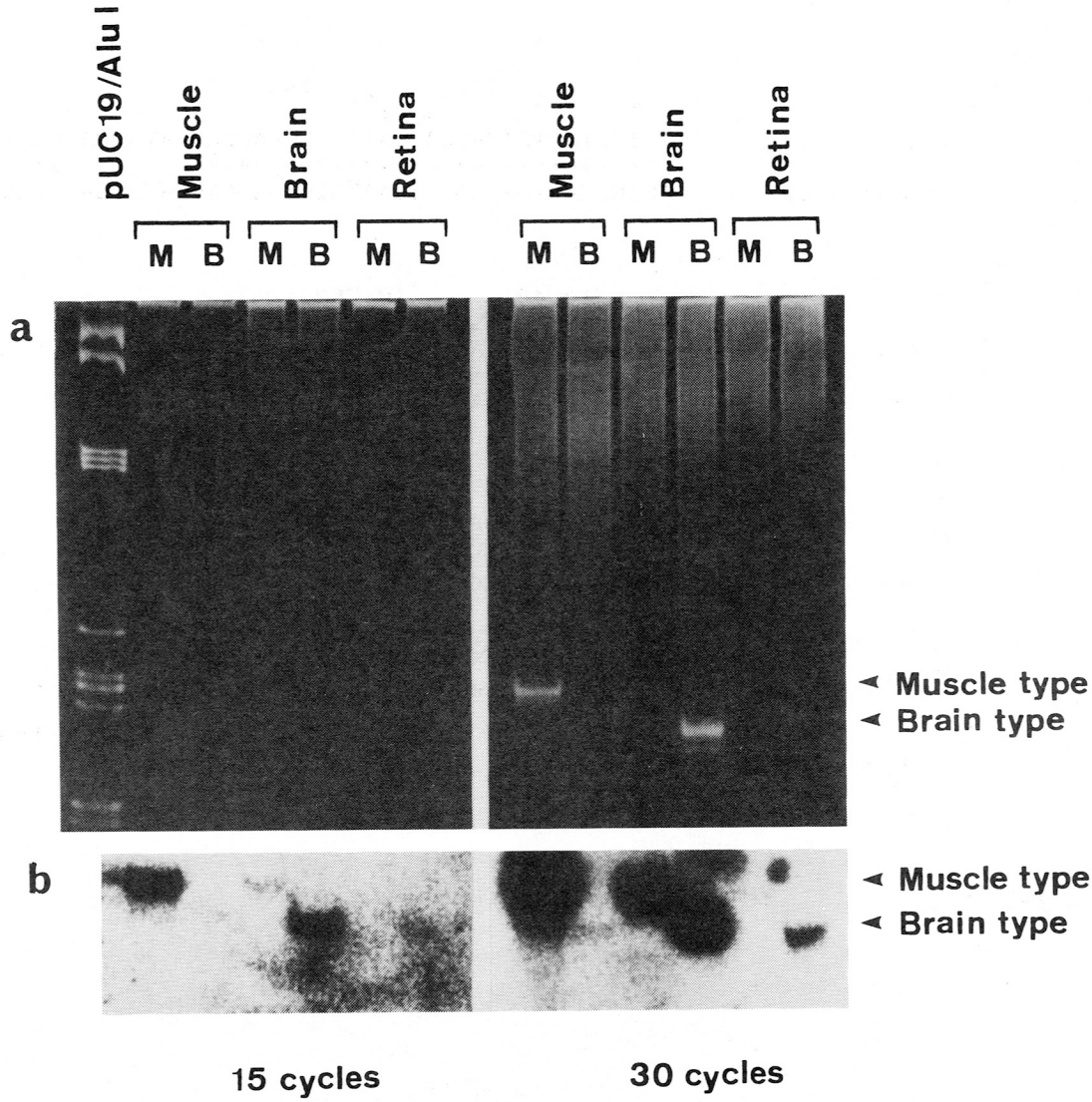
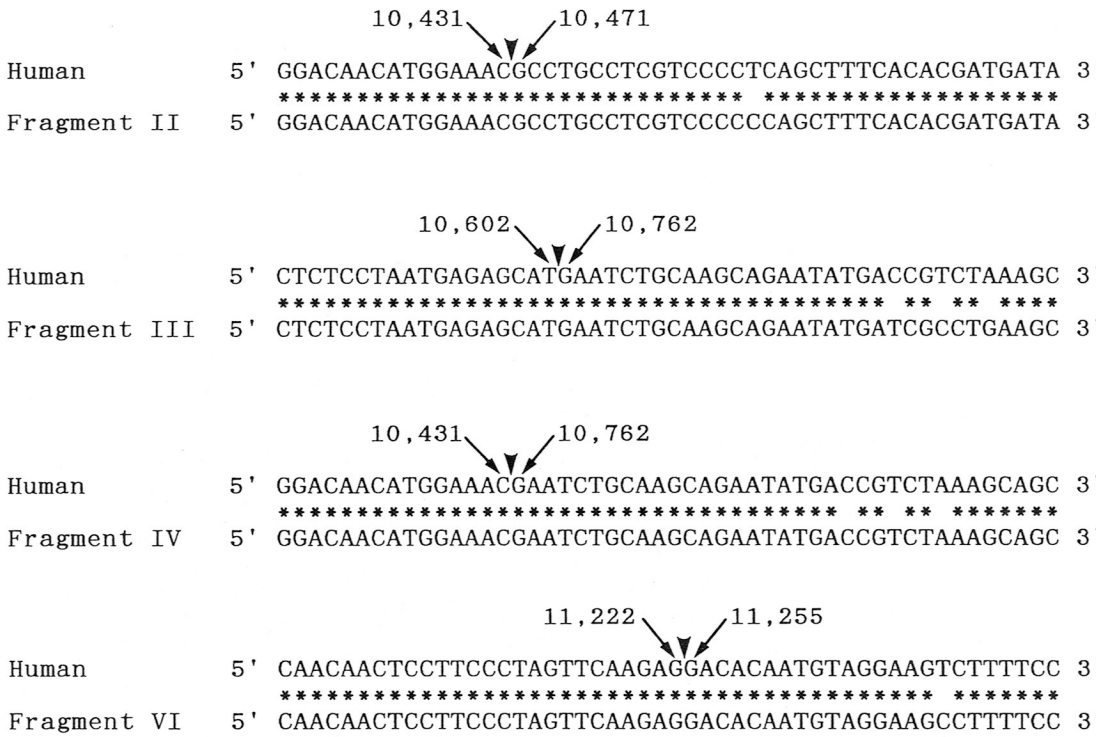


Fig. 4



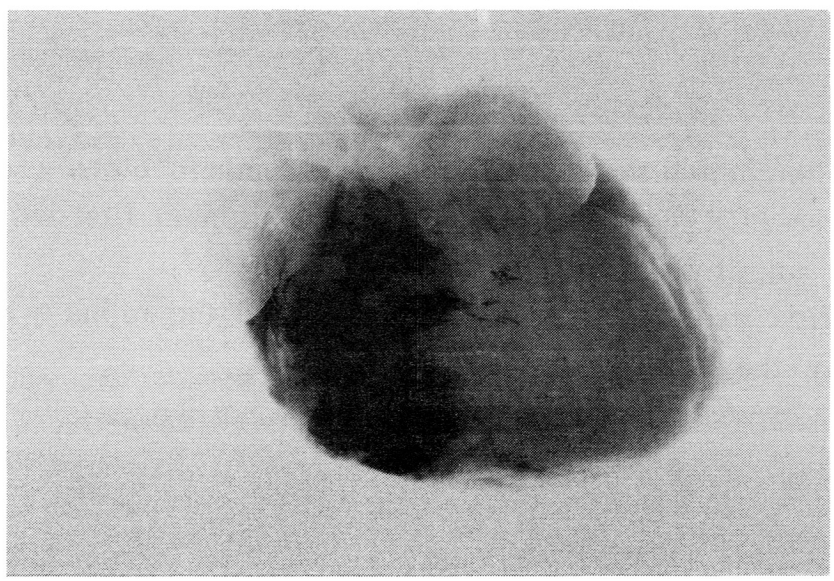
(a): Ethidium bromide staining. (b): Southern blots.
Reverse PCR products in which the dystrophin first exon was amplified.
The first exon of dystrophin transcript in the retina was mainly the brain type.

Fig. 5



Fragments II and III show the sense sequence of the mouse dystrophin cDNA, and fragments IV and VI the antisense sequence. Arrowheads indicate borders of deleted sequences. Asterisks show matched sequences between the human and the mouse cDNA.

Fig. 6



Expression of the transgene is seen only in the right ventricle of the heart.

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Modulation of splicing of intron by modifying an intra-exon sequence which is deleted from the dystrophin gene in dystrophin Kobe

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Together, Duchenne and Becker muscular dystrophies (DMD and BMD, respectively) are the most common inherited muscular disease. They are caused by mutation in the dystrophin gene, is the largest known human gene (1). Dystrophin, a large (427 kD) cytoskeletal protein localized to the sarcolemma, is encoded by a 14-kb RNA transcript that is processed from 79 exons (2). Results of a recent study conducted in our laboratory have extended this gene region by more than 500 kb through the identification of a new promoter/exon sequence upstream of the known 5' end of the gene and expanded the dystrophin gene to around 3,000 kb (3). At least seven promoters including ours have been identified in the dystrophin gene, and each promoter is expressed in a tissue-or development -specific manner, giving rise to multiple isoforms of dystrophin (4). Alternative splicing of introns further increases the number of dystrophin isoforms, each of which may have a specific, unique function. considering the larger number of both exons and alternative splicing sites, splicing abnormality of dystrophin pre-mRNA is supposed to be more complex. In one particular dystrophin gene mutation named dystrophin Kobe, we found that exon skipping during splicing was induced by the presence of intra-exon deletion mutation in the genome, although all of the consensus sequences known to be required for splicing were unaffected. The deletion was detected by PCR analysis, which revealed that the product amplified from the exon 19 encompassing region from the DMD case was smaller than normal. This result suggested the presence of a novel mutation within the amplified region. Sequence analysis confirmed that this was the case by showing that 52 bp out of

88 bp of exon 19 were deleted from 2 to 3 bp upstream from the splice donor site (5). This 52 bp deletion was considered to result in a frameshift mutation that would cause DMD. The dystrophin transcript of dystrophin Kobe was then analyzed using reverse-transcription PCR (RT-PCR). Surprisingly enough, the product amplified from the region encompassing exon 19 was smaller than predicted according to the results of the genomic DNA analysis. Sequence analysis indicated that the whole of exon 19 was missing from the dystrophin cDNA, causing an out-of frame mutation. In particular, this indicated that the deletion mutation within an exon sequence could induce a splicing error during maturation of messenger RNA, even though the known consensus sequences at the 5' and 3' splice sites of exon 19 were maintained (6).

These data suggest that the deleted sequence of exon 19 may function as a cis-acting element for exact splicing for the upstream and downstream introns. To investigate this potential role of exon 19, an *in vitro* splicing system using artificial dystrophin mRNA precursors (pre-mRNAs) was established. Pre-mRNA containing exon 18, truncated intron 18, and exon 19 was spliced precisely *in vitro*, whereas splicing of intron 18 was almost completely abolished when the wild-type exon 19 was replaced by the dystrophin Kobe exon 19 (7). Splicing of intron 18 was not fully reactivated when dystrophin Kobe exon 19 was restored to nearly normal length by inserting other sequences into the deleted site. These results suggest that the presence of the exon 19 sequence which is missing in dystrophin Kobe is more critical for splicing of intron 18 than the length of the exon 19 sequence. Characteristically, the efficiency of splicing of this intron seemed to correlated with the presence of a polypurine track within the downstream exon 19. More recently, several exons have been shown to included purine-rich regions, called exon recognition sequences or ERSs, which are necessary for splicing of the upstream intron (8). ERSs are likely targets for splicing factors that identify exon sequences and promote their inclusion in the mature mRNA.

The possibility that oligonucleotides could be used as modulators of gene expression, and hence as chemotherapeutic agents, is currently under intense investigation: The modulation of splicing by antisense oligonucleotides has recently attracted much attention (9, 10). Dominski and Kole recently described an elegant experiment in which aberrant splicing induced by a thalassemia mutation was corrected by an antisense 2'-O-methylribonucleotide

(2'-O-Me RNA) (10). This prompted us to test whether splicing of dystrophin pre-mRNA could also be modulated by an antisense RNA as the first step towards evaluating the potential therapeutic use of antisense RNA to correct aberrant splicing reactions in patients with DMD. An antisense 31 mer 2'-O-methyl ribonucleotide complementary to the 5' half of the deleted sequence in dystrophin Kobe exon 19 inhibited splicing of wild-type pre-mRNA in a dose- and time-dependent manner in vitro splicing system (7).

To simulated this inhibition in living cells, the antisense oligonucleotide mixed with LipofectAmine (GIBCO BRL) was added to the culture medium of EB-virus transformed lymphoblastoid cells. Resulting dystrophin transcript was analyzed by RT-nested PCR and sequencing. The result revealed that sequence of exon 19 disappeared from mRNA after a certain period of incubation of lymphoblastoid cells with antisense oligonucleotide. This clearly showed that exon skipping can be induced by antisense oligonucleotide.

This first *in vitro* evidence that dystrophin pre-mRNA splicing can be modulated by an antisense oligonucleotide raised the possibility of a new therapeutic approach for DMD. DMD would be transformed to mild BMD by changing a frame-shift mutation causing DMD into an in-frame mutation characteristic of BMD by modifying the dystrophin mRNA splicing (11). For example, the same antisense oligonucleotide will be used to treat a DMD case with a 242 nucleotide deletion of exon 20 (Fig. 1). If we are able to induce exon 19 (88 bp) skipping in vivo, the dystrophin transcript will lack both exons 19 and 20 but the translational reading frame will be restored. As a result, this modulation of splicing should transform DMD into BMD. Though this strategy seems to be an alternative way to retard progression of the clinical symptoms of DMD, more extensive studies are required before clinical trials can begin; for example, we need to confirm modulation of splicing by antisense oligonucleotide in an in vivo experiment. We also need to develop an efficient method to deliver antisense oligonucleotides to the nucleus.

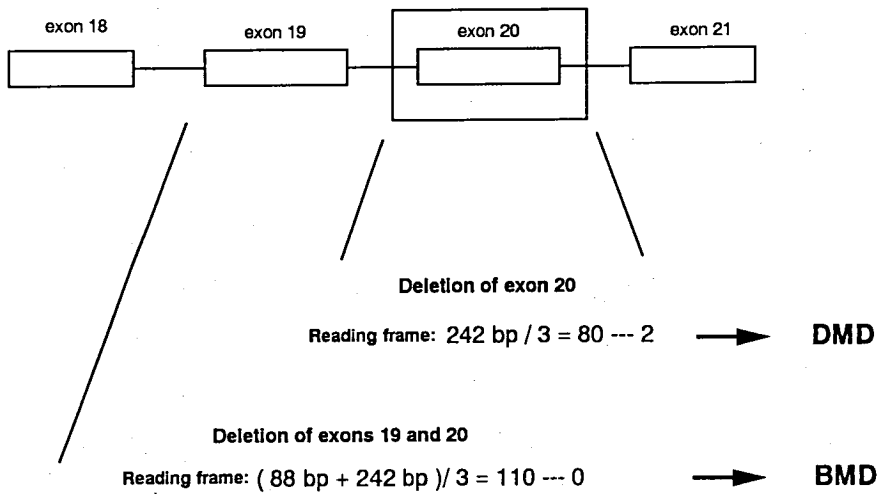

Fig. 1 Theoretical use of antisense oligonucleotide for treatment of DMD. The DMD phenotype of the patient with an exon 20 deletion would be transformed into a BMD phenotype by inducing the exclusion of exon 19 from the matured transcript in the presence of antisense 2'-O-Me RNA.

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Characterization of the Interaction between the Schwann Cell Dystroglycan Complex and Endoneurial Laminin

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Introduction

Dystroglycan is encoded by a single gene and cleaved into two proteins α- and β-dystroglycan by posttranslational processing (Ibraghimov-Beskrovnaya et al., 1992). In skeletal muscle, α-dystroglycan with a molecular mass of 156 kDa binds the basal lamina component laminin (Ibraghimov-Beskrovnaya et al., 1992; Ervasti and Campbell, 1993), and β-dystroglycan binds to cytoskeletal proteins dystrophin and utrophin (see Ozawa et al., 1995). Laminin is a heterotrimer made up of three chains of classes α, β and γ, and exists in numerous trimeric isoforms in different tissues (see Burgeson et al., 1994). Laminin α chains have globular domain (G) repeats at the C-terminus, and laminin-1, comprised of the α1, β1 and γ1 chains, binds to α-dystroglycan via the G repeats in the α1 chain (Gee et al., 1993). In peripheral nerve, laminin-2, comprised of the α2, β1 and γ1 chains, is expressed in the endoneurium (Leivo and Engvall, 1988; Sanes et al., 1990). Alpha-dystroglycan with a molecular mass of 120 kDa is expressed surrounding peripheral nerve fibers *in vivo* and binds both laminin-1 and 2 *in vitro* (Yamada et al., 1994). Recently, laminin α2 chain was shown to be deficient in congenital muscular dystrophy and *dy* mice, which have muscular dystrophy and peripheral dysmyelination (Arahata et al., 1993; Sunada et al., 1994; Tomé et al., 1994; Xu et al., 1994). Furthermore, the expression of laminin in the basal lamina is associated with Schwann cell development (Leivo and Engvall, 1988; Obremski and Bunge, 1995). These findings suggest a role for the interaction of dystroglycan with laminin in peripheral myelinogenesis. The goal of our laboratory is to prove this hypothesis. As

an initial step towards this goal, we have characterized the dystroglycan-laminin interaction in peripheral nerve (Yamada et al., 1996).

Characterization of the dystroglycan complex in peripheral nerve

The results of cLSM analysis of rabbit sciatic nerve are shown in Fig 1. Immunoreactivities for α-, β-dystroglycan and laminin-2 were localized surrounding the outermost layer of myelin sheath of nerve fibers (Fig. 1). On the other hand, immunoreactivities for utrophin and Dp116, a dystrophin gene product specific to peripheral nerve (Byers et al., 1993), resembled that for Schwann cell marker S-100 and was localized in the Schwann cell cytoplasm (Fig. 1).

Membrane-associated cytoskeletal and peripheral membrane proteins, but not integral membrane proteins, are known to be extracted from the membranes by alkaline treatment (Ervasti and Campbell, 1991). Based on this, we performed alkaline extraction of the crude bovine peripheral nerve membranes. Alpha-dystroglycan of 120 kDa was extracted at pH 11, while β-dystroglycan of 43 kDa was not extracted at pH 11 or 12 (Fig. 2). Together with the fact that α-dystroglycan corresponds to the N-terminal half of the dystroglycan precursor protein containing the signal peptide but no transmembrane domain and β-dystroglycan corresponds to the C-terminal half containing a transmembrane domain (Ibraghimov-Beskrovnaya et al., 1992), these results suggested that peripheral nerve α-dystroglycan was a peripheral membrane protein, while peripheral nerve β-dystroglycan was an integral membrane protein. The extracellular matrix protein laminin α2 chain of 320 kDa was partially extracted at pH 11 and almost completely extracted at pH 12 (Fig. 2), together with the β1 and γ1 chains (not shown). Dp116 and utrophin, of 116 and 395 kDa respectively, were both extracted at pH 11 (Fig. 2), in good agreement with the prediction from the primary structure that they are cytoskeletal proteins (Byers et al., 1993; Tinsley et al., 1994).

We isolated, using WGA and laminin affinity chromatographies, α-dystroglycan and its associated proteins from the digitonin extracts of the crude bovine peripheral nerve membranes. As expected, laminin-binding α-dystroglycan was isolated by these procedures (Fig. 3). Beta-dystroglycan, which did not bind laminin in blot overlay, was also isolated (Fig. 3),

suggesting that it was complexed with α-dystroglycan. On the other hand, Dp116 and utrophin were not isolated by these procedures (Fig. 3).

The data presented here, taken together, indicate that (1) α-dystroglycan is an extracellular peripheral membrane glycoprotein which links β-dystroglycan in the Schwann cell outer membrane with laminin-2 in the endoneurial basal lamina, and (2) dystrophin homologues, Dp116 and utrophin, are cytoskeletal proteins of the Schwann cell cytoplasm (see Fig. 7). It remains to be determined if a fraction of Dp116 and utrophin are complexed with β-dystroglycan beneath the Schwann cell outer membrane (see Fig. 7).

Characterization of carbohydrate chains of Schwann cell α-dystroglycan

We isolated, using WGA and laminin affinity chromatographies, α-dystroglycan from the alkaline extracts of the crude bovine peripheral nerve membranes. We tested its reactivity with monoclonal antibodies specific for carbohydrate chains. Alpha-dystroglycan was stained intensely with monoclonal antibody anti-HNK-1, indicating that it had 3-sulfated glucuronyl-substituted oligosaccharide (Fig. 4*a*) (Chou et al., 1986). On the other hand, it was not stained with monoclonal antibodies against heparan sulfate, keratan sulfate, sialyl Tn, Lewis X, sialyl Lewis X (Fig. 4*a*) or sialyl Lewis A (not shown).

The lectin-binding properties of Schwann cell α-dystroglycan are shown in Fig. 4*b*. Schwann cell α-dystroglycan was stained positive with WGA which recognizes cluster of sialic acids and/or bisecting GlcNAc (Fig. 4*b*), but was not stained with PHA-E4, a lectin specific for bisecting GlcNAc (not shown), indicating that it contained cluster of sialic acids. It was stained positive with both *Maackia amurensis* agglutinin (MAA), a lectin specific for sialic acid linked α2-3 to galactose, and peanut agglutinin (PNA), a lectin specific for unsubstituted Ser/Thr-linked disaccharide Galβ1-3GalNAc unit (Fig. 4*b*). It was also stained positive with Jacalin which also recognizes Galβ1-3GalNAc (Fig. 4*b*). It was not stained with *Sambucus nivalis* agglutinin (SNA) which recognizes sialic acid linked α2-6 to galactose (Fig. 4*b*). These results indicate that Schwann cell α-dystroglycan is sialylated α2-3 to galactose in O-glycans. Schwann cell α-dystroglycan was stained positive with VVA-B_4 (Fig. 4*b*), indicating that it contained non-reducing terminal β-

GalNAc. It was stained positive with both Con A and *Galanthus nivalis* agglutinin (GNA) (Fig. 4*b*), indicating that it contained N-linked mannose residues. It was not stained with *Datura stramonium* agglutinin (DSA) which recognizes Galβ1-4GlcNAc in complex and hybrid N-glycans, in O-glycans and GlcNAc in O-glycans (Fig. 4*b*). The results of staining with Con-A, GNA, PHA-E4 and DSA, when combined, suggest that the N-glycan(s) of Schwann cell α-dystroglycan could be high-mannose type. Schwann cell α-dystroglycan was not stained with lectins specific for various types of fucose residues, AAL, UEA-1 (Fig. 4*b*), LCA or Lotus (not shown).

Role of carbohydrate chains of Schwann cell α-dystroglycan in laminin binding

To see if carbohydrate residues of α-dystroglycan are involved in laminin binding, we tested, by blot overlay assay, the binding of laminin to Schwann cell α-dystroglycan treated with deglycosylation enzymes. Treatment of α-dystroglycan with both *A. ureafaciens* neuraminidase (Fig. 5) and *V. cholerae* neuraminidase (not shown) greatly reduced the binding of laminin. Treatment with O-glycosidase, together with neuraminidase, did not have further effects on the binding of laminin (Fig. 5). The binding of laminin was not affected by treatment with N-glycosidase F, chondroitinase ABC, heparinase, heparitinase or keratanase (Fig. 5). When nitrocellulose transfers of α-dystroglycan were treated with neuraminidase, the binding of laminin to α-dystroglycan was reduced (Fig. 6*a*). The presence of 0.1 M sialic acid, but not 0.1 M GlcNAc, in the blot overlay medium inhibited the binding of laminin to α-dystroglycan (Fig. 6*b*). Inhibition of laminin-binding by sialic acid was quantified by ELISA. Two forms of sialic acid, N-acetyl and N-glycolyl neuraminic acids, and colominic acid, polymer of sialic acids, inhibited the binding of laminin (Fig. 6*c*).

Our findings suggest a role for sialylation of α-dystroglycan in the binding of laminin. The results thus far indicate that α-dystroglycan is a mucin-type glycoprotein in brain (Smalheiser and Kim, 1995), cardiac muscle (Brancaccio et al., 1995) and peripheral nerve (this study). A mucin-like motif is detectable in the central region of α-dystroglycan, and, indeed, cardiac muscle α-dystroglycan was shown to consist of two globular domains connected by a rod-shaped segment (Brancaccio et al., 1995), consistent with

the prediction that a mucin domain is extended into a rigid rod. The densely clustered array of O-glycans in the mucin domain may present terminal sialic acids in a polyvalent manner for the interaction with laminin (Fig. 7*c*).

Besides α-dystroglycan, laminin binds a variety of glycolipids and proteins (see Kennedy et al., 1983; Gee et al., 1993). At present, it is unclear if sialylation alone is responsible for the binding of α-dystroglycan with laminin, because laminin was suggested to recognize highly charged oligosaccharides on proteins rather than monosaccharides (Kennedy et al., 1983). In this respect, it is of interest to note that, similar to brain α-dystroglycan (Smalheiser and Kim, 1995), Schwann cell α-dystroglycan is HNK-1 positive. Furthermore, the HNK-1 carbohydrate epitope, found in a number of neural cell adhesion molecules, plays important roles in cell adhesion and has been implicated in the binding to laminin (Künemund et al., 1988; Hall et al., 1993). It would be thus interesting to see if this carbohydrate structure is also involved in the interaction of α-dystroglycan with laminin.

ACKNOWLEDGMENTS

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Components of Dystroglycan Complex in Peripheral Nerve

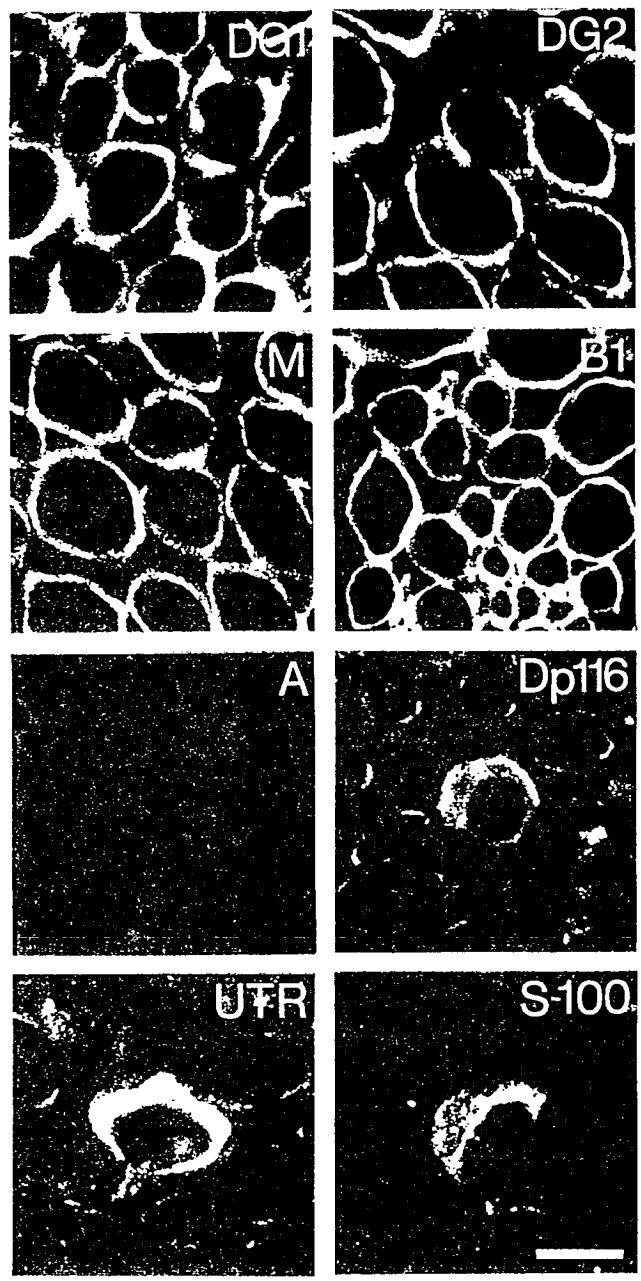


FIG. 1. cLSM immunofluorescent analysis of rabbit sciatic nerve

Shown are the cLSM immunofluorescent images of rabbit sciatic nerve. Alpha-dystroglycan (DG1), β-dystroglycan (DG2), laminin α2 chain (M), laminin β1 chain (B1), laminin α1 chain (A), Dp116, utrophin (UTR) and S-100 protein were detected by antibodies anti-FPD, 43DAG/8D5, 2G9, 4E10, 11D5, VIA4$_2$, DRP1 and 15E2E2, respectively. Bar, 10 μm.

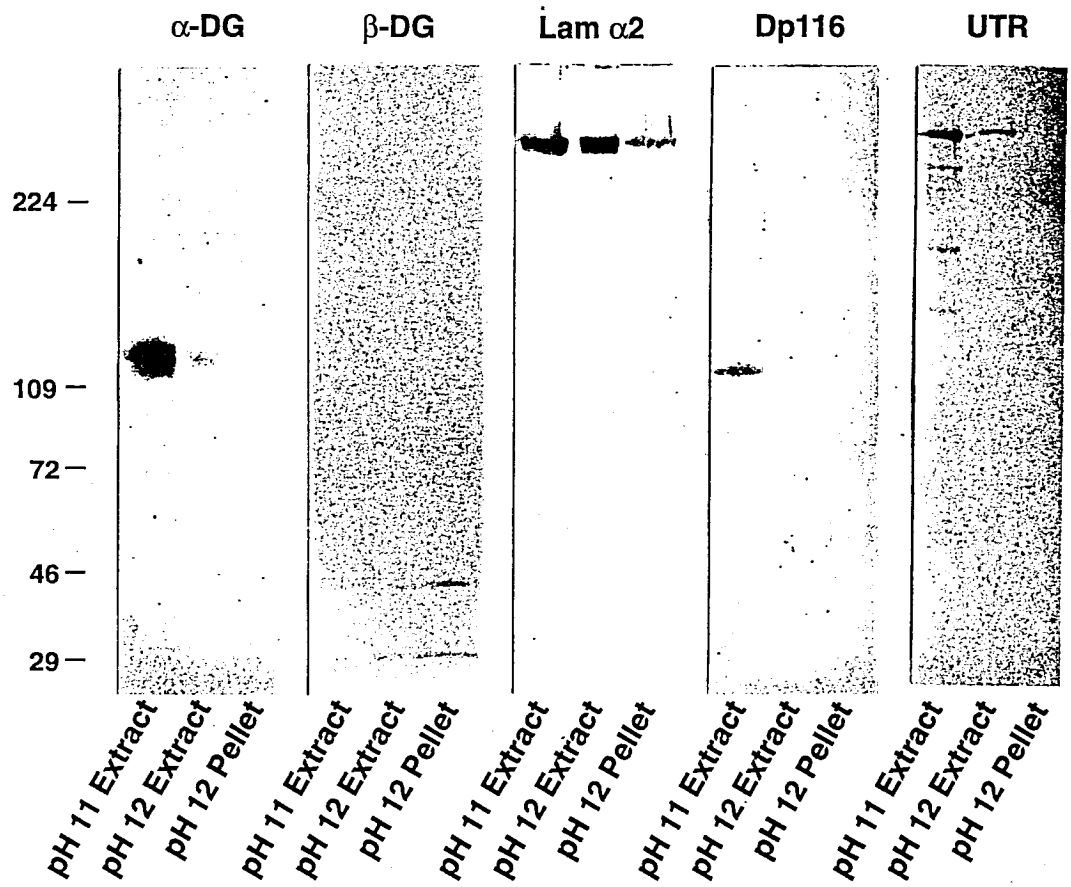


FIG. 2. Alkaline extraction of the crude bovine peripheral nerve membranes

120 µl of the pH 11 extracts (first lane), the pH 12 extracts of the pH 11 pellets (second lane) and the pH 12 pellets (third lane) of the crude bovine peripheral nerve membranes were separated by 3-12% SDS-PAGE and transferred to nitrocellulose membranes. Alpha-dystroglycan (α-DG), β-dystroglycan (β-DG), laminin α2 chain (Lam α2), Dp116 and utrophin (UTR) were detected by antibodies IIH6, 43DAG/8D5, 2D9, DYS2 and anti-DRP, respectively. The 30 kDa band detected by 43DAG/8D5 is presumed to be a proteolytic fragment or the unglycosylated form of β-dystroglycan. Molecular weight standards (Da x 10^{-3}) are shown on the left.

Components of Peripheral Nerve Dystroglycan Complex

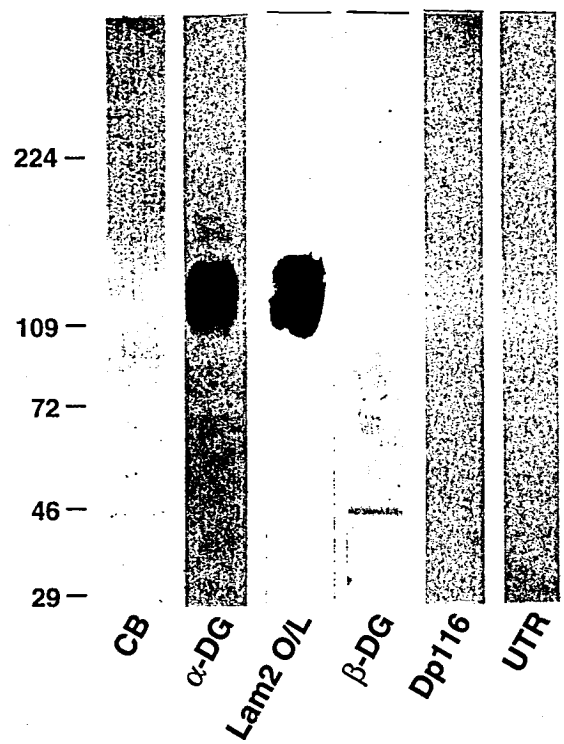


FIG. 3. Characterization of peripheral nerve α-dystroglycan and its associated proteins

Alpha-dystroglycan and its associated proteins were isolated from the digitonin extracts of the crude bovine peripheral nerve membranes by WGA and laminin affinity chromatographies. 120 µl of the EDTA eluates of the laminin-Sepharose were separated by 3-12% SDS-PAGE and transferred to nitrocellulose membranes. Alpha-dystroglycan (α-DG), β-dystroglycan (β-DG), Dp116 and utrophin (UTR) were detected by antibodies IIH6, 43DAG/8D5, DYS2 and anti-DRP, respectively. **Lam2 O/L** indicates the identical nitrocellulose transfer overlaid with laminin-2. Heavily-glycosylated α-dystroglycan was not identified clearly on the SDS gel stained with Coomassie blue (**CB**) (Yamada et al., 1994). Molecular weight standards (Da x 10^{-3}) are shown on the left.

Monoclonal Antibody Reactivity of Peripheral Nerve α-Dystroglycan

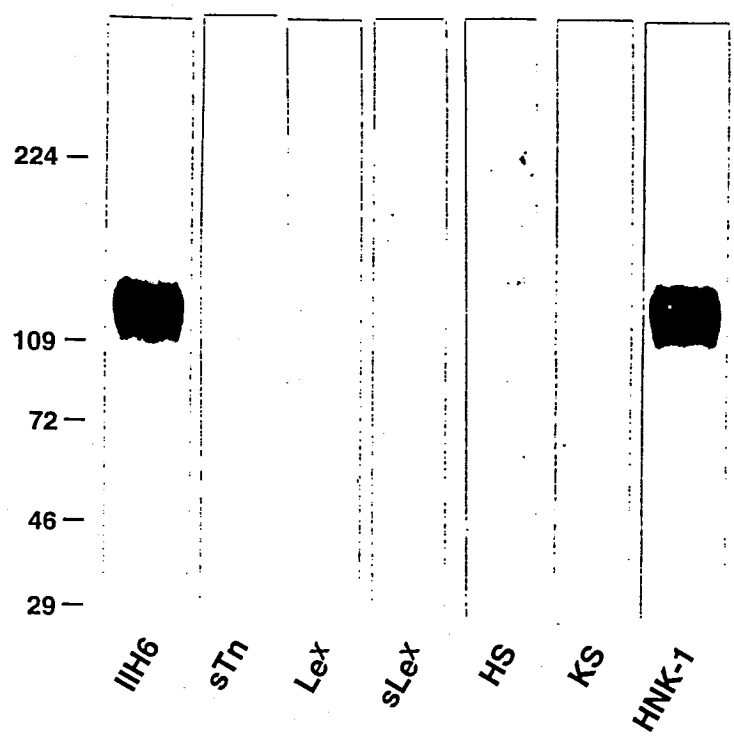
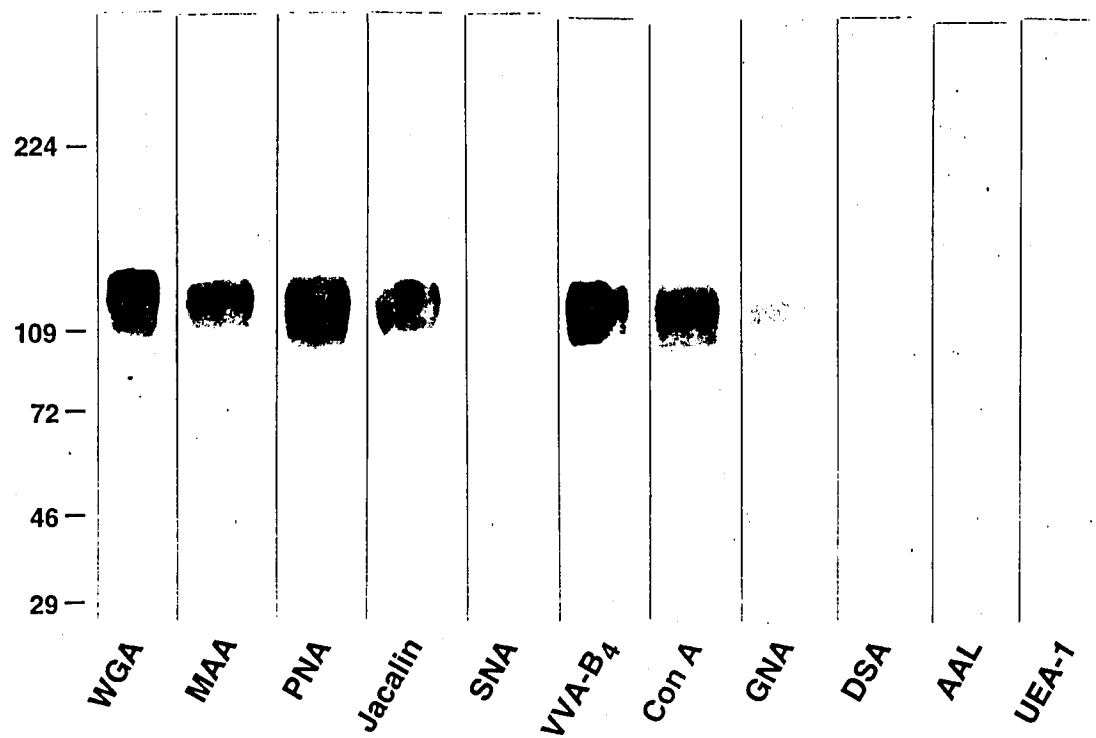


FIG. 4. Characterization of carbohydrate chains of Schwann cell α-dystroglycan

(*a*) Alpha-dystroglycan (3 μg) was separated by 3-12% SDS-PAGE, transferred to nitrocellulose membranes, and then reacted with monoclonal antibodies against α-dystroglycan (IIH6), sialyl Tn (**sTn**), Lewis X (**Le^x**), sialyl Lewis X (**sLe^x**), heparan sulfate (**HS**), keratan sulfate (**KS**), or monoclonal antibody anti-HNK-1.
(*b*) The identical nitrocellulose transfers as shown in (*a*) were reacted with various lectins. Molecular weight standards (Da x 10^{-3}) are shown on the left.

Lectin-Binding Properties of Peripheral Nerve α-Dystroglycan



(*b*) The identical nitrocellulose transfers as shown in (*a*) were reacted with various lectins. Molecular weight standards (Da x 10^{-3}) are shown on the left.

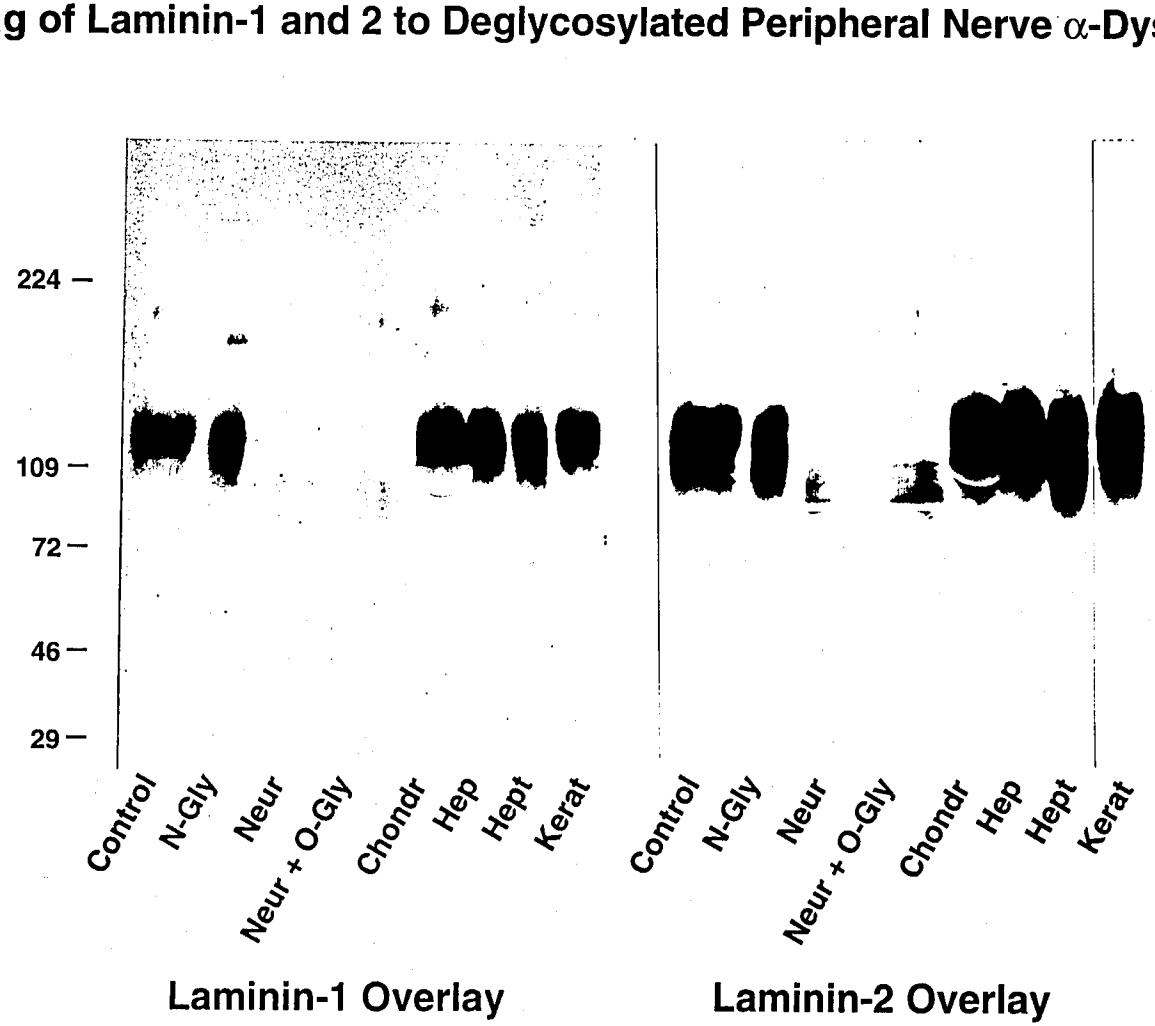


FIG. 5. Binding of laminin to deglycosylated Schwann cell α-dystroglycan

Alpha-dystroglycan was treated with N-glycosidase F (**N-Gly**), neuraminidase (**Neur**), neuraminidase and O-glycosidase (**Neur + O-Gly**), chondroitinase ABC (**Chondr**), heparinase (**Hep**), heparitinase (**Hept**), or keratanase (**Kerat**). **Control** indicates α-dystroglycan treated identically but in the absence of neuraminidase. Deglycosylated α-dystroglycan (3 μg) was separated by 3-12% SDS-PAGE, transferred to nitrocellulose membranes, and then overlaid with laminin-1 (left panel) or 2 (right panel). Molecular weight standards (Da x 10^{-3}) are shown on the left.

Binding of Laminin to Peripheral Nerve α-Dystroglycan

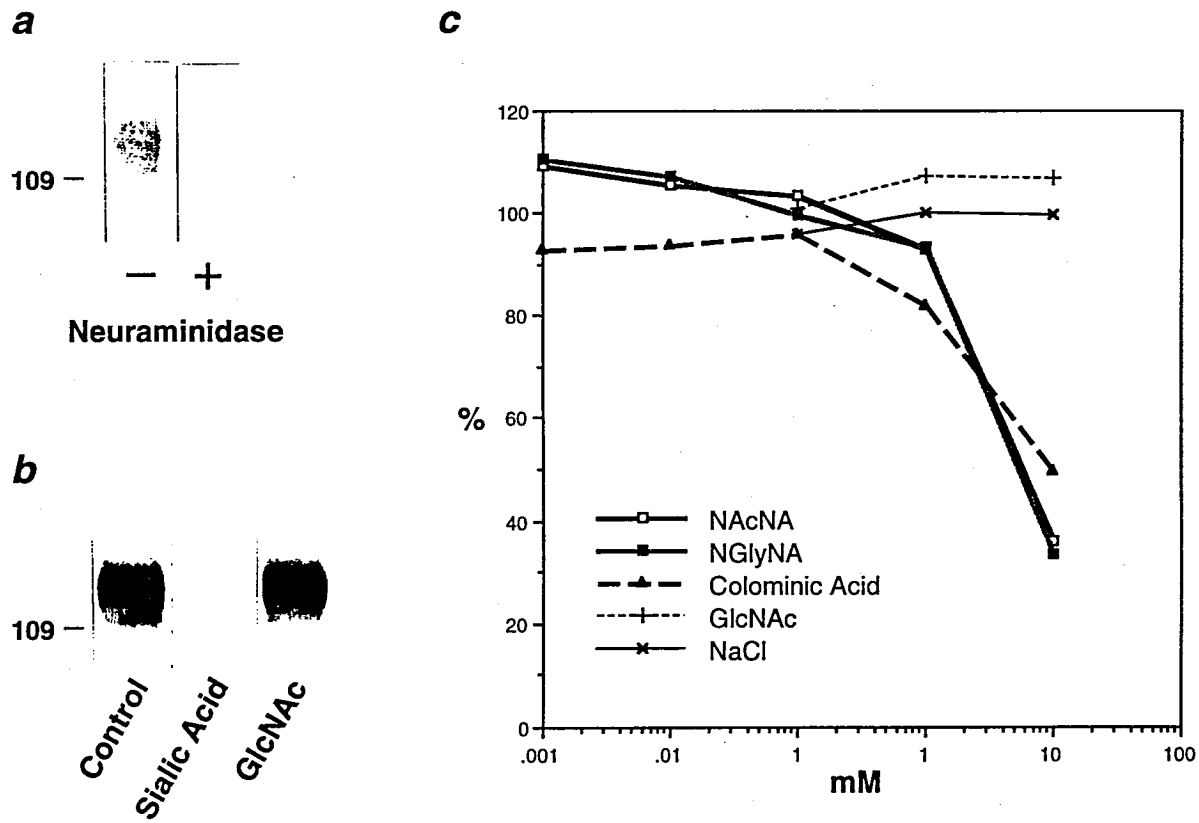


FIG. 6. (*a*) Effect of neuraminidase treatment on the binding of laminin to Schwann cell α-dystroglycan

Alpha-dystroglycan (3 μg) was separated by 3-12% SDS-PAGE and transferred to nitrocellulose membranes. The nitrocellulose transfers were incubated with or without neuraminidase (+ and -, respectively), and then overlaid with laminin-1. Molecular weight standard (Da x 10^{-3}) is shown on the left.

(*b*) Effect of sialic acid on the binding of laminin to Schwann cell α-dystroglycan (blot overlay)

Alpha-dystroglycan (3 μg) was separated by 3-12% SDS-PAGE, transferred to nitrocellulose membranes, and then overlaid with laminin-1 in the presence of 0.1 M sialic acid, 0.1 M GlcNAc or in the absence of these sugars (**Control**). Molecular weight standard (Da x 10^{-3}) is shown on the left.

(*c*) Effect of sialic acid on the binding of laminin to Schwann cell α-dystroglycan (ELISA)

In the presence of various concentrations of N-acetyl neuraminic acid (NAcNA), N-glycolyl neuraminic acid (NGlyNA), colominic acid, GlcNAc or NaCl, laminin-1 was incubated with α-dystroglycan (1 μg) coated onto microtiter wells. Bound laminin-1 was quantified by ELISA. Points are the percentage compared to the values of the wells incubated in the absence of inhibitors. Points represent the mean of triplicate wells in one experiment representative of three similar experiments.

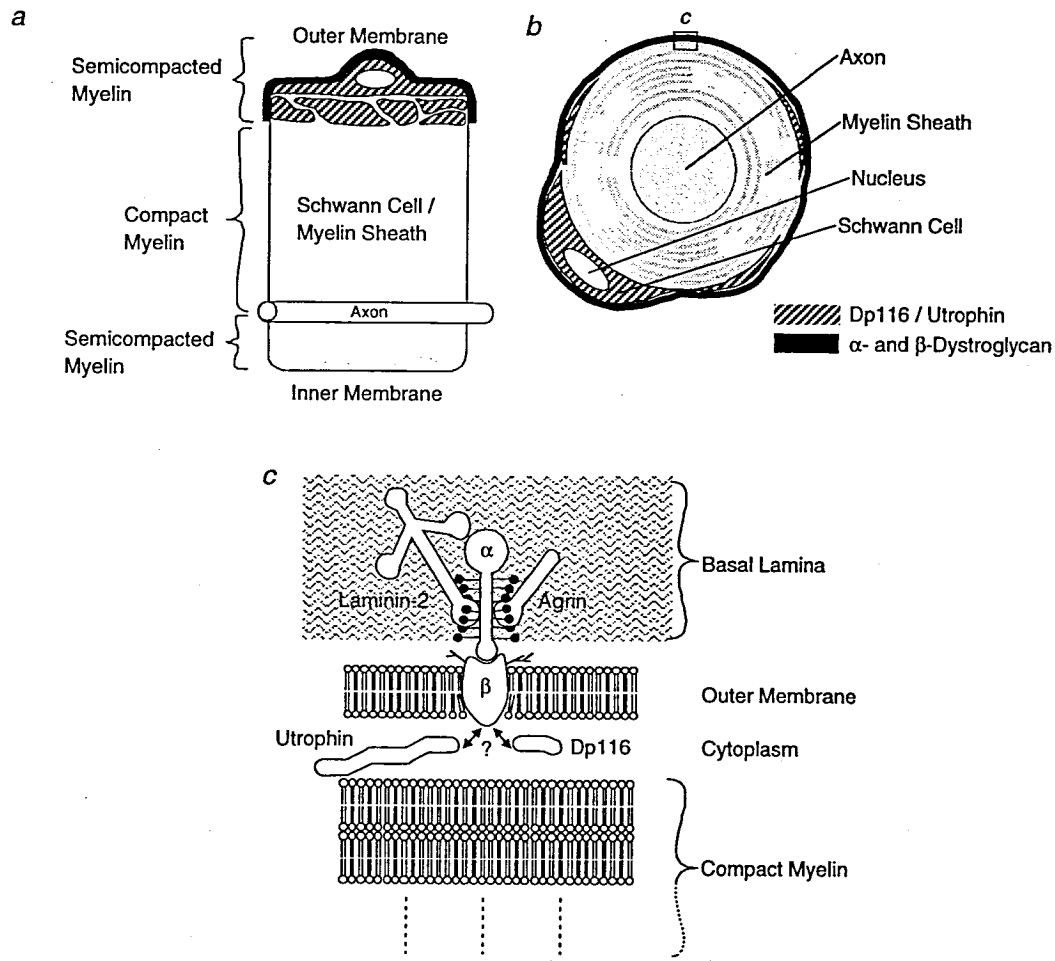


FIG. 7. Hypothetical model of dystroglycan-laminin interaction in peripheral nerve

(*a*), (*b*) Alpha- and β-dystroglycan are localized in the Schwann cell outer membrane, while Dp116 and utrophin are localized in the Schwann cell cytoplasm. (*c*) Alpha-dystroglycan (**a**), which is an extracellular peripheral membrane glycoprotein, links β-dystroglycan (**b**) in the Schwann cell outer membrane with laminin-2 in the endoneurial basal lamina. The mucin domain of α-dystroglycan is extended into a rigid rod. Clusters of sialic acid residues in O-glycans of α-dystroglycan are presumed to be involved in the interaction with the G-domain of laminin α2 chain.

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Dystrophin-associated protein, syntrophin (A1)

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Dystrophin, the protein responsible for Duchenne/Becker muscular dystrophy, is purified as a complex with several proteins called dystrophin-associated proteins (DAPs) from digitonin-solubilized rabbit skeletal muscle [1,2]. Among DAPs, the one with a molecular mass of about 60,000 is named A1 or 59DAP. We have previously shown that this DAP is actually a heterogeneous group of proteins consisting of an acidic (α-A1) and a distinct basic (β-A1) component [3]. Here we report the cloning of a cDNA for a human basic isoform of A1 and demonstrate by comparison between our results and others that there are at least three A1s (all of which will be henceforth named syntrophins) , namely two basic ones (β1 and β2) and one weakly acidic one (α). The heterogeneous natures of A1 we found are thus mostly clarified.

cDNA Cloning of β-A1

A1 is separated into triplet bands by 1-dimensional SDS/PAGE of purified rabbit dystrophin-DAP complex [1,2]. For cloning we at first determined the internal amino acid sequences of the triplet bands, respectively (Table 1) [4]. Oligonucleotides for probes were designed on the basis of the sequence No. 1 (or No.4) and a library of human fetal muscle λgt10 cDNA was screened by them. A single positive clone was plaque-purified, subcloned into EcoRI-cut Bluescript II SK(+) vector and sequenced. The 2979-bp sequence encodes a single large open reading frame, starting from an initiator ATG within the context of a Kozak consensus. The clone had no poly (A) tail but contained the sequence which seems to be the polyadenylation signal. The deduced protein is composed of 538 amino acids and considered to be a cytoskeletal protein, because no signal sequence and no transmembrane sequence were found. The calculated molecular mass of 58 kDa and the calculated pI of 9.0 agree well with the

electrophoretically determined size and charge of β-A1[3]. The deduced human amino acid sequence contains exact matches to the sequenced polypeptides 1-5 originated from the top two bands (Table I), despite the fact that the partial peptide sequences were derived from rabbit. On the contrary, the sequences 6-11 were not found in the deduced sequence.

Table 1. Partial polypeptide sequences of A1

Band	Sequence No.	Amino acid sequence
A1a	1	**ATPYVKKGSPVS**
	2	**KDLLI**
	3	**KQ*KP*LVVL**
A1b	4	Same as 1
	5	**SISNQK*GVK**
	6	Same as 7
A1c	7	**AK*VPLKMAYVS***TPSDP**
	8	**L*AGTGAGAGG**
	9	**I*SADGQD**
	10	**LLLYGGLPQTLIAT*L**
	11	**LAA*T*QLVDG**

Table 1. The DAP was resolved into a triplet A1a, -b, and -c by SDS/PAGE and digested with V8 protease. The polypeptides released were then separated by HPLC and microsequenced. An asterisk means the residue at that position was not determined. Oligonucleotides designed for this study are reverse translations of sequence: 5'-GCI ACI CCI TAK GTI AAR AAR GG-3'. Positions with R were synthesized with a combination of both dA and dG and those with a K were synthesized with a combination of dT and dG.

During the sequencing of this human β-A1 cDNA, the sequences of other candidate A1 genes in mouse, rabbit, and *Torpedo* became available [5,6] and are aligned with our sequence. The sequences 6-11, which are originated from the bottom band except No.6, were identical to the deduced amino acid sequence of rabbit candidate 59DAP-1[6] and are close to that of mouse syntrophin-1 [5]. These proteins are considered to be α-A1, because their calculated pIs both

are 6.4. Interestingly, we realized mouse syntrophin-2 [5] to be a basic protein by the isoelectric point calculated based on its partial published sequence (pI=9.4), but it did not match to our β-A1 as described below.

As the cDNA was being sequenced, we identified a relative within the human EST data base, a 210-bp EST from a human muscle cDNA library. Its deduced amino acid sequence had 48% identity at best to that of β-A1. However, we found that the EST is rather close to the deduced mouse syntrophin-2 sequence, because the amino acid identity reached to 82 % between them. We thus considered it most probable that the EST is the human homologue of mouse syntrophin-2. Using this EST sequencee, we have now cloned the human homologue of syntrophin-2, which is distinct from β-A1 [7]. We thus conclude that there are at least three A1s, two basic A1s and one weakly acidic A1. So far, several names were used for these proteins. To avoid confusion, we will hereafter use syntrophin as a standard name for A1s. We use β1- and β2- for the basic ones and α- for the acidic one to discriminate those. β-A1 we cloned and syntrophin-2 are thus named β1- and β2-syntrophins, respectively, while α-A1, syntrophin-1 and 59DAP-1 are named α-syntrophin.

We determined the gene locations of human syntrophins including α-syntrophin which we cloned by the use of published sequences of mouse and rabbit α-syntrophin [5,6]. They were 8q23-24, 16q23-24 and 20q11.2 for β1-, β2- and α-syntrophins, respectively [7].

Immunoblot Analysis of Syntrophins

We have now known the presence of β2-syntrophin by molecular cloning, but we have not detected it yet on the protein level. Using anti-β1-syntrophin antibody which we prepared and the other anti-syntrophin antibodies provided by Prof. S. C. Froehner of North Carolina University, we examined the dystrophin-DAP complex purified from rabbit skeletal muscle by immunoblot analysis [8]. In the blots separated by 1-dimensional SDS/PAGE (Fig.1), the antibody against "β1" reacts with the top two of the triplets bands, while antibody against "α" reacts with the bottom band. This result well agrees with the facts that the sequenced polypeptides derived from the top two bands and those from the bottom band were found in the deduced sequences of human β-A1 and rabbit 59DAP-1, respectively. On the other hand, we

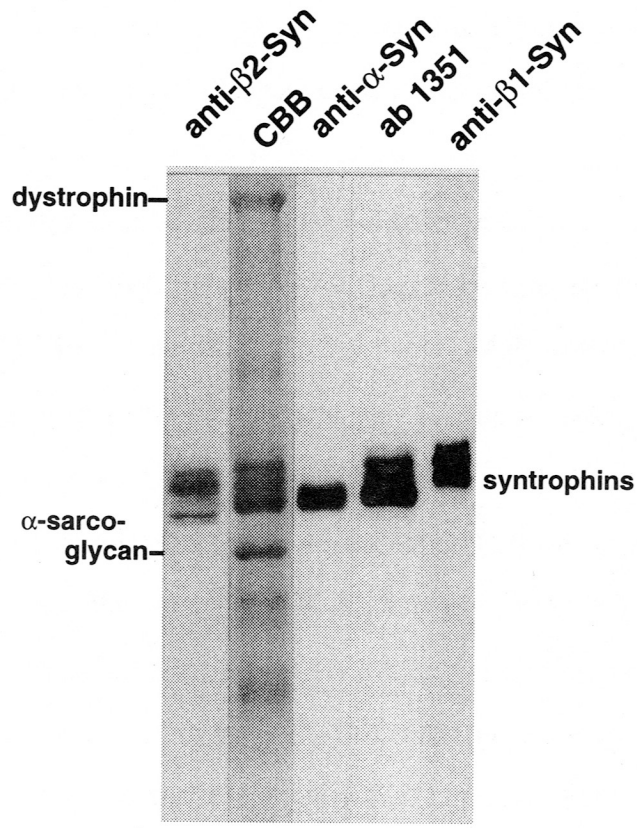

Fig.1. Immunoblot patterns of dystrophin-DAP complex with various antibodies. Dystrophin-DAP complex was separated by 1-dimensional SDS/PAGE. From the left lane, 6.5, 4, 1.3, 0.1, and 0.5 μg of samples were loaded. Anti-β1-Syn is the antibody against human β1-syntrophin [8]. Anti-α- and anti-β2-Syns are the antibodies against syntrophins "α" and "β2", respectively, which were prepared based on the mouse syntrophin sequences [10]. ab 1351 is the monoclonal antibody against *Torpedo* syntrophin [10].

found that the monoclonal antibody against *Torpedo* syntrophin (ab1351) reacted with all the triplet bands, although the binding ability to α-syntrophin was much stronger than β1-syntrophin. We barely detected β2-syntrophin with its specific antibody, when a large quantity of sample is loaded on the gel, suggesting that β2-isoform is present in less minor fraction than others. Therefore it is natural that we failed to detect the isoform on the protein level formerly. Several bands were stained, most of which apparently overlap the other syntrophin bands, but it seems that there is a slight difference between β1- and β2-syntrophins.

The blots separated by 2-dimensional PAGE are shown in Fig.2. As expected from the above results, the antibody against "β1" reacts with the basic series of spots, while antibody

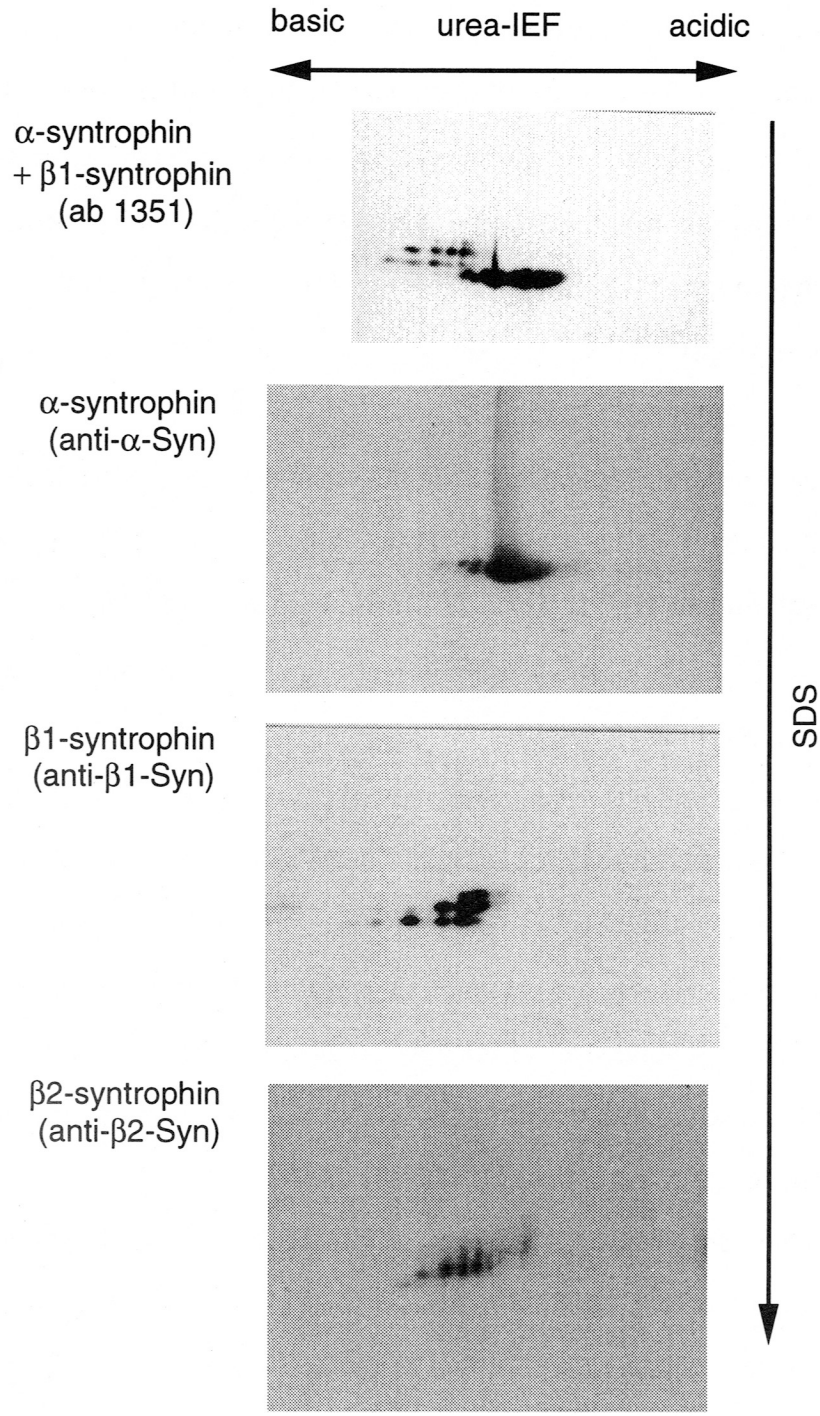

Fig.2. Immunoblot patterns of dystrophin-DAP complex separated by 2-dimensional PAGE with various antibodies. From the top panel, 1, 10, 4 and 12 μg of samples were loaded on the gels. At the 1st electrofocusing, samples were loaded from the basic side except the bottom panel, in which β2-syntrophin was focused only when the sample was loaded from the acidic side. Antibodies used are the same as those in Fig.1.

against "α" reacts with the acidic series of spots. On the other hand, ab1351 reacted with both acidic and basic series of spots in agreement with the result shown in Fig.1. We could detect β2-syntrophin as a basic series of spots similar to that of β1-syntrophin. By these blots, however, we could not clearly show the difference between both basic syntrophins. Then we attempted to analyze them on a sheet of blot by the double staining method, and we found that the average position of β2-syntrophin spots was slightly more basic than that of β1-syntrophin (data not shown) [8].

Here, we showed by molecular cloning that there are at least three kinds of syntrophins and identified them on the protein level in rabbit skeletal muscle. We determined the gene locations of human β1-, β2- and α-syntrophins as 8q23-24, 16q23-24 and 20q11.2, respectively [7]. These locations exclude syntrophins from the human neuromuscular diseases with a known position.

Curiously, each syntrophin was shown to be still composed of many spots which were separated by 2-dimensional PAGE. A part of the spots can be explained by phosphorylation, because they disappeared by the alkali phosphatase treatment of dystrophin-DAP complex [3]. Other spots may suggest the presence of isoforms derived from the alternatively spliced mRNAs, because several sizes of mRNAs are observed by Northern analyses of syntrophins [4, 7] and because the gene structures of mouse syntrophins composed of several exons were recently reported [9]

We have shown elsewhere that α- and β-isoforms of syntrophins tandemly associated in this order with dystrophin at the very limited area in the neighborhood of the C-terminal [7, 11, 12]. We have also shown that a 90-kDa DAP, A0, also associates with dystrophin in the site where β-isoforms associate (β-site) [11]. Since it might be difficult for these proteins to associate with dystrophin in this site at the same time, we at present speculate that β-syntrophins (and A0) may alternately associate with dystrophin in the β-site. Recently, it is reported by immunohistochemistry that α-syntrophin is detected on the cell surface membrane including the neuromuscular junction (NMJ) of rat skeletal muscle, but β2-syntrophin is only

detected at NMJ [10]. This result may suggest that β-site of dystrophin at NMJ is occupied with β2-syntrophin. We attempted to examine the subcellular localization of β1-syntrophin, but our antibody was not useful for histochemistry. Since the subcellular localization of each isoform of syntrophin over development may lead to a better appreciation of the function of syntrophins, good antibodies against syntrophins including "β1" are desired.

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SARCOGLYCANOPATHY HYPOTHESIS

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Recently, a new word "sarcoglycanopathy" has been introduced to represent a group of limb-girdle muscular dystrophy in the place of severe childhood autosomal recessive muscular dystrophy, namely SCARMD[1].

Present themes are how the sarcoglycanopathy concept has been formed and what problems are left to be solved.

1. Dystrophin-associated proteins

Since we discovered that dystrophin is present in association with muscle membrane in 1988[2], we have studied the problem how dystrophin molecule is present in subsarcolemmal undercoat of cytoskeleton. During the study of this subject, new problems were raised concerning the molecular architecture of dystrophin and dystrophin-associated proteins or briefly DAPs[3].

DAPs were assorted almost simultaneously by Campbell's group and our group in 1990 and each group gave the different names to DAPs[4,5]. On this occasion, it is necessary to emphasize the difference in the names of the 43kD protein which looked doublets in 1-dimensional gel electrophoresis. Campbell's group reported that the doublets are immunologically the same[6]. And they named it 43DAG.

On the other hand, we considered that the doublets represent different two proteins, and then named A3a and A3b, respectively.

Later, we found that these two are completely different proteins. They differ in immunological reactivities, isoelectric points, peptide maps, complex formation patterns and tissue distribution patterns[7]. Recently, they are shown to be different at the cDNA level. And now, A3a and A3b are called β-dystroglycan and β-sarcoglycan, respectively.

When dystrophin and DAP complex extracted from muscle membrane fraction by digitonin-treatment, are further treated with octyl-glucoside and

fractionated with gel filtration column, we found that the complex is separated into roughly three fractions[7]. The void fraction contained dystrophin binding with syntrophin group. The first fraction was composed of dystroglycan complex, namely, α- and β-dystroglycans. The second fraction contained 50DAG (adhalin), A3b and 35DAG. Since they are found to be present as a complex, we named this complex sarcoglycan complex[7]. However, there are still some other proteins to be classified such as 25DAP as members of sarcoglycan complex. 25 DAP is considered as a transmembrane protein and present very close to either dystroglycan complex or sarcoglycan complex.

Taking the experimental results mainly of our group and also a few of other groups into consideration, we constructed a model how dystrophin and DAPs are associated with muscle membrane. Fig.1 shows our updated model. Dystrophin binds actin filaments at its N-terminus[9], and binds at its carboxyl side of the molecule with β-dystroglycan[10], which in turn binds with α-dystroglycan[7]. α-dystroglycan is an extracellular protein which binds with laminin[6], one of the main components of basal lamina. Thus, these components construct an axis connecting the extracellular matrix and subsarcolemmal cytoskeleton network[1].

Sarcoglycan complex, on the other hand, does not seem to serve as the member of the connecting axis[1]. Presumably, sarcoglycan complex plays a role other than the connecting axis.

Concerning syntrophin group, the previous speaker, Dr.Yoshida has reviewed the present status of the research.

The nomenclature of the sarcoglycan complex so far known is compared. This complex is composed of at least 3 proteins. They are adhalin, A3b and 35DAG[7]. Recently, all the cDNAs are cloned and these proteins are renamed to be α-, β- and γ-sarcoglycans[8,11,12]. The order of α, β and γ is the decreasing order of molecular mass of the proteins and at the same time the order of cloning of cDNA.

2. SCARMD and its responsible genes

SCARMD is a group of the muscle dystrophies due to the defect of sarcoglycan complex[13]. Although this name bears severe and childhood, this disease is now considered not necessarily to be severe and restricted to the

patients in childhood, and is sometimes called Duchenne-like autosomal recessive muscular dystrophy.

In 1992, Matsumura et al.[14] showed that in SCARMD muscle, 50DAG or adhalin is missing. This was the first indication of the loss of a certain protein in the SCARMD muscle. This gave strong impression to the researchers that SCARMD is a disease due to the defect of adhalin.

At the same year, namely in 1992, Ben Othmane et al.[15] showed that Tunisian SCARMD is linked to 13q12. But in the next year, Passos-Bueno et al.[16] in San Paulo showed that Brazilian SCARMD is not linked to 13q. And they claimed that SCARMD is not a single disease but is a group of diseases which are genetically heterogeneous.

In 1994, we found using immunological staining of SCARMD muscles what is lacking in SCARMD muscle is not confined to adhalin, but A3b and 35DAG are also lacking. Like other groups, we confirmed other components such as dystrophin, dystroglycan complex etc. are normal in SCARMD muscle. This showed that the whole members of sarcoglycan complex are lost in SCARMD muscle, although other components are intact[17]. Based on these findings as well as the finding that SCARMD is genetically heterogeneous, we proposed a hypothesis that SCARMD is caused by the loss of sarcoglycan complex[17].

The hypothesis is as follows: First, when any one of the sarcoglycan gene is defective, this may result in the loss of the protein product of the gene. Second, when the protein is missing which is the gene product, this causes failure in forming the complex and the incomplete complex is not sufficient enough to be fixed to the sarcolemma. And loss of the whole complex ensues. Third, the loss of the complex causes the SCARMD phenotype. Therefore, gene defect of any one component of sarcoglycan complex causes the same disease. This may be the reason of genetic heterogeneity of SCARMD.

When we proposed this hypothesis, no sarcoglycan gene had been known to be responsible to SCARMD.

As soon as our paper presenting this hypothesis was accepted, Roberds et al.[11] reported cloning of human adhalin cDNA. It is mapped at 17q21 and the gene is responsible for SCARMD. They showed a single base replacement in the each allele of adhalin gene of SCARMD patient linked to 17q. Thereafter, on these basis, the word adhalinopathy was popularly used. Some

people extending the usage of this word by using the word "secondary adhalinopathy" for SCARMD which were not linked to the adhalin gene.

Our next problem was whether the other sarcoglycan genes are responsible for SCARMD or not. It was in February of 1995, when we started the experiments to clone the cDNAs of A3b and 35DAG. At that time, adhalin had been shown to be responsible for SCARMD, and classified to be LGMD 2D[11,18]. SCARMD linked to 13q12 was classified to be LGMD 2C but its responsible gene was not known.

By the beginning of June 1995, we succeeded to clone A3b cDNA together mainly with Kunkel's group in Boston and Hoffman's group in Pittsburgh. Using our data on partial polypeptide sequences of A3b, Kunkel's group cloned the cDNA and determined gene locus to be at 4q12. And Hoffman's group found mutations on A3b gene of SCARMD patient. We further examined A3b in 4q12-linked gene defect muscle at protein level either biochemically and immunohistochemically. These results were published in the November issue of Nature Genetics in 1995[8].

Since Campbell's group also obtained the same results, their paper appeared in tandem with ours[19]. On the publication, the name of the protein A3b was determined to be β-sarcoglycan by the proposal of Lou Kunkel and with the agreement of related researchers. At the same time, the name of adhalin was changed to α-sarcoglycan.

Until the end of June, Noguchi succeeded to clone rabbit cDNA of 35DAG using our monoclonal antibody as a probe. Then our group cloned human gene and found that the mRNA is expressed exclusively in the skeletal and cardiac muscles, namely striated muscles, as we had previously shown by immunoblot analysis. Kunkel's group mapped the gene at 13q12 and found the mutations on the gene of the SCARMD patients linked to 13q12. We renamed 35DAG to be γ-sarcoglycan. This was published in November 3rd issue of Science last year[12].

In both β- and γ-sarcoglycan gene mutations, the muscles were found to be absent from whole sarcoglycan complex as our hypothesis[17] predicted. Therefore, we consider that SCARMD can be called sarcoglycanopathy.

3. On new classification of LGMD

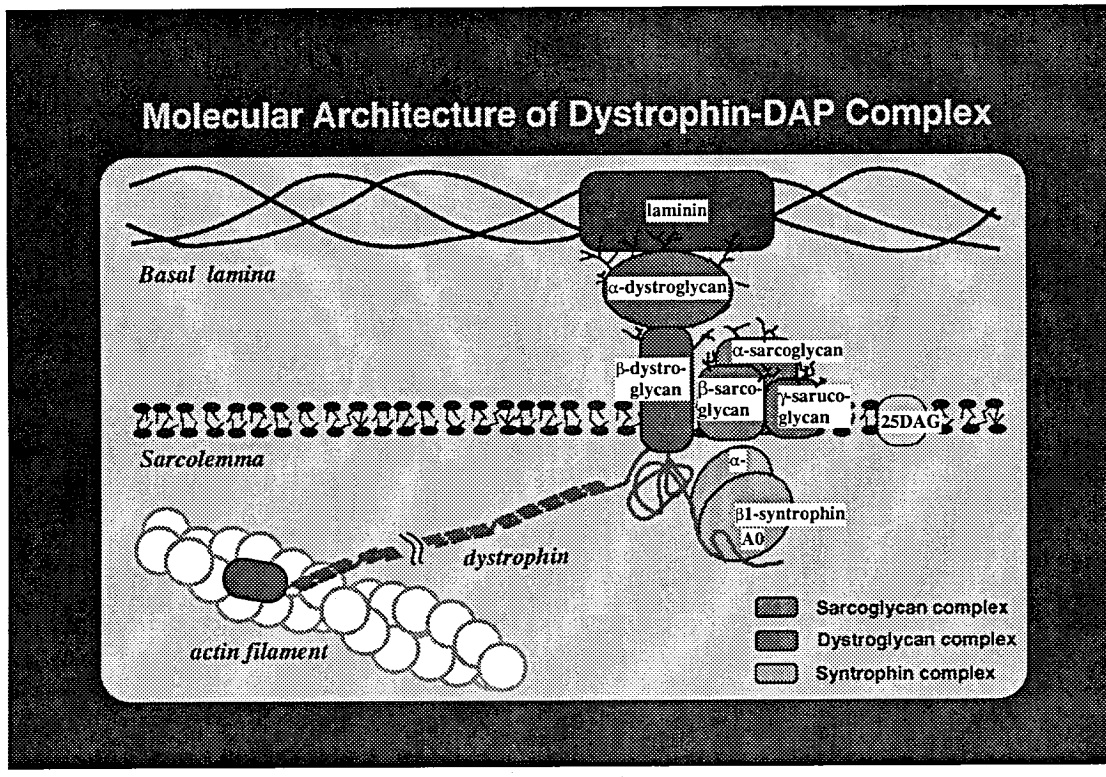
It has been described that limb-girdle muscular dystrophies are classified

on the genetical point of view. According to this classification, γ-sarcoglycanopathy is LGMD 2C, α-sarcoglycanopathy is LGMD 2D and β-sarcoglycanopathy should be 2E.

There is, however, a problem whether there are still some types of limb-girdle autosomal recessive muscular dystrophy to be classified as sarcoglycanopathy. Hoffmann's group found that there is at least one more component of the complex to be called δ-sarcoglycan and the dystrophy whose responsible gene is δ-sarcoglycan gene, namely, δ-sarcoglycanopathy.

In the above-mentioned classification of limb-girdle muscular dystrophy[18], it is the rule to give the succeeding alphabet to the newly discovered gene mutation. Therefore, the next autosomal recessive limb-girdle should be 2F. On the other hand, there are still some other sarcoglycanopathy and there may be some additional autosomal recessive limb-girdle muscular dystrophies which do not belong to sarcoglycanopathy. If we continue to use the rule of the present classification, it is possible that the phenotypically different muscular dystrophies will be arranged in the intermingled manner. We cannot predict that the next member of limb-girdle belongs to sarcoglycanopathy or not.

Therefore, the following problem should be seriously considered that the sarcoglycanopathy is classified separately from the other autosomal recessive limb-girdle muscular dystrophies and be given the number 3. In this context, α-, β- and γ-sarcoglycanopathy should be called LGMD 3A, 3B and 3C, respectively. In this way the classification may become clearer and more useful.


Molecular Architecture of Dystrophin-DAP Complex
laminin
Basal lamina
α-dystroglycan
α-sarcoglycan
β-dystro-glycan
β-sarco-glycan
γ-saruco-glycan
25DAG
Sarcolemma
α-
β1-syntrophin
A0
dystrophin
actin filament
Sarcoglycan complex
Dystroglycan complex
Syntrophin complex

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β- and γ-sarcoglycan

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Dystrophin-associated glycoproteins are clarified into two groups, dystroglycan complex and sarcoglycan complex, on the basis of biochemical properties and the tissue distribution of these proteins. Sarcoglycan complex is composed of 50-kDa α-sarcoglycan (adhalin), 43-kDa β-sarcoglycan (A3b) and 35-kDa γ-sarcoglycan (35DAG). Sarcoglycan components were shown to be selectively deficient in the muscles of patients of severe childhood autosomal recessive muscular dystrophy (SCARMD). Based on these findings, we postulated the sarcoglycanopathy hypothesis that SCARMD is a disease caused by the primary defect of one of the sarcoglycan genes[1]. A defect of anyone of the sarcoglycan component causes the secondary destabilization of the sarcoglycan complex resulting in the common histological phenotype of the loss of a whole sarcoglycan complex. Recently α-sarcoglycan was shown to be primarily responsible to SCARMD[2]. Therefore the other components of sarcoglycan complex, β- and γ-sarcoglycan, were candidates for the responsible genes for SCARMD.

Now we isolated rabbit and human cDNAs encoding β-sarcoglycan with an oligonucleotide derived from a part of a determined internal peptide

sequence from β-sarcoglycan[3]. β-Sarcoglycan was 318 amino acid residues and this molecular mass is well-consistent with apparent molecular mass for the glycopeptidase-F digested β-sarcoglycan on the SDS-PAGE. Rabbit and human γ-sarcoglycan cDNAs were isolated with a specific monoclonal antibody[4]. γ-Sarcoglycan was 291 residues. These sequences were well-conserved between human and rabbit with 97% identity for β-sarcoglycan and 87% identity for γ-sarcoglycan, respectively. Both proteins are novel type II single transmembrane proteins that contain large extracellular domain at C-terminal side and small intracellular domain at N-terminal side. There are three and one conserved potential asparagine-linked glycosylation sites in C-terminal region of β- and γ-sarcoglycan, respectively. The sequence homology between β- and γ-sarcoglycan was not detected but these proteins had some characteristic common structures, which are a potential phosphorylation site in intracellular domain and a cluster of four conserved cysteine residues in the distal portions of the extracellular C-termini. Furthermore the putative secondary structures of these proteins were similar to each other as shown in Figure. The extracellular domains of all three components are rich in the β-turn structure. A couple of α-helices is commonly present in the center of the extracellular domains in three proteins.

The Northern analysis showed that mRNA of β-sarcoglycan is expressed at the highest level in skeletal muscle and heart and at the substantially lower level in brain, kidney, placenta, pancreas and lung. On the other hand, the expression of mRNA of γ-sarcoglycan was confined in skeletal muscle and heart which is consistent with our previous data of the immunochemical survey of the γ-sarcoglycan protein in primate tissues.

The human genes for β- and γ-sarcoglycan were mapped to chromosome 4q12 and 13q12, respectively. This same region, 13q12 was implicated in North African SCARMD families by linkage analysis. Another region, 4q12 didn't fit

the locus reported to relate to muscular dystrophies for which a chromosomal assignment is known without a defined primary genetic defect.

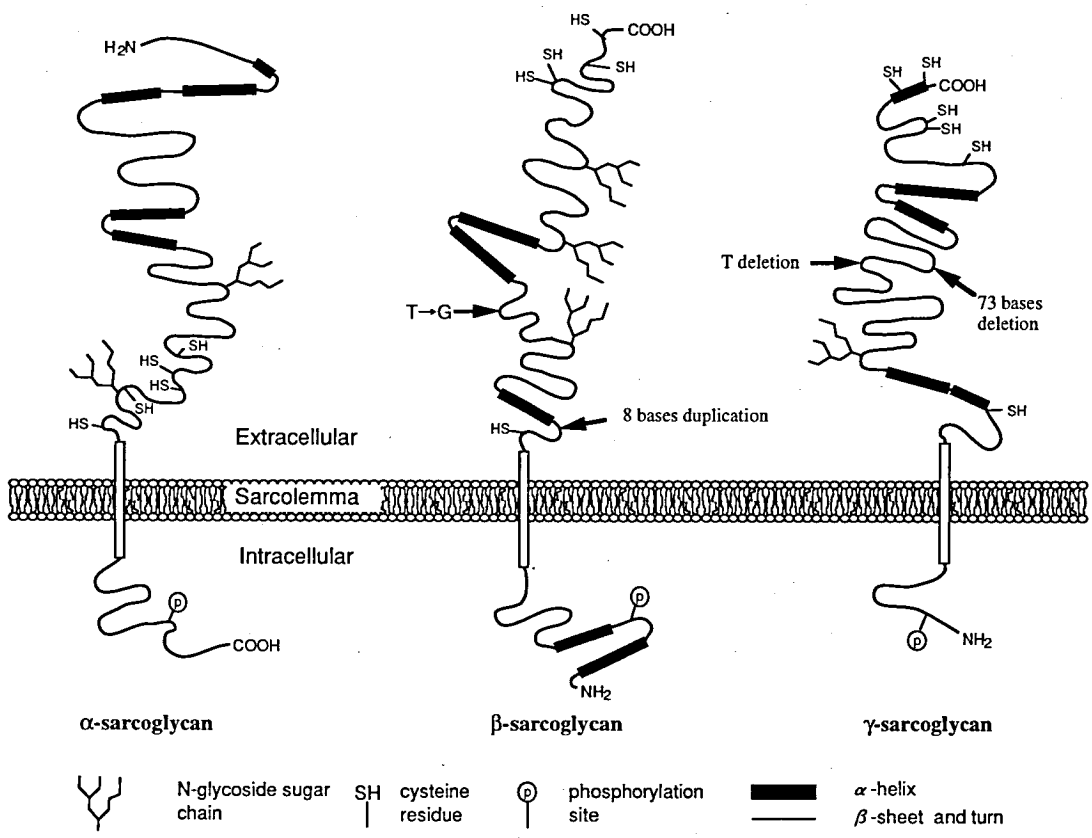

Figure. The predicted structures of the sarcoglycan components.

The secondary structures were predicted by the method of P. Y. Chou and G. D. Fasman (1978). The predicted structures of the three components are similar to each other. They have single transmembrane domains, and larger extracellular and smaller intracellular domains. Five conserved cysteine residues are present in the extracellular domains of all the components. The mutation positions identified in the β- and γ-sarcoglycan genes in SCARMD patients are shown by arrows.

Therefore we tried to find out the muscular dystrophy patients with the mutation of β-sarcoglycan gene. We selected 62 muscle biopsies in which to analyze β-sarcoglycan mRNA for mutations. These biopsies were chosen from a muscle biopsy bank of over 2,400 patients, and met the criteria: 1) H&E staining of cryosections showing histopathological features consistent with muscular dystrophy; 2) normal dystrophin molecular mass and amount; 3) variable immunostaining patterns of the either dystrophin, adhalin of merosin

on cryosection; and 4) serum CPK values in excess of 1,000 IU/l. For initial screening, we performed SSCP analysis on the RT-PCR product amplified from RNA isolated from each of the 62 biopsies. We found a single patient with gives four aberrant conformers. In one conformer-pair, a T to G conversion was identified which resulted in a nonsense codon (Y184X). In the second conformer-pair, an 8 base pair duplication after amino acid position 125 was found which caused a frame shift and an immediate stop-codon at nucleotide 129. The mutations were confirmed by PCR analysis of genomic DNA from the patient and both parents. Analysis of genomic DNA from the parents showed the patient's mother to be heterozygous for the T to G conversion and father to be heterozygous for the duplication, confirming autosomal recessive inheritance of this muscular dystrophy. These premature stop mutations are predicted to severely truncate the protein, ablating most of its extracellular domain, including the two or three asparagine glycosylation sites and the cluster of cysteine residues in C-terminal (see Figure).

The minimal candidate region for SCARMD was recently refined to a small region surrounding D13S232 and linkage disequilibrium with the marker D13S232 suggests that both alleles of the responsible gene should carry an identical mutation in North african families. To determine the homozygous mutation responsible for SCARMD, we prepared RNA from a muscle biopsy taken from an affected Tunisian SCARMD patient whose family is consanguineous and demonstrates linkage disequilibrium with D13S232 and performed PCR for γ-sarcoglycan gene followed by direct sequencing. One of the thymine residues from nucleotides 645 to 649 was homozygously deleted in the patient. This deletion was confirmed in the genomic DNA from this patient. This mutation was also found in an affected sibling in this family and in two additional Tunisian SCARMD families. The deletion of one thymine changed the reading frame after amino-acid 175, creating a premature stop codon at amino acid 193. This aberrant γ-sarcoglycan would retain the its

transmembrane anchor and its asparagine-linked glycosylation site but lose the cluster of cysteine residues present in the distal portion of the protein as shown in Figure. To ascertain whether γ-sarcoglycan mutations contribute to sporadic cases of SCARMD, we analyzed γ-sarcoglycan cDNA from muscle biopsies of four Japanese patients whose muscle displayed dystrophic architecture on H&E staining, deficiency of sarcoglycan components, and preservation of normal dystrophin staining. SSCP analysis of the region from nucleotide 516 to 847 of the γ-sarcoglycan gene showed a single patient gives two aberrant conformers and a conformer band was sequenced. It corresponded to a deletion of 73 base pairs , producing a premature stop codon at amino acid 170 of the aberrant γ-sarcoglycan. In this case, the C-terminal one third of the γ-sarcoglycan protein was lost, including the distal cysteine cluster (see Figure).

The immunostaining patterns showed the common phenotype that sarcoglycan complex is selectively lost from muscle membrane. Immunostaining of the muscle biopsy of the patients with affected β-sarcoglycan gene demonstrated a primary deficiency of β-sarcoglycan and secondary deficiencies of α- and γ-sarcoglycan in contrast to the preservation of dystrophin, β-dystroglycan and merosin staining. Immunostaining of 13q12-linked patients muscle showed α- and β-sarcoglycan deficiencies beside the deficiency of γ-sarcoglycan. This suggests that one component of sarcoglycan complex is required for the stabilization and/or localization of two other components, thereby maintaining the integrity of the sarcoglycan complex in the membrane.

These results show that β-and γ-sarcoglycan are responsible to SCARMD as well as α-sarcoglycan. The mutations in β-and γ-sarcoglycans not only affected the primarily-mutated proteins but also disrupt the integrity of the entire sarcoglycan complex. These facts further supports our hypothesis. We proposed the name "sarcoglycanopathy" as a collective name for these types of the disease called as SCARMD

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From adhalinopathies to sarcoglycanopathies

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Dystrophin, the defective gene product in Duchenne and Becker muscular dystrophy, is associated with a complex of sarcolemmal glycoproteins believed to link the cytoskeleton of muscle fibers to laminin, a major component of the extracellular matrix[1-2].

It has been suggested that deficiencies in any component of the transmembrane linker may disrupt the link between the cytoskeleton and extracellular matrix and lead to associated exercise induced muscle necrosis[3-4].

The large oligomeric complex with dystrophin is comprised of three sub-complexes[5].

1) Dystroglycan complex
   - α-dystroglycan (156DAG or A0)
   - β-dystroglycan (43DAG or A3a)

2) Sarcoglycan complex
   - α-sarcoglycan (Adhalin or 50DAG or A2)
   - β-sarcoglycan (43DAG or A3b)
   - γ-sarcoglycan (35DAG or A4)

3) Syntrophin complex
   - α-syntrophin (59DAP or A1)
   - β1-syntrophin (59DAP or A1)
   - β2-syntrophin (59DAP or A1)

An additional sarcolemmal protein (25DAP or A5) has been shown to copurify with these complexes but has not been extensively studied.

The three components of the sarcoglycan complex are muscle-specific glycoproteins unlike the ubiquitous dystroglycan complex[6-8].

A dramatic reduction of α-sarcoglycan (Adhalin or 50DAG) was observed in patients affected with autosomal recessive muscular dystrophy (SCARMD) [9-10], with some reduction of the other members of the sarcoglycan complex. Actually the disease is phenotypically and genotypically heterogenous, since in some cases the α-sarcoglycan (adhalin) gene itself is mutated (LGMD2D at 17q21.2)[11-12], whereas in other cases the adhalinopathy is secondary, the morbid locus being either the β-sarcoglycan gene (LGMD2E at 4q12)[13-14] or the γ-sarcoglycan (LGMD2C at 13q12)[15-17]. The respective incidence of the three categories is unknown yet. SCARMD was initially described in Tunisia[18] and further reported in patients from other North-African and Middle-Eastern countries. SCARMD stands for the most severe muscular dystrophies, and is often considered as the worst end of a continuum rather than a discrete entity, distinct from the milder forms[19].

We present a series of 32 unrelated families with mutations in the alpha-sarcoglycan gene, therefore qualifying as "primary adhalinopathies". They were found after primary DGGE screening of genomic DNA from 100 unrelated patients with proximal muscle dystrophy. In our sample α-sarcoglycanopathies account for about 50% of adhalin deficient patients. We found 22 different mutations (2 nonsens, 3 frame shift insertions or deletions, 4 splice mutations, 13 missense). All the missense mutations are in the extracellular domain, and were found in 2/3 of the mutated chromosomes.

Five missense mutations (4 of which involving a CpG dinucleotide) were found more than once with a striking prevalence of the Arg(77)Cys mutation (30% of the mutated chromosomes), and of the Arg(284)Cys mutation (13 % of the mutated chromosomes). The **recurrent mutations** are carried by unrelated subjects on different haplotype backgrounds. The dinucleotide CpG is a "hotspot" for mutation in the human genome as a result of the modification of the 5' cytosine by cellular DNA methyltransferases and the consequent high frequency of spontaneous deamination of 5-methyl cytosine (5mC) to thymidine[20]. The Arg(77)Cys mutation has also been described in Japan[21] and Brazil[22]. The Arg(98)His has been also identified in an Afro-American girl

with childhood-onset muscular dystrophy[23]. This girl was a compound heterozygote, and the other mutation was Arg(98)Cys that could be explained by a distinct mutation of the same CpG dinucleotide.

The **severity** of the phenotype varies greatly. Patients with null mutations are more severely affected. A broad range of severity is observed in missense mutations. Homozygous patients for the Arg(284)Cys mutations exhibit a milder disease than those with the Arg(77)Cys mutation. Very mild limb-girdle muscular dystrophies could be underdiagnosed since some patients could only complain of being easily tired, muscle weakness being not readily evident upon physical examination. In these mild cases, muscle biopsies are not routinely performed and sarcoglycan deficiencies not diagnosed. The actual frequency of α- and γ-sarcoglycan mutations could thus be higher than estimated from investigation of Severe Childhood-onset Autosomal Recessive Muscular Dystrophy cases[23-25].

The **widespread geographic origin** of the 32 patients of this series (22 from Western Europe, 6 from North Africa, 4 from North America) suggests that α-sarcoglycanopathies are produced by world-wide independent mutations. In North-Africa where SCARMD had been primarily described this severe phenotype is due either to α- or to γ-sarcoglycanopathies. The proportion of sarcoglycan deficiency in muscular dystrophies varies from 2% to 12%[24-25]. This variation could be explained by sample bias or could mirror actual population differences in muscular dystrophies.

It will be interesting to understand the impact of the different missense mutations on the interactions of adhalin with the other member of the sarcoglycan complex.

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Identification of muscle-specific calpain and β-sarcoglycan genes in progressive muscular dystrophies

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Introduction

Limb-girdle muscular dystrophies (LGMD) are a group of inherited neuromuscular diseases presenting clinical heterogeneity. LGMDs are characterized by progressive weakness of the pelvic and shoulder girdle muscles, and their genetic etiology has yet to be elucidated. These disorders may be inherited either as an autosomal dominant or recessive trait, the latter being more common with an estimated prevalence of 10^{-5} (Emery, 1991). In view of the lack of a consensus on the specific nosological definition of each entity, it was proposed at the 30th-31th ENMC workshop to temporarily lump these autosomal recessive entities under the common denomination of LGMD2 (Bushby and Beckmann, 1995). The fact that this decision is far from having generated a unanimous support among clinicians involved in this area, illustrates the complexity of this medical and scientific problem.

The first demonstration of a genetic basis for any form of LGMD was obtained in 1991 using biallelic RFLP markers (Beckmann et al., 1991). This study was performed in a genetic isolate from the Isle of La Réunion, localising by linkage a recessive form (LGMD2A, MIM number 253600) to chromosome 15q. Confirmation of this localization was subsequently reported in northern Indiana Amish (Young et al., 1992) and Brazilian (Passos-Bueno et al., 1993)

pedigrees. The latter and subsequent work demonstrated that the autosomal recessive forms (LGMD2) constitute a genetically heterogeneous group involving five genes. These were shown to map to either chromosomes 2p13-p16 (LGMD2B, Bashir et al., 1994), 13q12 (LGMD2C, Ben Othmane et al., 1992; Azibi et al., 1993; Noguchi et al., 1995), 15q15.1 (LGMD2A, Beckmann et al., 1991), 17q12-q21.33 (LGMD2D, Roberds et al., 1994; McNally et al., 1994) and 4q12 (Lim/Duclos/Broux et al., 1995; Bönnemann et al., 1995). The LGMD2D gene was recently identified: it encodes for the 50 kDa adhalin glycoprotein (Roberds et al., 1994; McNally et al., 1994; Picollo et al., 1995), now known as α-sarcoglycan. The LGMD2C locus was also just identified: it encodes for the 35 kDa γ-sarcoglycan (Noguchi et al., 1995). Both sarcoglycans are members of the dystrophin glycoprotein complex.

Our efforts focussed mainly on the LGMD2A and LGMD2E loci. These genes were identified following either a classical positional cloning (for LGMD2A) or a combined positional and functional cloning (for LGMD2E) strategies. The various steps involved in this quest are reviewed here.

Mapping of a Chromosome 15 Region Involved in LGMD2A

Four years ago, few highly informative genetic markers were available. Fortunately, the Généthon program had started and had already produced a score of new markers (Weissenbach et al., 1992; Gyapay et al., 1994). In parallel, we also set to develop in a targetted fashion markers from the LGMD2A region (Fougerousse et al., 1994; Pereira de Souza et al., 1994). After genotyping the newly available chromosome 15 microsatellite markers, two were found to flank the disease locus within an interval that was assessed, based on segregation studies on the CEPH reference families, as spanning 7 centiMorgans. The screening of the CEPH YAC libraries with the corresponding probes allowed the isolation of YACs which were used in fluorescence *in situ* hybridization to define the LGMD2A cytogenetic interval as 15q15.1-15q21.1 (Fougerousse et al., 1994). Different approaches were pursued for the establishment of the physical map of this area. This allowed eventually the assembly of an uninterrupted YAC contig spanning an estimated 10-12 megabases (Figure 1), with a current average STS resolution of 50 kb, or for the 36 polymorphic microsatellites on this map, of 300 kb. Twelve genes and

25 genetic markers were initially positioned in this contig, which is constituted of a minimum of 10 clones.

Preferential Localization of LGMD2A in the Proximal Part of a 1 cM 15q15.1-q15.3 Interval

New microsatellite markers developed using the YACs from the 10-12 Mb LGMD2A YAC contig were genotyped on large, consanguineous LGMD2A pedigrees from different origins (Allamand et al., 1995a). The identification of recombination events in these families allowed the restriction of the LGMD2A region to an estimated 1 cM interval, equivalent to approximately 3-4 Mb (Figure 1), bracketed by D15S514 and D15S222. Linkage disequilibrium data on genetic isolates from the Isle of La Réunion and from the Amish community suggested a preferential location of the LGMD2A gene in the proximal part of this region, an area covered by YAC 774G4.

Furthermore, analysis of the interrelated pedigrees from the Isle of La Réunion revealed the existence of at least six different carrier haplotypes. This allelic heterogeneity is incompatible with the *a priori* presumed existence of a founder effect, and suggests that multiple LGMD2A mutations may segregate in this population.

An STS Map of the LGMD2A Region

As YAC clone 774G4 spans a region showing significant linkage disequilibrium with LGMD2A, this 1.6 Mb long YAC was tentatively organized into a contig of overlapping cosmid clones, mainly by STSs screening and inter-Alu PCR hybridization. A total of 70 STSs, including 13 transcription units and 7 microsatellites covers this 1.6 Mb region with an average of 3 cosmids per STS and one STS every 25 kb (Richard et al., 1995a).

A primary expression map of chromosome 15q15 region containing the recessive LGMD2A gene

In order to progress toward the identification of the gene involved in LGMD2A, a primary transcription map of the LGMD2A genomic region was generated (Chiannilkulchai et al., 1995). The direct cDNA selection strategy was used with 3 YACs covering the candidate region and two different muscle cDNA libraries. Seventeen different transcription units were identified among

171 cDNA fragments analysed. Five sequences corresponded to known genes, and twelve to new ones. They were characterized for their sequences, physical positions within the YAC and cosmid contigs, and expression patterns. Among those specifically transcribed in muscle, the calpain gene - whose cDNA sequence was known since 1989 (Sorimachi et al., 1989) - appeared to be a good positional candidate for LGMD2A, although it was not considered, *a priori*, as a functional candidate.

Mutations in the proteolytic enzyme, calpain 3, cause LGMD2A

The CANP3 gene, encoding the large subunit of the muscle-specific calcium-activated neutral protease 3. This cysteine protease belongs to the family of intracellular calpains, requiring calcium for their catalytic activities (for a review see Saido et al. 1994). Its genomic organisation was determined, by sequencing cosmids containing this gene, which is composed of 24 exons spread over more than 40 kb (Richard et al. 1995b).

Validation of a candidate gene requires the identification of the pathogenic mutations. A total of 16 nonsense, splice site, frameshift or missense mutations in this muscle-specific gene were initially found to cosegregate with the disease in LGMD2A families, six of which were found within Réunion island LGMD2A patients (Richard et al., 1995b). A digenic inheritance model was proposed to account for the unexpected presence of multiple independent mutations in this small inbred population. One of the mutations encountered in the isle of La Réunion, was found to be a synonymous missense mutation leading to a predicted Gly-to-Gly substitution. As a matter of fact, to reveal this mutation, we had to screen lymphocyte illegitimate transcription products (Chelly et al., 1989), since the CANP3 locus shows a tissue-specific pattern of expression and for lack of muscle tissues from this like from most patients. In this manner, we could demonstrate that this apparently neutral mutation creates a new splice site, and is thus effectively equivalent to a null mutant (Richard and Beckmann, 1995). Last but not least, the finding that calpain mutations cause LGMD2A is the first demonstration of an enzymatic rather than a structural protein defect causing a muscular dystrophy, a defect that may have regulatory consequences, perhaps in signal transduction (Richard et al., 1995b).

Having identified the role of the calpain gene in LGMD2A, we next analysed the segregation of markers flanking the LGMD2A locus and carried out a search for CANP3 mutations in LGMD2 pedigrees from various geographic origins. Altogether, 42 distinct mutations were uncovered (23 missense, 11 frameshift, 2 nonsense, 4 splice site, and 2 small in-frame deletions), dispersed throughout the entire length of the CANP3 gene (unpublished data). These data indicate that muscular dystrophy caused by mutations in the muscle calpain gene are found in patients from all countries so far examined, with no predominant mutation, and further support the wide heterogeneity of molecular defects in this rare disease.

Characterisation of the murine canp3 gene

cDNA clones encoding mouse muscle-specific calpain were selected by hybridization and sequenced (Richard and Beckmann, 1996). The mouse gene encodes for an mRNA of similar size to the human CANP3 mRNA, directing the synthesis of an 821 amino acids long protein. A partial characterization of the genomic organisation of the canp3 gene suggests that it follows the same overall structure as its human homologue. The results obtained on somatic cell hybrid panel indicates either a localization on mouse chromosome 2 or on chromosome 4. Information of synteny conservation with human support the chromosome 2 localization. Interspecies comparison of the canp3 DNA sequences shows a high level of identity both at the DNA and amino acid levels. The deduced amino acid sequence shares 98.7%, 93.5% and 77.3% identity with the rat, human and chicken coding sequences, respectively (Richard and Beckmann, 1996). The degree of similarity is indicative of the fundamental biological role of this protein. Together with the other known animal sequences, the mouse canp3 sequence would be useful to assess missense mutations in LGMD2A patients. This sequence information can be used to study the spacio-temporal and tissue-specific expression as well as to develop knock-out mouse models. It represents thus a useful tool for gene-regulation, physiopathological studies of the disease as well as for therapeutic investigations.

Genetic heterogeneity of autosomal recessive LGMD in a genetic isolate (Amish) and evidence for a new locus

Cases of autosomal recessive limb-girdle muscular dystrophy among members of the old order of Amish of northern and southern Indiana were already described by Jackson and Carey (1961) and Jackson and Strehler (1968). The families of these communities are interrelated by multiple consanguineous links and common ancestry which can be traced back to the 19th century in the Canton of Bern, Switzerland. It is upon studying these families that Young et al. (1992) confirmed the chromosome 15 localisation of the LGMD2A locus. All Amish LGMD patients from northern Indiana were subsequently shown to be homozygous for the same Arg769Glu missense CANP3 mutation (Richard et al., 1995b).

Allamand et al. (1995b) reported the exclusion of the LGMD2A locus in six Amish kindreds from southern Indiana that are related by multiple consanguineous links to the same northern Indiana families in which the involvement of the chromosome 15 locus was previously demonstrated. These findings disclosed genetic heterogeneity of LGMD in this Indiana Amish isolate. In view of the high consanguinity and the similar clinical presentation of all Amish LGMD patients, this result was totally unexpected. Furthermore, genetic analyses also ruled out the possible involvement of the other known LGMD2 loci, thus demonstrating that a mutation within at least one additional locus leads to this condition. Several candidate genes putatively involved in neuromuscular disorders were also excluded. A systematic genome-wide genetic study undertaken with six of these families eventually uncovered genetic linkage to the pericentromeric region of chromosome 4 (Lim/Duclos/Broux et al., 1995), thereby providing legitimacy to the LGMD2E locus.

Identification of the LGMDE locus

Meanwhile, the human cDNA of β-sarcoglycan, a 43 kDa dystrophin-associated glycoprotein (also known as A3b), was cloned and mapped to the same chromosome 4 region (Lim/Duclos/Broux et al., 1995). Several chromosome 4 pericentromeric markers and a β-sarcoglycan intragenic polymorphic CA repeat cosegregated perfectly with the disease in Amish families from Southern Indiana with autosomal recessive limb-girdle muscular dystrophy. In addition, reconstruction of haplotypes allowed identification of chromosomes descending

from a common ancestor. Furthermore, a Thr-to-Arg missense mutation, present in a homozygous state in all patients, was identified within the β-sarcoglycan gene that leads to a dramatically reduced expression of β-sarcoglycan in the sarcolemma and a concomitant loss of α-and γ-sarcoglycan, thereby disrupting the integrity of the dystrophin glycoprotein complex (Lim/Duclos/Broux et al., 1995). Similar evidence were also obtained by Bönneman et al. (1995). These data demonstrated that a defect in β-sarcoglycan is responsible for the recessive muscular dystrophy in southern Indiana Amish families. Thus the β-sarcoglycan gene is the fifth locus (LGMD2E) involved in autosomal recessive limb-girdle muscular dystrophy, and the third sarcoglycan involved in a myopathy (Table I).

Conclusion

The year that just ended seems to have been a good vintage year for geneticists and clinicians interested in autosomal recesive progressive muscular dystrophies. Indeed in a little over a year, four LGMD2 genes have been identified, three of which belong to the dystrophin-associated protein complex, the fourth one being an enzyme (Table I). These discoveries raise more questions than answers. But this is not a matter of complaint. They are also a new source of hope. This knowledge can, on the one hand, already be used to learn more about the physio-pathological processes involved. On the other hand, careful examination of the phenotype-genotype relationships (e.g. Fardeau et al., 1996) will enable a clearer définition of the specific nosological boundaries of each one of these similar, yet different, clinical entities, a knowledge that could be of great immediate benefit to the patients themself. And last but not least, it opens prospects for new therapeutic avenues.

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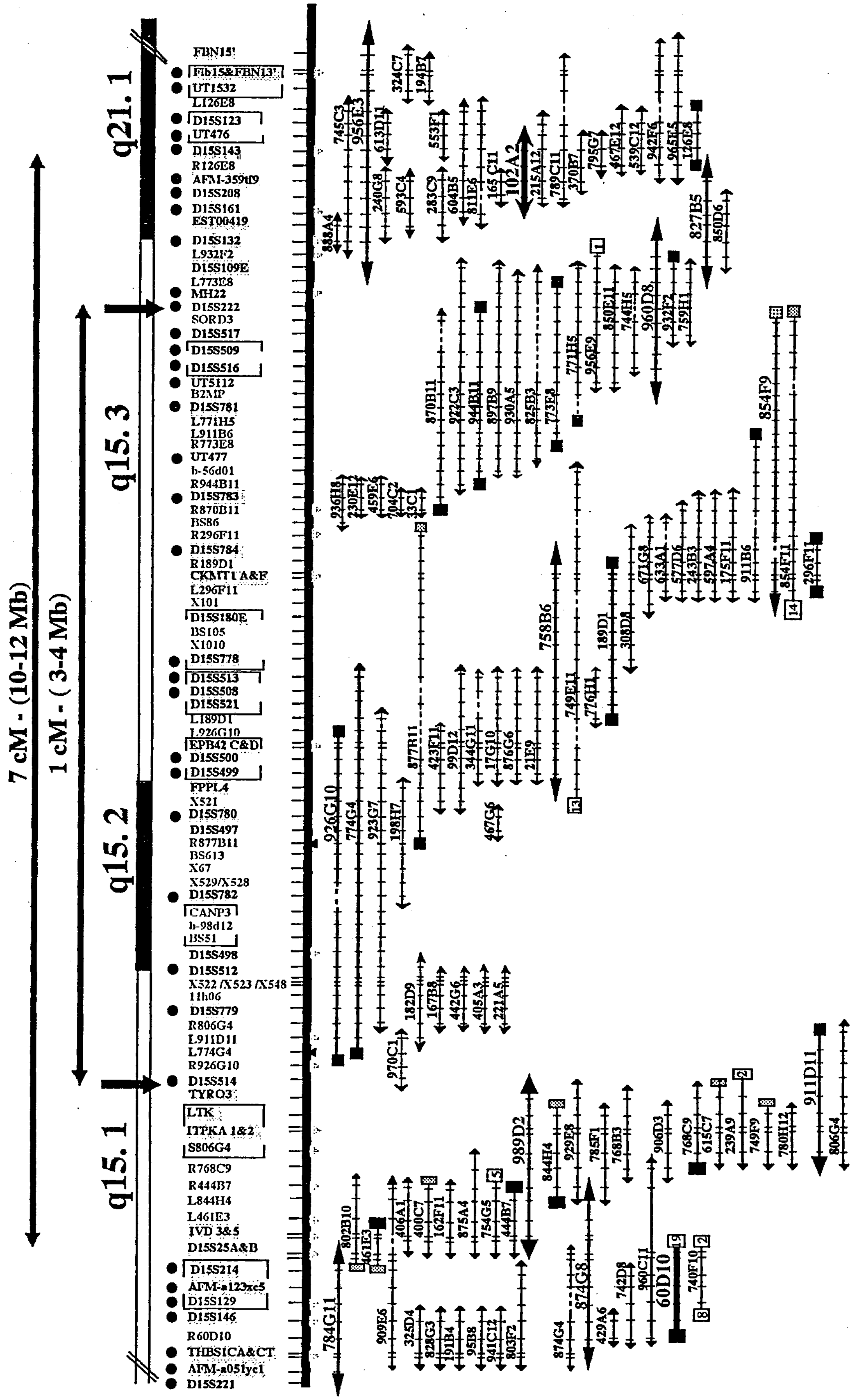

q21.1
q15.3
q15.2
q15.1
7 cM - (10-12 Mb)
1 cM - (3-4 Mb)
FBN15'
Fib15&FBN13'
UT1532
L126E8
D15S123
UT476
D15S143
R126E8
AFM-359tf9
D15S208
D15S161
EST00419
D15S132
L932F2
D15S109E
L773E8
MH22
D15S222
SORD3
D15S517
D15S509
D15S516
UT5112
B2MP
D15S781
L771H5
L911B6
R773E8
UT477
b-56d01
R944B11
D15S783
R870B11
BS86
R296F11
D15S784
R189D1
CKMT1 A&F
L296F11
X101
D15S180E
BS105
X1010
D15S778
D15S513
D15S508
D15S521
L189D1
L926G10
EPB42 C&D
D15S500
D15S499
FPPL4
X521
D15S780
D15S497
R877B11
BS613
X67
X529/X528
D15S782
CANP3
b-98d12
BS51
D15S498
D15S512
X522/X523/X548
11h06
D15S779
R806G4
L911D11
L774G4
R926G10
D15S514
TYRO3
LTK
ITPKA 1&2
S806G4
R768C9
R444B7
L844H4
L461E3
IVD 3&5
D15S25A&B
D15S214
AFM-a123xe5
D15S129
D15S146
R60D10
THBS1CA&CT
AFM-a051yc1
D15S221

Figure 1: LGMD2A YAC contig

The map shows the contiguous array of YAC clones which are presented as lines (Only clones with more than one characterized STS are drawn). The names of the STSs are given above the line representation of the contig. The position of polymorphic microsatellite markers is indicated by the presence of a red dot above the marker name. The STSs are drawn equidistant except when they are issued from the same gene (and hence are arbitrarily ordered), but for the 5' end of the fibrillin gene. Adjacent markers which cannot be oriented with respect to this map are placed within brackets. Inverted triangles: STSs having supplied YACs upon screening of the CEPH libraries. Upright triangles: STSs which failed to yield YACs in this screening. Bars on full lines indicate positive STSs. Dotted lines mean that a clone was not found to be positive for a given internal STS. Arrows at the end of the clone indicate that a clone was found to be negative for the adjacent STSs. The red lines represent the minimal tiling path consisting of 10 YAC clones spanning the contig. The chromosome 15 YAC end-STSs (R or L) are shown in full squares, and YAC end constituted of repeated sequences as dotted squares. The ends are arbitrarily drawn whenever both ends failed to map within the contig. One extremity of clone 911B6, a double insert and mitotically unstable YAC, is shown as a hatched square, in the middle of the contig, as this clone was shown to be positive for external STSs. A drawing showing approximate G-banding pattern of the corresponding region of chromosome 15 is placed above the contig presentation. The smallest recombinant interval defining the LGMD2A region is shown by the blue vertical arrows on the regional chromosome map, and the distance spanning the recombinant intervals is given above.

Table I: Molecular etiology of autosomal recessive progressive myopathies

Locus symbol (synonym)	MIM	chromosomal localisation	protein (size, kDa)	sub-cellular localisation	identification method	function
LGMD2A	253600	15q15	calpain (94)	?	pos. cloning	?
LGMD2C (SCARMD1)	253700	13q12	γ-sarcoglycan (35)	membrane	C.G.S.	link between
LGMD2D (SCARMD2)	600119	17q12	α-sarcoglycan (50)	of muscle	C.G.S.	laminin-2
LGMD2E	600900	4q12	β-sarcoglycan (43)	cells	pos. + funct. clo.	& dystrophin
CMD[1] lamina	156225	6q22	laminin a-2 (342)	extracell.	C.G.S	component of basal
EDMD[2]	310300	Xq28	emerin (28)	membrane	pos. cloning	vesicular transport ?

Table I: Molecular etiology of autosomal recessive progressive myopathies.

[1] Helbling-Leclerc et al, 1995; [2] Bione et al., 1994. The following abbreviations stand for: C.G.S.: candidate gene strategy; pos. cloning: positional cloning; funct. clo.: functional cloning.

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Skeletal Muscle Specific Novel Calpain, p94: Properties and Function

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The activity of intracellular proteases must be regulated strictly in order to prevent co-existing cytosolic proteins from random degradation. If this activity regulation system does not work properly, the level of protease activity increases or decreases than the normal level resulting in rapid degradation or accumulation of proteins leading to various disorders. Muscular dystrophy is an example of the former case and deposition of amyloid proteins in Alzheimer's disease is the latter case. Understanding of the activity regulation system of intracellular proteases is therefore essential to clarify the molecular mechanisms of various diseases arising from the abnormal level of protease activity.

Calpain is a most typical cytosolic cysteine proteinase in animal cells and the activity is regulated by calcium ions and a proteinaceous inhibitor, calpastatin (for reviews and references see (1-3)). It plays a pivotal role not only as a modulator in calcium-dependent cellular events but also as an initiator of degradation of proteins, especially short lived proteins. Calpain is believed to be responsible for muscle atrophy in Duchenne muscular dystrophy. We have been studying physiological function and regulation of activity of calpain. Quite recently, several new calpain species have been found. Some of them are expressed exclusively in certain tissues in contrast to ubiquitous expression of calpain thus far known. These tissue specific novel calpain species are more suitable than ubiquitous calpains in analyzing physiological function and activity regulation system. Here we summarize our recent studies performed mainly using skeletal muscle specific calpain, p94.

1) New Family Members of Calpain

For a long time, two isozymes, μ and m-calpains with distinct calcium sensitivities are known. These are composed of a distinct catalytic 80kDa subunit (80K) and a common 30kDa regulatory subunit (30K). Recently several novel calpain species including a third ubiquitous calpain, μ/m type[4], have been identified, indicating that calpain constitutes a large family. These are homologs of 80K of μ and m-calpains as summarized in Fig. 1. Skeletal muscle specific calpain, p94 or nCL-1 (novel calpain large subunit), contains three insertion sequences, IS1, IS2 and NS. It must be mentioned that an alternative splicing short product without the calcium binding domain is found for stomach specific calpain (nCL-2/nCL-2') and drosophila calpain (calp A/calp A'). These novel calpain species including alternative splicing products are not minor species at least as far as the mRNA level is concerned. For instance, the mRNA levels of μ, m, nCL-2, and nCL-2' in stomach are essentially the same, indicating that these species must have their own important function. Further, the amount of p94 mRNA in skeletal muscle is at least one order of magnitude higher than those for μ and m-calpains. Nevertheless, none of them except for μ/m-calpain has been isolated as a protein and characterization of these calpains remains to be an important issue.

2) The large 80kDa Subunit is an Active Calpain Species

Previous dissociation and reassociation studies of calpain have shown that 80K has intrinsic protease activity, but 30K is essential for expression of full enzyme activity, suggesting that both subunits are required for full activity. Since novel calpain seems to exist as a monomer of an 80K homologue, we reexamined renaturation of 80K after dissociation from 30K in urea and found that 80K alone could regain full enzyme activity after prolonged incubation (ca.30 days at 4°C)[5]. Addition of 30K to 80K accelerated the rate of renaturation but did not affect the final level of activity recovery. An *E.coli* chaperonin GroE had similar effect but only in the presence of ATP, suggesting that 30K functions as a chaperon rather than as an activator of 80K. In addition, dissociated 80K showed enhanced calcium sensitivity identical to the that of activated N-terminally modified calpain prepared by preincubation in calcium[6]. The calcium sensitivity of μ and m-calpains is not high enough to be active in vivo and must therefore be increased by a certain mechanism. Autolysis which

modifies the N-terminal region of both subunits has long been proposed as an activation mechanism. The N-terminally modified 80K prepared by autolysis showed the same enzymatic properties as those of intact 80K including calcium sensitivity. Moreover, it has been shown that calpain comprising N-terminally modified subunits tends to dissociate more easily. These results clearly indicate that autolysis is not the cause of activation but dissociation of 80K from 30K corresponds to activation. In conclusion, 30K can now be interpreted as a regulatory subunit essential for regulation of 80K activity in response to changes in calcium concentrations.

3) Properties of Muscle Specific Calpain p94

Although the mRNA level of p94 is significantly higher that μ and m-calpains, identification of p94 as an enzyme is very difficult. In vitro transcription and translation experiment of p94 cDNA indicates that newly translated p94 degrades very rapidly with a half-life of ca.30min. This degradation is autocatalytic, because various point mutants of the active site Cys, His and Asn residues are stable. Experiments with truncation mutants revealed that IS2 is responsible for this rapid auto-degradation. IS2 might contain a signal for rapid protein degradation. Although isolation of intact p94 is hardly possible, p94 can be detected histochemically in cells after transfection of its cDNA. p94 exists in both cytosol and nucleus which is controled by a nuclear translocation signal located in IS2. When rat skeletal muscle is stained with antibody, a similar staining patter is observed. Since the p94 message can not be detected in cultured muscle cells but exists in skeletal muscle, the message must appear at a certain stage of muscle cell differentiation. p94 in the nucleus might regulate differentiation by regulating the level of muscle specific transcription factors such as MyoD, myogenin, etc.

4) p94 Binds to Connectin/Titin

To identify a regulatory subunit of p94, if any, a human skeletal cDNA library was screened by the yeast two-hybrid system using an inactive point mutant of p94 where the active site Cys-129 is replaced with serine. We used the μ and m-calpain large subunit (μCL and mCL) and calpastatin as a control for this screening. The results showed that μCL and mCL interact with 30K but calpastatin which is known to bound calpain only in the presence of calcium

does not bind to calpain. The results suggest that the yeast two-hybrid system works in low calcium conditions in yeast so that only the interaction which occurs in the absence of calcium can be detected. Under the conditions used, p94 did not bind to 30K but 32 positive clones were obtained[7]. Comparing the sequences of the clones with the complete amino acid sequence of human connectin[8], it was identified that 31 clones encode sequences identical to the C-terminal sequence of connectin and that the remaining one clone corresponds to the connectin sequence of the N_2 line position. Connectin is a giant elastic muscle protein of ca.3000kDa spanning the Z line to M line in myofibrils (Fig. 2). The N- and C-termini of connectin locate at the Z- and M-lines, respectively. Connectin is susceptible to split at the N2 line which is 1200kDa down stream from the N-terminus of connectin or the Z-line. Hydrolysis of connectin at this position is often observed during preparation of connectin and one of typical features observed with muscles of Duchenne muscular dystrophy. Although the physiological meaning of this splitting is not clear, calpain or calcium is reported to be involved. The present results that p94 binds to the N_2 line suggest that p94 might be responsible for the splitting. Since binding of p94 to the N_2-line is much stronger than that to the C-terminal region, further precise experiments were performs with the former case. Using various deletion mutants the binding site of p94 for connectin was identified as IS2. This is consistent with the fact that μ and m-calpains without IS2 do not bind connectin. It should be noted that p94 specific sequence IS2 is responsible for nuclear localization, rapid autolysis and binding to connectin. IS2 thus plays a crucial role in the physiological function of p94.

To examine p94-connectin binding in vivo, we isolated myofibrils and examined colocalization of p94 and connectin. SDS-gel elecetrophoresis and Western blottings using p94 antibodies indicated that intact p94 existed in the myofibril fraction. However, once connect was extracted from myofibrils by a buffer containing high salt, p94 degraded very rapidly into fragments. Since isolated p94 is unstable, the presence of intact p94 suggests that its activity is regulated when bound to connectin. Further, histochemical detection of p94 by its specific antibodies, gave signals at the Z-band and at positions corresponding to the N_2-line[7], being consistent with the results of the two hybrid system, although we have not yet identified clones corresponding to the Z-line staining. Since connectin is one of the biggest proteins, to obtain clones for the N-

tertminal region is very difficult.

5) Correlation between p94 and Muscular Dystrophy

Quite recently mutations in the p94 gene are shown to cause limb girdle type 2A muscular dystrophy, LGMD2A[9]. Various mutations including point mutations and nonsense mutants which yield shorter p94 without the protease domain are found. Thus a loss-of-function of p94 must be the cause of LGMD2A. In the case of Duchenne type muscular dystrophy, defects in dystrophin changes the membrane permeability of muscle cells resulting in increase in the intracellular calcium concentration which triggers activation of calpain and degradation of muscle proteins. In the case of LGMD2A, similar degradation of muscle proteins is observed, suggesting participation of calpain in this case as well. It is therefore essential to elucidate a signaling pathway from inactivation of p94 to activation of calpain and degradation of skeletal muscle proteins. This signal transduction system will involve p94 binding proteins, neighboring proteins and substrate proteins, such as connectin, calpastatin, myotonin protein kinase, etc. It is hoped that precise analysis of this signal transduction system will eventually clarify the mechanism of muscle degradation for various muscular dystrophies.

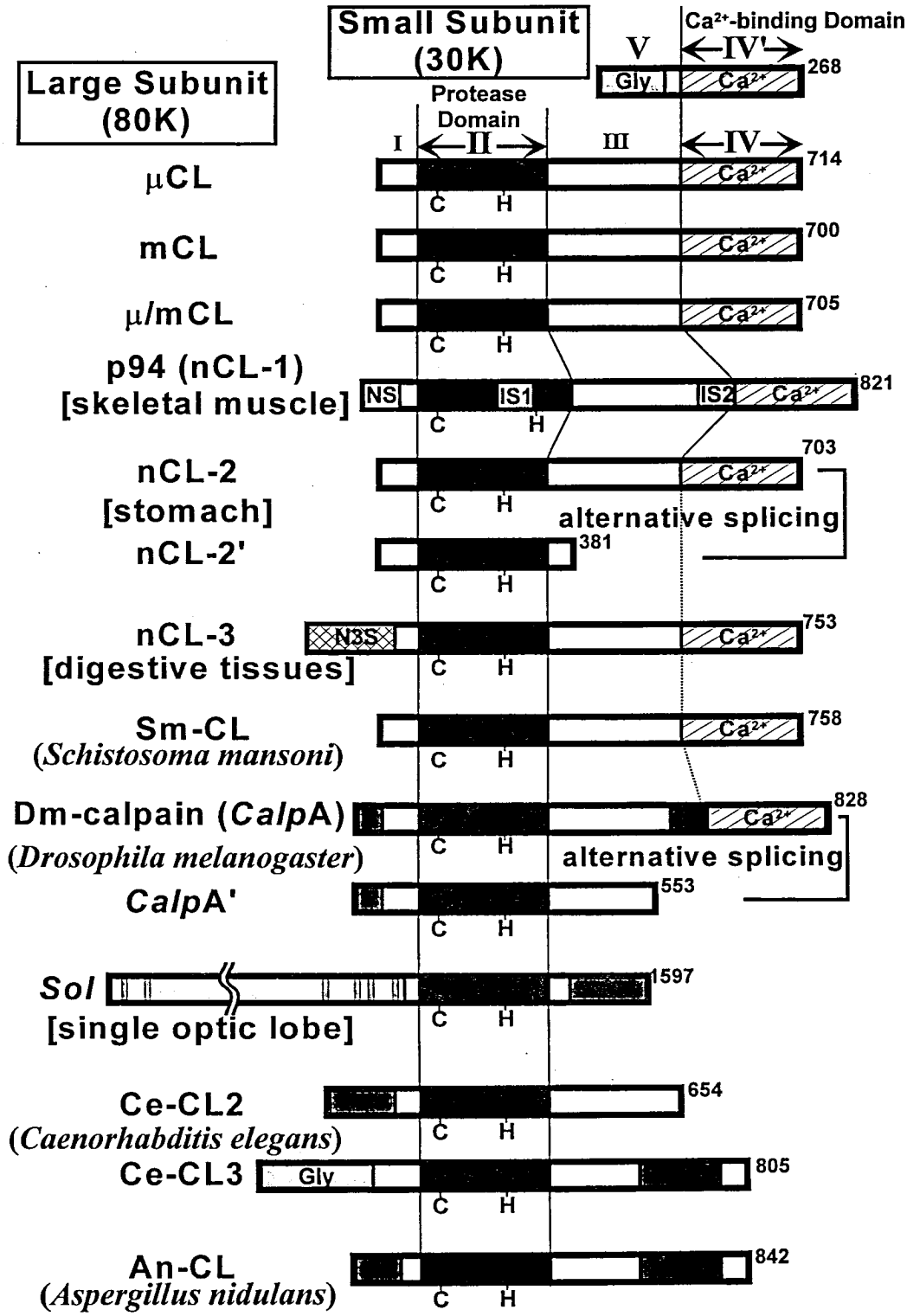

Fig. 1. Schematic domain structure of calpain family members.

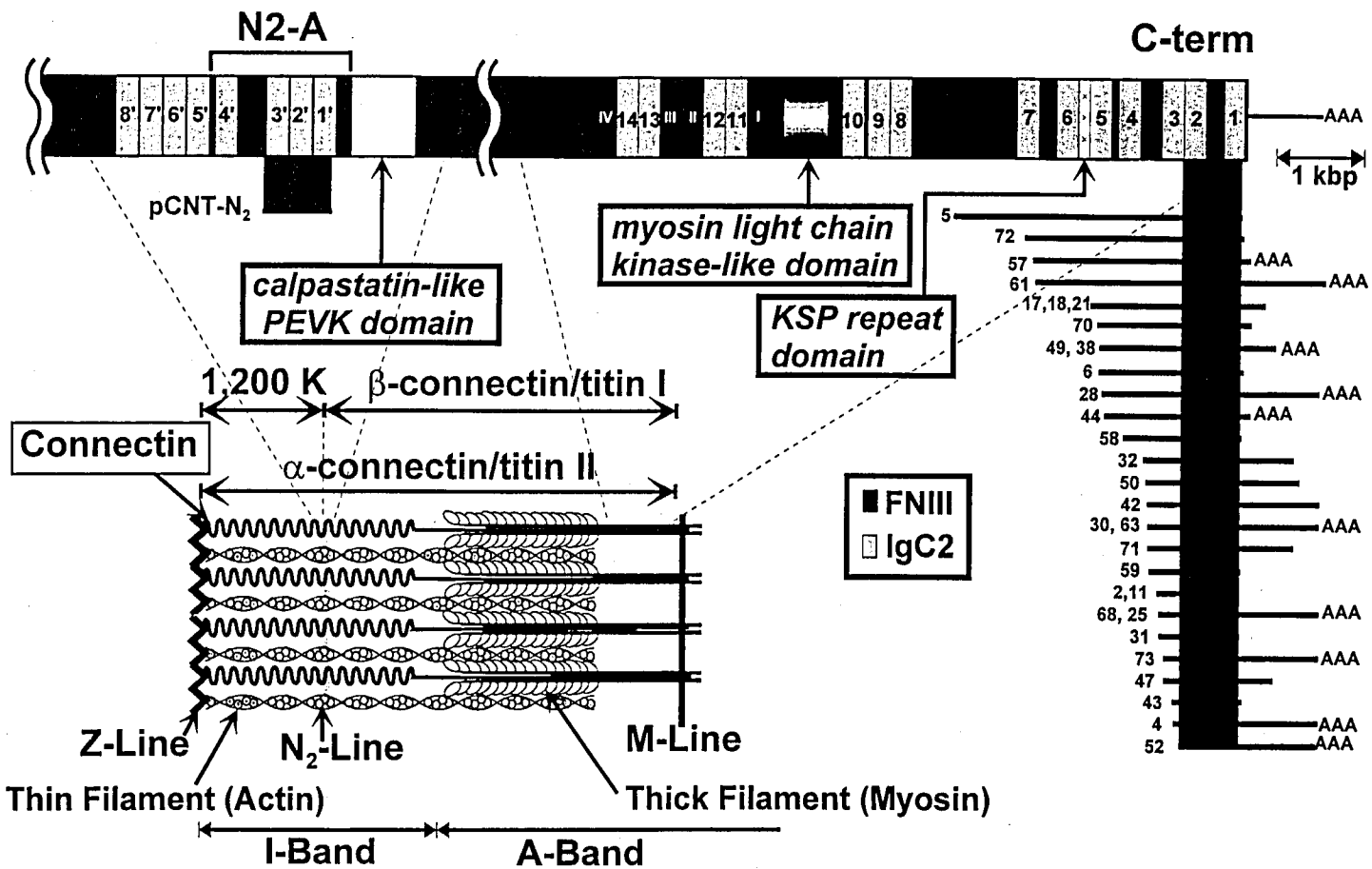

Fig. 2. Binding of p94 to connectin.
The positions of isolated 32 clones are shown along the schematic structure of connectin.
Schematic myofibril structure is also shown.

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Familial Hypertrophic Cardiomyopathy: A disease of the cardiac sarcomere

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Familial hypertrophic cardiomyopathy (FHC) is inherited as an autosomal dominant disorder that is characterized by hypertrophy, often of the left ventricle, with predominant involvement of the interventricular septum in the absence of other cause of hypertrophy such as hypertension or valvular heart disease. The predominant cardiac pathology is myocyte hypertrophy and sarcomere disarray, the former is found in most cardiac diseases while the latter is a hallmark of FHC. The ventricular diastolic function is usually impaired and the clinical manifestations range from a benign asymptomatic course to severe heart failure and sudden death.

FHC is not only heterogeneous phenotypically, but it is also genetically heterogeneous.

In 1989, the first locus (CMH1) was identified on chromosome 14q11-q12[5]. Since the α and β myosin heavy chain genes are localized in the same region, they became obvious candidates as disease genes. Indeed, a missense mutation was thereafter detected at codon position 403 in the gene which codes for the β myosin heavy chain[3]. Since this finding, more than 29 different point mutations have been described(for review see (9)).

Besides this allelic heterogeneity, it became very rapidely obvious that FHC was also heterogeneous in term of loci and 4 loci were subsequently identified on chromosome 1q3 (CMH2)[13], 15q2 (CMH3)[10], 11p13-q13 (CMH4)[2] and 7q3 (CMH5)[7].

The disease genes corresponding to CMH2 and CMH3 were identified and shown to code for cardiac troponin-T (cTnT) and α-tropomyosin (α-TM) respectively[11]. Lastly, the gene corresponding to CMH4 was also identified annd shown to code for the cardiac Myosin-Binding Protein C (MyBP-C)[1, 12].

I) - β-Myosin Heavy Chain

Myosin is the molecular motor which drives shortening of the sarcomere, hence muscle contraction. Myosin consists of two heavy chains and two pairs of light chains all of which exist as various isoforms that are specific for the various type of muscles. In the adult human heart, the major myosin heavy chain is the β isoform which is also expressed in slow fibers of skeletal muscle.

From a structural point of view, myosin heavy chain is a complex molecule. The N-terminus forms a globular head which contains the fixation sites for the myosin light chains as well as all the necessary elements to generate movement of actin relative to myosin during ATP hydrolysis. The C-terminus part of the molecule has a rod shape and is involved in the polymerisation of the molecule to form the thick filaments.

All mutations associated with FHC are missense point mutations and are localized in the globular head or in the head-rod junction of the molecule. The three-dimensional structure of the β myosin heavy chain is unknown but because the primary sequence of myosin is highly conserved, it can be expected that the three-dimensional structures of all myosin molecules will be very similar hence making it possible to use the three-dimensional structure of chicken skeletal myosin to interpret the effects induced by the various mutations. All the mutations in the cardiac amino acid sequence map to well-defined parts of the three-dimensional structure of chicken skeletal myosin[8]. They are clustered around specific locations in the head and in particular, one group is associated with the actin binding interface, a second is close to the active site and a third group is close to the interface of the heavy chain with the essential light chain. Recently, taking advantage of the fact that β myosin heavy chain is also expressed in slow twitch muscle fibers, a study was conducted on skeletal muscle biopsies obtained from patients with three distinct β myosin heavy chain mutations[6]. Two of these mutations, one near the binding of essential light chain and one at the actin interface, show depressed velocity of shortening while the third, at the end of the ATP-binding pocket, shows no modification of the myosin properties. Although there is variability in the nature and the extend of functional impairments in skeletal fibers containing different β myosin heavy chain mutation, it is very likely that these functional alterations constitute the primary stimulus for the cardiac hypertrophy.

II) - α-Tropomyosin and Cardiac Troponin T

Tropomyosin and Troponin T interact with one another and are components of the thin filament in the sarcomere. As for the myosin, multiple isoforms exist which show muscle type specificity.

Two mutations have been described for the α tropomyosin which both alter the net charge of the molecule and affect a region close to one of the binding sites of Troponin T to tropomyosin.

As for the cardiac troponin T, five out of seven mutations change the net charge of the molecule and are localized in a part of the molecule involved in the interaction with tropomyosin. A sixth mutation induces aberant splicing which results in a premature termination of the molecule which, again, will not interact with tropomyosin.

As for the β myosin heavy chain, it is very likely that all these mutations alter the kinetic of the interaction between thin and thick filaments which results in a modification in the speed of contraction of the cardiac sarcomere leading to compensatory hypertrophy.

III) - Cardiac Myosin Binding Protein C

The myosin-binding protein C is also found in the sarcomere where it is arrayed transversely in the sarcomere A-bands and bind myosin heavy chain in the thick filament, possibly actin in the thin filament and titin in elastic filaments. In particular, the domain of myosin interaction is localized at the C terminus end of the molecule.

The function of the MyBP-C is not well understood, however, recent studies have shown that partial extraction of MyBP-C from rat skinned cardiac and rabbit skeletal muscle fibers leads to an increased rate of contraction and increases the Ca^{++} sensitivity of the force-velocity curve[4]. These results tend to indicate that MyBP-C plays an important role in regulating muscle contraction

Three distinct mutations have been identified, two of which affect splicing of the transcript and result in premature terminaison of the protein thus leading to the formation of a variant lacking the myosin binding domain. These mutations could induce cardiac hypertrophy through a perturbation of the sarcomere function.

In conclusion, all the genes which have been identified so far as being responsible for FHC code for a component of the sarcomeric machinery which reinforce the hypothesis that FHC is a pathology of the sarcomere and that hypertrophy is a compensatory mechanism in response of an alteration of the muscle contraction. It remain to be seen if the unidentified genes also belong to the same family.

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Identification and characterization of a determining gene in spinal muscular atrophy

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Spinal muscular atrophies (SMA) are a group of neuromuscular disorders characterized by degeneration of anterior horn cells of the spinal cord leading to limb and trunk paralysis with muscular atrophy. They represent the second most common fatal recessive autosomal disorder after cystic fibrosis with an incidence of 1 in 6000 live births. Based on clinical criteria from the International SMA Consortium that include age of onset, milestones of development and age of survival, the SMA are classified into 3 forms. The most acute form or Werdnig-Hoffmann disease (type I) is characterized by a severe hypotonia with an onset early after birth or within the next 3 months. This disease may be distinguished from the intermediate form (type II), and juvenile disease (type III or Kugelberg-Welander). Type II children will acquire the ability to sit unaided but will never walk. Type III SMA has a later onset and will usually progress to a chronic course. The underlying biochemical defect(s) remains unknown. It leads us to develop a positional cloning strategy that allows the identification of a disease gene by its position on the genome.

By means of linkage analysis, we and others have shown that all three forms of SMA map to chromosome 5q11.2-q13.3 (1-4), suggesting that they are allelic disorders. These results were the starting point for a high resolution genetic map. The genetic interval between the flanking markers was successively reduced from 9 centimorgans cM) to 2 cM between markers AFM265wf5 and AFM281yh9 (5). This interval was cloned into a 4-Megabase yeast artificial chromosome (YAC) contig spanning the disease locus by combining chromosomal walking with the identification of new highly

polymorphic markers. The 5q13 chromosomal region is characterized by the presence of specific low-copy repeats (6). Indeed, polymorphic markers detected either 2 (C212 and C272) or 3 (C161) loci within the 5q13 region. In addition, no recombination event was detected between loci identified by markers C212-C272 and the SMA locus, indicating that these markers are close to the disease gene.

To test the hypothesis that the low copy repeats of the 5q13 region might trigger large-scale rearrangements, we analyzed the allele segregation at loci detected by C212 and C272 in 201 non-consanguineous SMA families. Absence and incomplete parental contributions were observed in 9 affected children. Southern blot analysis confirmed the presence of either inherited or de novo deletions in these patients. In addition, an heterozygosity deficiency at loci detected by markers C212 and C272 was observed in 18% of type I patients. These results indicated that deletion events of the 5q13 region in SMA are statistically associated with the severe form of the disease (type I). Interestingly, *de novo* deletions might account for the low segregation ratio in SMA (7). They might also account for the apparent genetic heterogeneity of the disease. The presence of low-copy repeats on chromosome 5q13 may account for the instability of the region and trigger frequent deletions by means of unequal crossing-over events in SMA. A genetic map allowed to characterize the extent of the 5q13 region involved in these rearrangements. Studying all polymorphic DNA markers derived from the YAC contig, we observed that the smallest deletions occurred within a region bordered by loci detected by marker C161 and the cluster C212-C272. These results suggested that the SMA locus lies within an interval of 1200 Kb entirely contained in YAC clone 903D1.

The two strategies undertaken consisted in characterization of the smallest deletion in SMA patients with the identification of candidate genes within the critical region. PFGE (Pulse Field Gel Electrophoresis) analysis of the critical region revealed a high degree of length variation of restriction fragments in both controls and SMA individuals using different restriction enzymes. Clearly, this variability hampered our ability to recognize abnormal restriction fragments in SMA patients (8). This observation emphasizes the genomic complexity of the critical region. In regard of this complexity, a fine physical map of the YAC contig covering the SMA critical region was

established. The position of the restriction sites and DNA markers provided evidence for a large inverted duplication of an element that was divided into Etel and Ecen according to location. Southern blot analysis using probe He3 enables to distinguish Etel from Ecen. The analysis of SMA patients born to consanguineous parents provided evidence for genomic rearrangements in Etel on both mutant chromosomes and supported the location of the SMA gene in Etel between probe He3 and marker C272. This critical region is entirely contained in a 140 Kb fragment.

We isolated a candidate gene named SMN (Survival Motor Neuron) gene by the detection of interspecies conservation using a subclone from a phage containing marker C272 (8). Screening of human fetal brain cDNA library allowed the isolation of 7 overlapping cDNA clones. Northern blot analysis revealed the presence of a widely expressed 1.7 Kb transcript. This gene is also expressed in the spinal cord. Sequence analysis revealed an open reading frame encoding a putative protein of 32 Da with no homology with sequences in databases. Specific hybridization with non-overlapping YACs containing only Ecen or Etel provided evidence for the duplication of SMN gene. But, only the telomeric version was highly candidate by its position.

The organization and structure of both SMN gene and its highly homologous counterpart (centromeric copy) were determined (9). The gene is about 20 Kb in length and is composed of nine exons. The sequence data of the 5'end of the gene show that marker C272 is within a putative region of gene regulation. The sequence analysis of each exon and its flanking regions allowed the detection of 5 discrepancies between the centromeric and SMN genes in introns 6, 7 and exons 7 and 8.

The analysis of SMN exons 7 and 8 revealed that all controls carried the SMN gene and 95% of them also carried the copy gene (8). On the other hand, SMN exon 7 is absent in 98,6% of patients independently of the clinical subtype. In addition, the three SMA patients retaining SMN exon 7 carried either a point mutation (Y272C) or short deletions in the consensus splice sites of SMN introns 6 and 7. Furthermore, in a recent collaboration with the group of Montserrat Baiget in Barcelona 54 unrelated Spanish SMA families were analyzed (10). Ninety one percent of patients lacked SMN exon 7 on both mutant chromosomes. Four patients retaining SMN exon 7 carried a 4 bp deletion resulting in a frameshift in exon 3 with a premature stop codon. The last patient retaining

SMN exon 7 had a limited gene conversion changing SMN exon and intron 7 into its highly homologous copy. These observations give strong support to the view that mutations of the SMN gene are responsible for the SMA phenotype. These findings have direct clinical applications in the SMA diagnosis. The absence of SMN exon 7 in patients makes molecular diagnosis easy in typical and variant SMA (11,12). Furthermore, direct prenatal diagnosis becomes feasible.

These results strongly suggest that the SMN gene and its copy must be different in some respects. Northern blot and RT-PCR analyses revealed that both genes are transcribed and that a differential splicing of exon 7 enables the SMN gene and its copy to be distinguished(8). Indeed, RT-PCR analysis from exon 6 to 8 revealed that the centromeric copy gene but not SMN gene undergoes alternative splicing of exon 7 to produce transcripts lacking this exon and a putative protein with a different C-terminal end. Investigations at the protein level should help addressing this question.

We investigated the correlation between the clinical phenotype and the genotype at these loci (13). A total of 106 patients were classified into type I (44), type II (31) and type III (31) and analyzed using SMN, markers C212 and C272, and NAIP mapping upstream and downstream from SMN respectively. NAIP is the neuronal apoptosis inhibitor protein gene characterized by Alex Mackenzie's team (14). NAIP exon 5 is specific to Etel. The combined analysis of all markers showed that a large proportion of type I (43%) carried large scale deletions involving both SMN and its flanking markers (C212/C272 and NAIP exon 5), as compared with none of the patients with type II or III SMA. The presence of large scale deletions involving these loci is specific to Werdnig-Hoffmann (type I) and allows one to predict the severity of the disease in our series. However, smaller rearrangements can still result in a severe phenotype as 27% of SMA type I patients lacked the SMN gene but not the C212-C272 or NAIP loci. These data suggest that other genetic mechanisms might be involved in the variable clinical expression of the disease. Elucidating the function of the gene products will be important for the understanding of the pathogenesis of SMA.

Recently, we and others (15,16,17) analyzed unusual SMA pedigrees. In these families, both the affected and the apparently non-affected siblings carried the same haplotype. These observations suggested either a misdiagnosis

or genetic heterogeneity of the disease. However, the absence of SMN gene confirmed the diagnosis of SMA patients with a disease locus linked to the SMN. The fact that the SMN gene was lacking in the non-symptomatic sibling may be due to either an incomplete penetrance of the gene or an additional genetic event taking place in the non-affected but not in the affected siblings. This situation is observed only in the mildest forms of the disease. This situation is rare and represents less than 1% out of 302 SMA families.

Careful analysis of the SMN gene and its copy at the genomic, the transcriptional and the protein level should contribute to the understanding of this extreme clinical variability. The creation of an animal model by SMN gene targeting will be very helpful to understand the physiopathology of the disorder.

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Molecular genetic diagnosis of Emery-Dreifuss muscular dystrophy

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X-linked Emery-Dreifuss muscular dystrophy (EDMD: MIM 310300) is first described by Drs. Dreifuss and Hogan in a large Virginian family in 1961. Soon after, in 1966, Dr. Emery, together with Dr. Dreifuss, reported this disease as a new form of X-linked muscular dystrophy after detailed clinical, biochemical, and clinical genetic observation (1,2). EDMD is a disease characterized by the triad of; (i) slowly progressive muscle wasting and weakness with a humero-peroneal distribution, (ii) contractures of the elbows, Achilles tendons and posterior neck, and (iii) cardiomyopathy with conduction block which is often life-threatening. In 1994, mutations in the *STA* gene at the Xq28 locus have been found in patients with EDMD (3). The name *STA* is originated from Dr. Stefano Rivella's clone-A. Importantly, this gene encoded a new protein named 'emerin' (3). We raised antibodies against synthetic peptide fragments predicted from emerin cDNA, to elucidate the subcellular localization of the protein (4). By these antibodies, we found positive nuclear membrane staining in skeletal, cardiac and smooth muscles in the normal controls and in patients with a wide range of neuromuscular diseases other than EDMD who had a novel nonsense mutation in the *STA* gene (4). In the normal controls but not in EDMD, a protein immunoreactive with the antibodies which had a relative molecular weight of 34 kDa exist. These findings suggest the specific deficiency of emerin in the nuclear

membrane of muscle cells in EDMD and the potential involvement of emerin in the pathological process of the disease.

Emerin

The human *STA* gene in the teromeric region of Xq28 locus is 2147 bp long, and has an open reading frame of 762 nts which encodes a serine-rich protein of 254 amino acids named "emerin" (3, 5). Emerin mRNA shows ubiquitous tissue distribution with the highest expression in skeletal and cardiac muscles (3). The cDNA sequence of emerin predicts a new tail-anchored membrane protein that has similarities in amino acid sequence to thymopoietins (TMPO) in the N- and C-terminal regions (~41%).

Development of anti-emerin antibodies

Two peptide fragments SRSSLDLSYYPTSSST (peptide ED1: 173-188) and FMQAEEGNPF (peptide ED2: 245-254) with an extra Cys to the N-terminus were synthesized, and conjugated with KLH (4). The peptide ED1 has no homology to other known protein, The peptide ED2 has little homology with thymopoietins β and γ. Polyclonal antisera were raised in New Zealand white male rabbits (anti-ED1 and ED2). Monoclonal anti-ED1 and ED-2 antibodies were also established using a conventional technique.

Specificity of the antibodies was examined by immunoblot, indirect ELISA

and immunocytochemical analyses. The polyclonal anti-ED2 antiserum was passed through an affinity column (ProtOnTM Kit) coupled with a synthetic peptide CNTF to avoid the cross-reactivity with thymopoietins β and γ.

Immunocytochemistry

Frozen skeletal and cardiac muscle biopsy (or autopsy) specimens were processed for immunocytochemistry. The specificity of the immunostaining was examined by replacement of the primary antibodies with preimmune rabbit serum or immunoglobulin, and pre-absorption of the primary antibodies with the antigen peptides ED1 and ED2. For the specific identification of nuclear membrane, each tissue section was double stained for immunofluorescence with anti-ED1 (or ED2) antibody and anti-human nuclear membrane antibody MAB1274.

Cellular localization of emerin (Figs. 1 - 3)

We found deficiency of the immunostaining for nuclear membranes in the EDMD skeletal and cardiac muscles. The emerin-positive neurological diseases included; Parkinsonism, motor neuron diseases, metabolic myopathies, inflammatory myopathies, and muscular dystrophies other than EDMD. The negative staining of muscle fiber nuclear membrane against the antibodies in patients with EDMD was quite distinct from other diseases examined.

Tissues other than skeletal and cardiac muscles

The positive nuclear membrane staining of the control sections was not restricted to the skeletal and cardiac muscles. It was also found in other cell types, i.e., vascular and visceral smooth muscles (Fig. 1d), primary spermatocyte and follicular cell of the thyroid gland (data not shown). On the other hand, neurons, spleen cells, renal tubular cells and hepatocytes had no clearly delineated staining around the nuclear membrane (data not shown), indicating that these cells have undetectable amount of the protein at the nuclear membrane or have different subcellular distribution from myogenic cells..

Immunoblot analysis (Fig. 4)

Subcellular fractionation studies revealed that the 34 kDa protein was associated with the nuclear fraction of control sample, but was absent in EDMD. The anti-ED2 antibodies also detected the 34 kDa protein in the control muscle, but was again absent in EDMD.

A mutation of the *STA* gene

Our EDMD patients had a nonsense mutation at position 735 (G to A) of the emerin cDNA that changed a tryptophan codon (TGG) to a stop codon (TAG). The G to A mutation at nucleotide 735 was confirmed by the restriction enzyme (BfaI) digestion of the amplified genomic DNA. The

mother of the brother patients was considered to be a heterozygous asymptomatic carrier of the disease. SSCP analysis of the PCR amplified products of genomic DNA of the *STA* gene also revealed that the brother patients and their mother had mobility shifts of the conformers bands. None of the twenty five controls had any nucleotide changes at cDNA position 735, indicating that this mutation is not a polymorphism.

Summary and Conclusions

First, paired immunofluorescence staining of tissue sections with the anti-ED1 (or ED2) antibody and the MAB1274 marker revealed a specific deficiency of emerin in the nuclear membrane of skeletal, cardiac muscles, and vascular smooth muscle in EDMD. The significance of this observation was further determined by the immunoblot analysis. These findings indicate that emerin is localized in the nuclear membranes of normal skeletal, cardiac muscle, and smooth muscle cells, and suggest a structural and/or functional role for emerin in the nuclear membranes.

Second, not all kinds of cell types show nuclear membrane staining, equally. Among the control human non-muscle tissues, neurons, spleen cells, renal tubular cells and hepatocytes exhibited markedly diminished or undetectable immunostaining of the nuclear membranes. These results imply the (i) presence of tissue-specific alternative emerin transcript(s), or (ii) other tissue restricted protein(s) that links emerin to the nuclear membrane which is

absent in non-muscle tissues. Ubiquitous expression of emerin mRNA and protein will be attributed, at least in part, to the presence of emerin in the nuclear membrane of vascular smooth muscle cells.

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Fig. 1

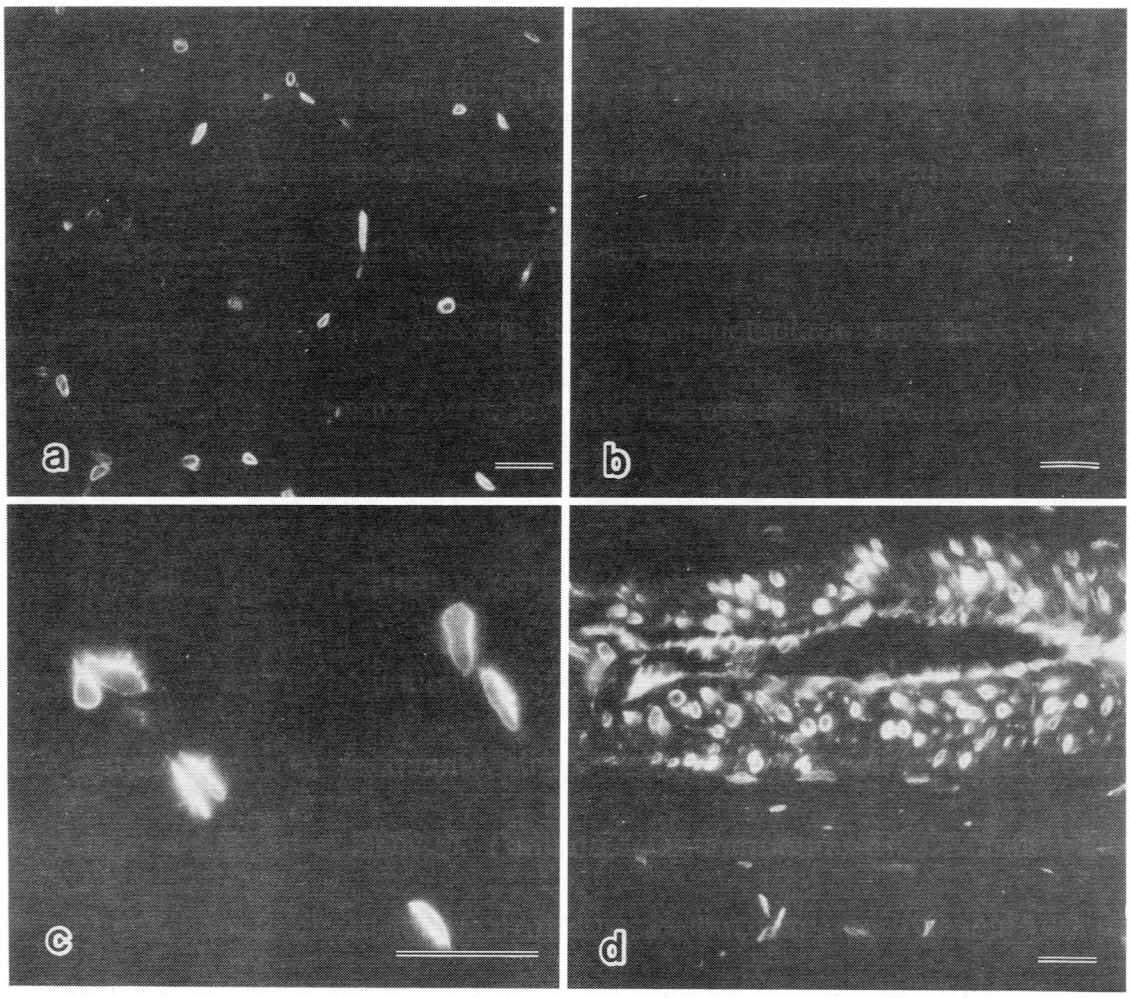

Fig.2

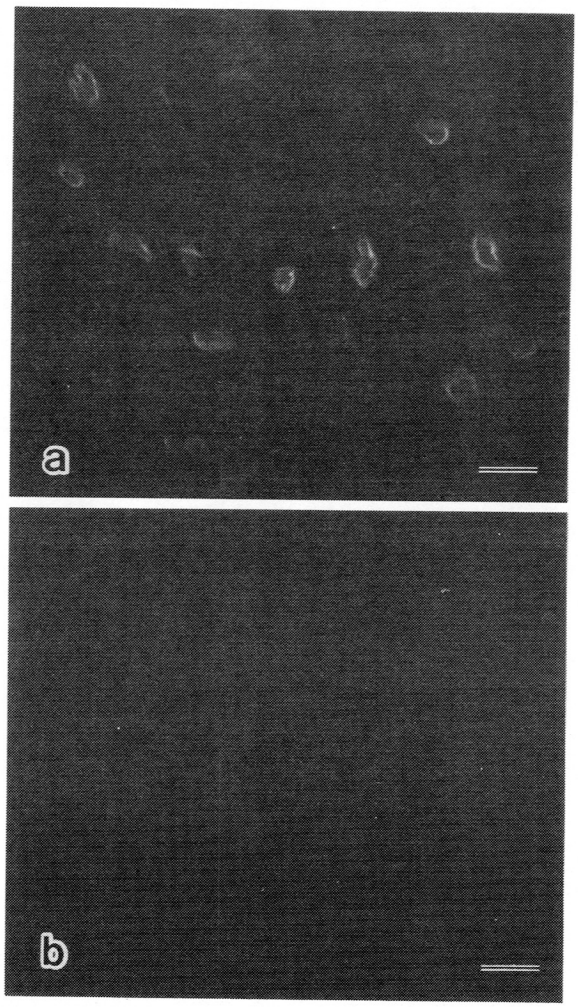

Fig. 3

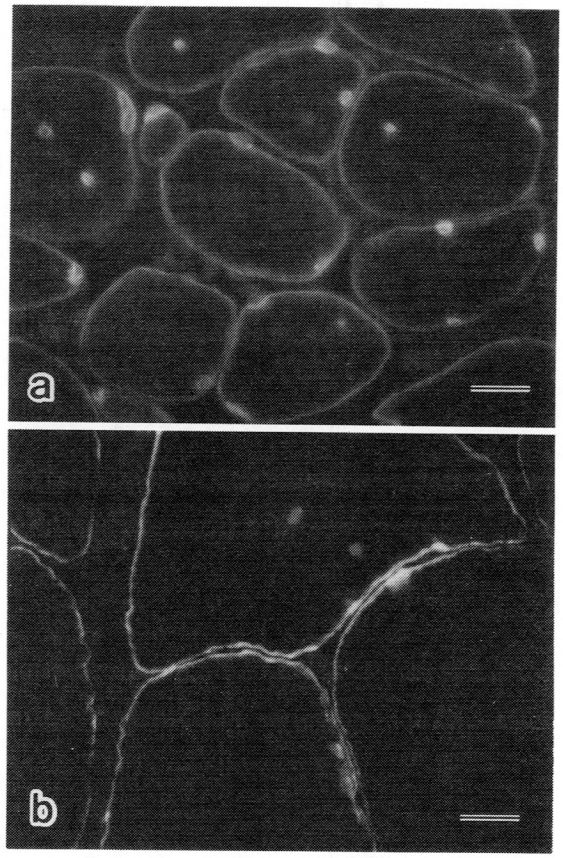

Fig.4

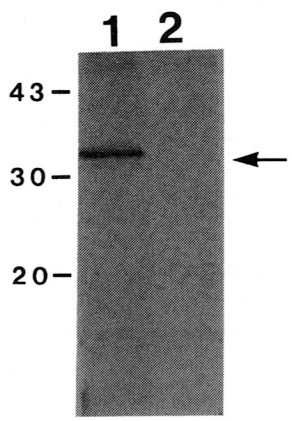

Fig. 5

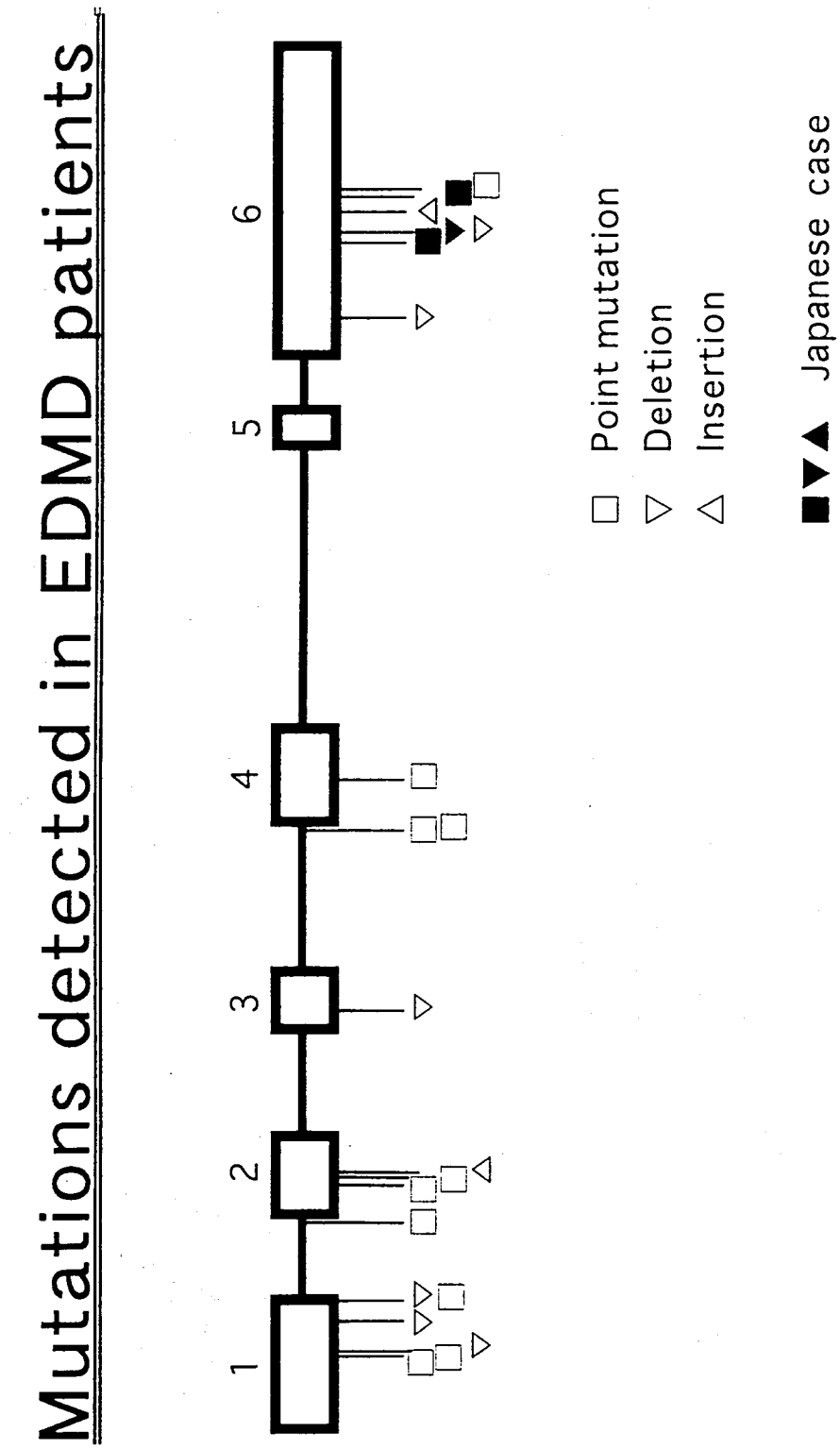
Mutations detected in EDMD patients
1
2
3
4
5
6
Point mutation
Deletion
Insertion
Japanese case

Figure Legends

Fig. 1

Indirect immunofluorescence staining of skeletal muscle sections with monoclonal anti-ED1 antibody. (a, c-d) Control normal muscle sections from a patient with non-neuromuscular diseases (taken at the time of orthopedic surgery with informed consent). In (a) $ED1^+$ nuclear membrane of skeletal muscle fibres is visualized, while muscle sections from a patient with Emery-Dreifuss muscular dystrophy who had a mutation in the *STA* gene, the ED1 marker showed no detectable immunostaining of the myonucleai (b). At a higher magnification, nuclear membrane indentation can be visualized as spike-like projections (c). Nuclear membrane of vascular smooth muscle cells show positive immunostaining for the antibody (d). Bar=20 microns.

Fig.2

Frozen sections of human cardiac muscles stained with monoclonal anti-ED1 antibody by indirect immunofluorescence. Note the cleat nuclear membrane staining of control (a) cardiac muscle. By contrast, there was no immunostaining of the nuclear membrane of EDMD (b). Bar=20 microns.

Fig. 3

Paired immunofluorescence staining of muscle sections with polyclonal anti-ED1 rabbit antiserum (visualized by green fluorescence) and monoclonal anti-human nuclear membrane antibody MAB1274 (visualized by red rhodamine fluorescence). On double exposure, in control muscle section from a patient with polymyositis, $ED1^+$ nuclear membrane is visualized in yellow color while sarcolemmal membrane remains green (a). On the other hand, muscle section from a patient with Emery-Dreifuss muscular dystrophy, the myonucleai remains red color due to negative immunostaining of the green ED1 marker (b). Bar=20 microns.

Fig.4

Immunoblot analysis of emerin. The immunoreactive component of a molecular weight of 34 kDa protein in a control muscle (lane 1; arrow) was not detected in Emery-Dreifuss muscular dystrophy (lane 2).

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Toward identification of the Fukuyama-type congenital muscular dystrophy (FCMD) gene

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Fukuyama type congenital muscular dystrophy (FCMD), the second most common form of muscular dystrophy in Japan, is an autosomal recessive severe muscular dystrophy, associated with brain anomalies due to a defect in the migration of neurons[1].

As a first step to elucidate the genetic defect of FCMD, we performed genetic linkage analyses using consanguineous FCMD families mainly, and localized the FCMD locus to around D9S58 on chromosome 9q31-33[2] (fig. 1). However, this region ranged over 20 cM and was presumed to include more than 400 genes.

Following our initial mapping, we further defined the locus within a region of approximately 5 cM between loci D9S127 and CA246, by homozygosity mapping in patients born to consanguineous parents and by recombination analyses in other families[3-4]. We also found evidence for linkage disequilibrium between FCMD and mfd220 (D9S306) in this candidate region. We suspected that the FCMD gene could lie within 1 Mb of the mfd220 locus located on 9q31[3] (fig. 1).

Since FCMD is prevalent among the Japanese which is genetically isolated population, linkage disequilibrium mapping is feasible as in Finland[5-6]. However, no markers have been discovered very close to mfd220. We constructed a yeast artificial chromosome (YAC) contig[7] containing mfd220, developed new microsatellite markers from the contig, determined their position on YACs, and allele-typed FCMD and normal individuals with these new markers[8]. "P excess" values[9], representing the strength of linkage disequilibrium, were 0.32, 0.81, 0.95, 0.60, and 0.45 at mfd220, M8, J12,

GATA, and A4, respectively. Distances between FCMD and each marker were presumed to be 1Mb, 160kb, 30kb, 450kb, and 740kb, respectively. These putative distances were consistent with each marker's position on YACs. Thus, the FCMD gene could lie within a region of less than 100kb containing J12 and we are able to perform highly-focused positional cloning[8] (fig. 2).

We further examined haplotypes of FCMD chromosomes at a few loci around J12, which showed maximum value of P excess. The results indicated that 77% of FCMD-bearing chromosomes carried an ancestral haplotype and that 88 % of FCMD patients carried two ancestral haplotypes homozygously, or one ancestral haplotype and one putative newly mutated chromosome heterozygously. So far, we have not seen this haplotype in the normal chromosomes, yet. These data verify our hypothesis of a single disease founder and suggest that these markers can be used for the diagnosis of sporadic FCMD, to a great extent, although they are polymorphic markers[8].

During the aforementioned process, new findings are as follows:

1) There are few known proteins located on 9q31. The FCMD gene product may be a) an unknown adhesion molecule related to neuronal migration or b) an unknown protein linked with the dystrophin-dystroglycan-laminin complex, because immunostaining of the constituents of this complex is reduced in FCMD muscles.
2) As described above, most FCMD patients at present are derived from a single ancestral founder[8].
3) Using these linked markers, prenatal and carrier diagnoses are feasible in FCMD families[10-12].
4) The progress of linkage disequilibrium mapping allowed us to diagnose whether a sporadic case has FCMD or not, by haplotype analysis using very close markers[8].
5) Atypical FCMD (who could walk) families have been linked to the 9q31 markers. These cases can belong to a series of clinical spectrum of FCMD.
6) It has been discussed whether FCMD and Walker-Warburg syndrome (WWS) belong to the same disease entity or not. We analyzed a family in which 3 siblings were affected with either FCMD or WWS. The results suggested that both FCMD and WWS siblings shared the identical combination of mutations on either allele of the FCMD locus. Although

WWS could be genetically heterogeneous, some WWS cases could be caused by mutations in the FCMD gene[13].

7) A neuropathological study of an fetus with prenatal diagnosis of FCMD revealed that the breaches of the glia limitans may be the primary cause of micropolygyria seen in this disease[10].

In summary, following our initial linkage analysis of the FCMD gene to chromosome 9q31-33, we defined the locus within a 5 cM, by homozygosity mapping and recombination mapping, and then found linkage disequilibrium. Furthermore, by linkage disequilibrium mapping, we are about to come down on the FCMD gene itself.

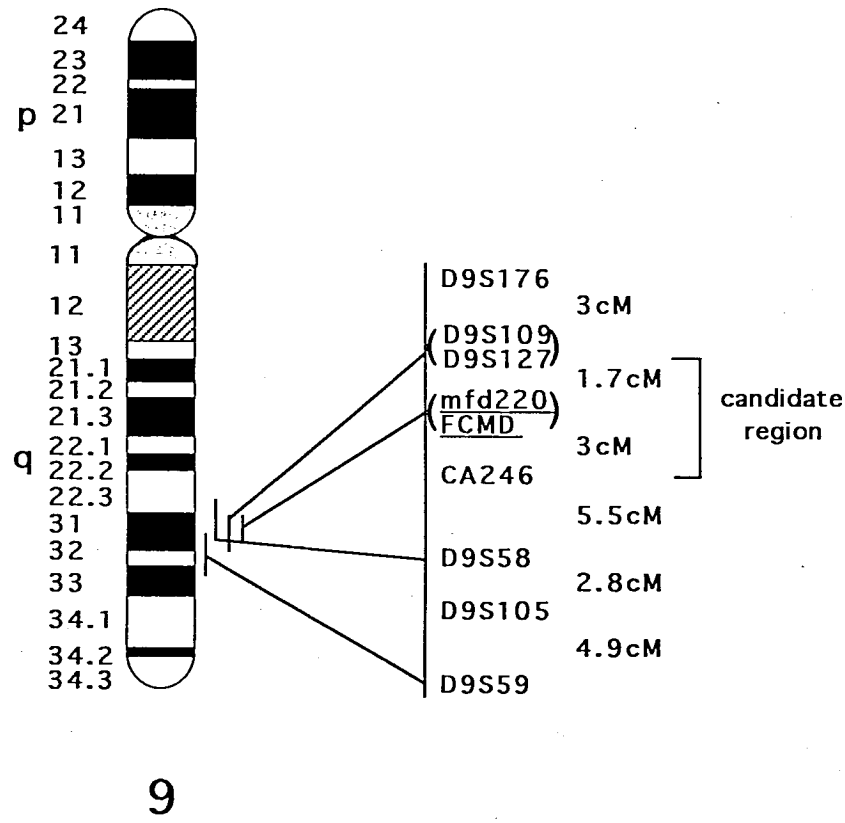
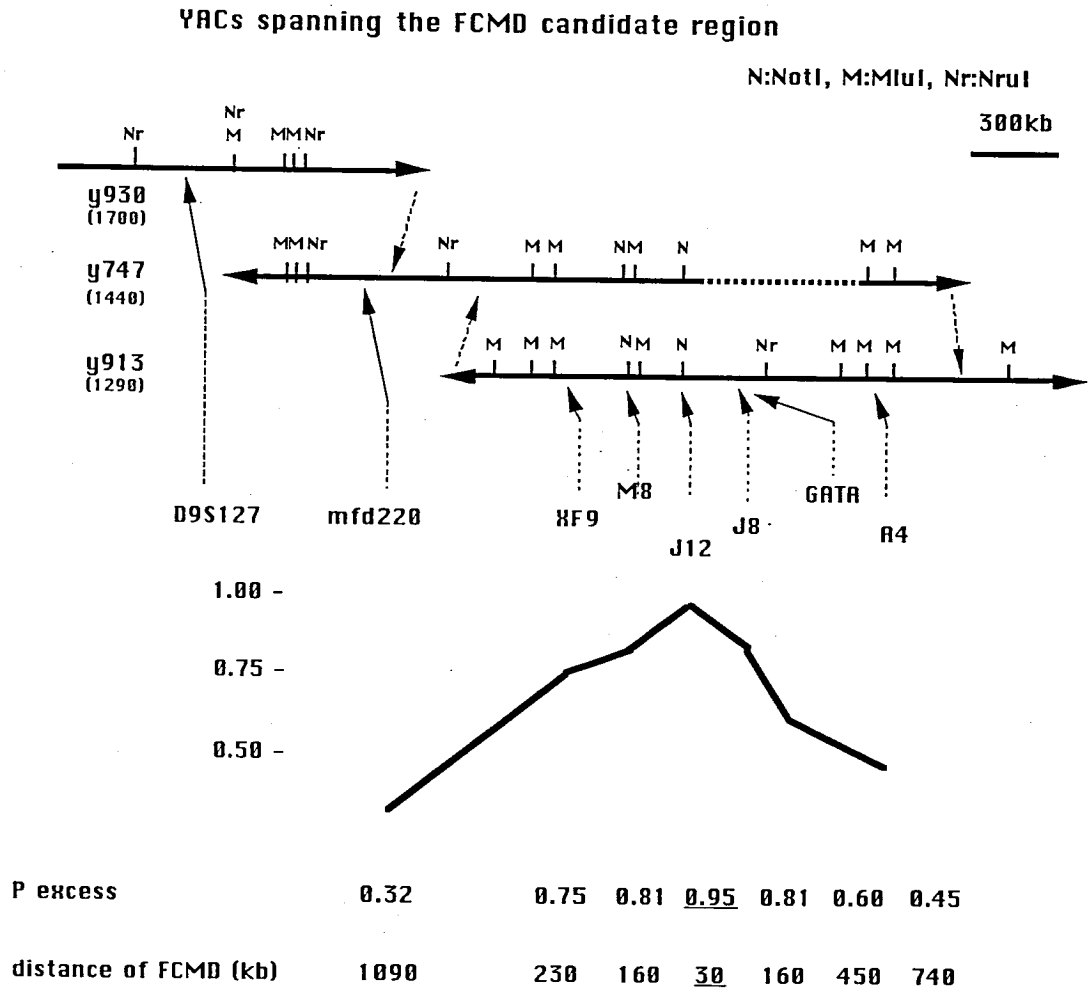


Fig 1. Physical and genetic locations of linked markers on chromosome 9q. 1 cM = about 1 Mb. The FCMD gene could lie within a region between D9S127 and CA246 and within 1 Mb of the mfd220 locus on 9q31 showing linkage disequilibrium.



P excess	0.32	0.75	0.81	0.95	0.81	0.60	0.45
distance of FCMD (kb)	1090	230	160	30	160	450	740

Fig 2. A YAC contig of the FCMD gene region and linkage disequilibrium mapping. Newly developed microsatellite markers are positioned. The maximum P excess value is obtained at J12. Putative distances between FCMD and each marker are also represented. The FCMD gene could lie within a region of less than 100kb containing J12.

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MEROSINOPATHIES

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Merosin is the laminin isoform specific of muscle fiber basement membrane [Leivo and Engvall. 1988]. Laminin is a heterotrimer made up of one heavy chain and two light chains, which are arranged in various combinations in different basement membranes [Engvall 1993, Tryggvason 1993]. The merosin (or laminin-2) is formed by a heavy M (or $\alpha 2$) chain and two light chains: B1 (or $\beta 1$) and B2 (or $\gamma 1$) [Burgeson et al. 1994]. A partial deficiency of merosin was first demonstrated in Fukuyama congenital muscular dystrophy (FCMD), using antibodies against laminin $\alpha 2$-chain [Hayashi et al. 1993]. FCMD is a form of congenital muscular dystrophy (CMD) characterized by its association with severe central nervous system disturbances [Fukuyama et al. 1960 and 1981, Nonaka and Chou 1979, Osawa et al, 1991]. Several hundreds of affected children were detected in Japan, and only very few outside. The disease is generally very severe, the highest motor level reached by the children being generally crawling on the buttocks or the knees. The children have a very low IQ, often associated with epilepsy. The CT scan shows decrease in density of the white matter, ventricular dilatation and cortical atrophy; the neuropathological examination consistently reveals marked brain changes, with pachygyria or micropolygyria of the cerebral and cerebellar cortex [Fukuyama et al, 1981; Osawa et al, 1991]. An autosomal recessive inheritance of FCMD was considered as highly probable [Osawa, 1978] and this disease was mapped on chromosome 9q31-33 [Toda et al. 1993 and 1994].

In Western countries the CMD is a frequent cause of severe neonatal hypotonia of neuromuscular origin, and is often referred to as classical or occidental form of CMD [Fardeau 1992, Banker 1994, Dubowitz 1995]. Its clinical manifestations are noticed at birth or in the first months of life and consist of muscle hypotonia and weakness, markedly delayed motor milestones,

severe and early contractures, often associated with joint deformities. Serum creatine kinase is usually raised, up 10 times normal, in the early stages of the disease, and then rapidly decreases. The occidental CMD has been considered as being usually clinical characterized by "pure" muscular involvement. However, when non-invasive techniques of brain imaging were used, white matter changes were detected in a significant proportion of CMD cases [Bernier et al. 1979; Egger et al. 1983; Echenne et al. 1986; Trevisan et al. 1991]. The histopathological changes reported in muscle biopsies consisted in large variation in muscle fiber size, a few necrotic and regenerating fibers, marked increase in endomysial collagen tissue, variable amount of fat tissue, and no characteristic ultrastructural features [Afifi el al. 1969; Zellweger et al. 1967a, 1967b]. These histological changes lack of specificity, as other muscle disorders may present with similar features. The frontiers of CMD were ill-defined and the diagnosis of CMD was often made after exclusion of the different types of structural congenital myopathies. It was clear, from the different series of CMD described in Western countries, that a marked clinical heterogeneity existed in this group. An autosomal recessive inheritance was generally postulated.

The marked increase in connective tissue observed in muscle biopsies of patients with CMD has suggested that an extracellular matrix abnormality could account for the pathogenesis of this disease [Duance et al, 1980, Fidzianska et al, 1982] but early studies failed to reveal significant changes of collagen and some other extracellular components [Stephens et al. 1982; Hantaï et al. 1985].

We have investigated whether one component of the complex of sarcolemmal glycoproteins associated with dystrophin (dystrophin associated complex - DGC) or one of subunits of the laminin, which is linked to this complex [see Campbell 1995 and Ozawa 1995] could be involved in the classical (occidental) form of CMD. We did not observe any significant changes of the proteins of the DGC but we have found, by both immunocytochemical (Fig. 1) and immunoblot techniques, complete merosin (laminin α2-chain) deficiency in 13 cases but not in other 7 cases, also diagnosed as CMDs [Tomé et al. 1994]. In all those merosin-deficient cases, as well as in others subsequently reported [Hayashi et al. 1995, Sewry et al. 1995, Fardeau et al. 1996, Fardeau and Tomé, 1996, Topaloglu et al. 1996, Trevisan et al. 1996], laminin α2-chain was absent not only from the basement membrane of muscle fibers but also from the basement membrane of Schwann cells of intramuscular nerves and

neuromuscular junctions. In contrast, the laminin A (or α1) chain (heavy chain of laminin-1) was overexpressed, to varying degrees, around muscle fibers in all merosin-deficient CMD cases - Fig. 1 [Tomé et al. 1994]. In muscle biopsy specimens from normal controls, with the antibodies against laminin α1-chain, the labeling was almost exclusively confined to the blood vessel basement membrane (Fig.1). In most merosin-non-deficient cases the expression this protein was identical to that of controls. However, in several cases, there was overexpression of this protein around a few muscle fibers [Fardeau and Tomé 1996]. The anti-laminin β1-chain and γ1-chain antibodies showed some variation in the intensity of the labeling in both groups of patients, but similar variations were seen in other pathologies. The anti-dystrophin antibodies did not show significant changes in the dystrophin expression either in the merosin-deficient or in the merosin-non-deficient cases.

This study suggested that the laminin α2-chain gene (LAMA2) could cause the merosin-deficient CMD, and as this gene had been localized to chromosome 6q22-q23 [Vuolteenaho et al. 1994], a genetic study involving homozygozity mapping and linkage analysis was undertaken on a panel of consanguineous families living in France and Turkey. This allowed the localization of the merosin-deficient CMD locus to a 16 cM region of chromosome 6q2 [Hillaire et al. 1994]. Recently we have readjusted this localization to a 3cM region (Fig. 2), more centromeric than previously thought [Helbling-Leclerc et al. 1995a]. This localization was also found in three patients with typical CMD of occidental type having trace of merosin: a faint staining of varying intensity was observed by immunocytochemistry around most muscle fibers [Helbling-Leclerc et al. 1995a]. Mapping to 6q2 locus was excluded in merosin non-deficient CMD patients [Hillaire et al. 1994]. The merosin-non-deficient CMD families studied by us did not map either to chromosome 6q2 or to chromosome 9q31-33, region where the FCMD gene was localized by Toda et al. (1993).

These findings led to clinical reevaluation of the two groups of CMD patients, defined according the normal presence or the deficiency of merosin, and who fulfilled the criteria agreed by the International Consortium on Congenital Muscular Dystrophy [Dubowitz 1994]. The initial evaluation suggesting that these two groups could be distinguished by their clinical patterns and their severity [see the reference to Fardeau presentation at the 22nd ENMC 1994 workshop on CMD, in Dubowitz and Fardeau 1995], was largely

confirmed by our subsequent studies [Dubowitz 1995, Fardeau et al. 1996, Fardeau and Tomé 1996]. They showed that the clinical phenotype was homogeneous and typical in merosin-deficient CMD group but it was heterogeneous and often atypical in merosin-non-deficient CMD. This demonstrated that these two groups were distinct and suggested that the merosin-deficient CMD was a clinically well-individualized entity, usually characterized by severe congenital hypotonia, contractures, delayed motor development and white matter hypodensity by RMI or scan brain imaging [Fardeau et al. 1996, Fardeau and Tomé 1996]. The severity of the disease was reflected by the fact that 4 (out of a series of 15 of our cases) died before the age of 6 years [Fardeau and Tomé, 1996]. In contrast, a series of 15 merosin-non-deficient CMD cases formed a more heterogeneous group, generally with less severity [Fardeau and Tomé 1996]. Neonatal hypotonia was rarely marked, contractures were constant, but rarely severe. Motor development was rarely severely delayed. None of the children in this series were reported to have died. It should be noted that similar differences between merosin-deficient and merosin-non-deficient patients were found in other series of cases [Philpot et al. 1995, Topaloglu et al. 1996].

The search for mutations in LAMA2 gene in merosin-deficient CMD patients allowed to find splice site and nonsense mutations in this gene in two families [Helbling-Leclerc et al. 1995b]. These mutations lead to truncated laminin-$\alpha 2$ proteins and suggested that mutations in the LAMA2 gene cause this severe form of muscular dystrophy.

It can be concluded from our studies that merosin-deficient CMD should be considered as is a well-defined clinical, morphological and genetic entity.

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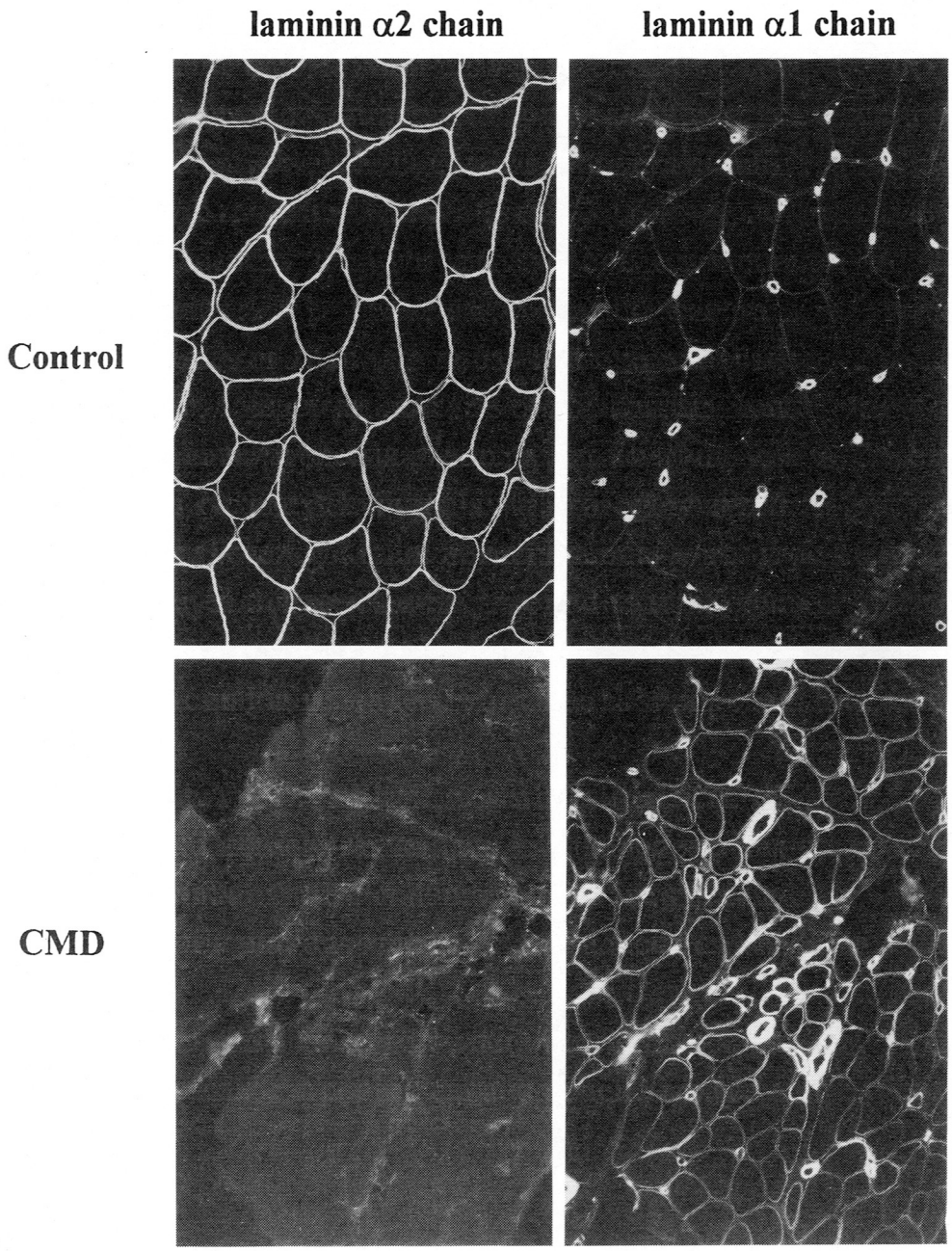

Fig. 1. Immunocytochemical analysis of laminins α2 or M (right micrographs) and α1 or A (left micrographs) chains in serial sections from the muscle biopsies of a normal control (upper micrographs) and a child with congenital muscular dystrophy - CMD (lower micrographs). Shown is absence of laminins α2 chain and overexpression of laminins α1 chain around muscle fibers in the CMD patient. Magnification X 265.

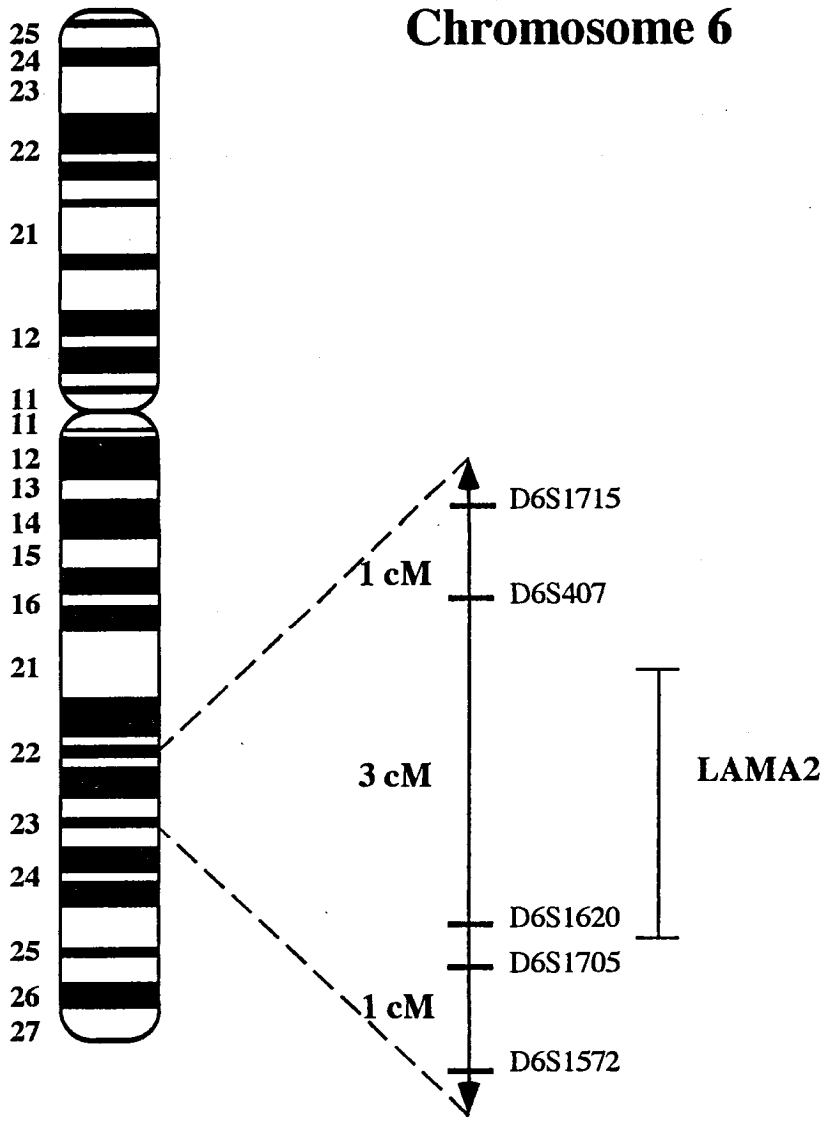

Fig. 2. Schematic representation of chromosome 6, showing the localization of the merosin-deficient CMD in a 3cM interval, between D6S407 and D6S1620 markers. The D6S1620 was homozygous for all the patients studied (Helbling-Leclerc et al. 1995a).

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Muscle Regeneration in Progressive Muscular Dystrophy

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Introduction

It is well-known that the muscle fiber is capable of regeneration after necrosis not only in normal but in dystrophic muscles[1-2]. Despite the presence of numerous regenerating fibers, the muscle weakness is "progressive" in progressive muscular dystrophies. To understand the reason why the regenerative process cannot compensate for muscle degeneration in progressive muscular dystrophies, we performed a comparative study between *dy*$^+$/*dy*$^+$*(dy)* and *mdx* mice. The former has progressive muscle weakness leading to death at approximately 3-4 months of age, while the latter has no apparent clinical symptom, even though there is obvious muscle fiber necrosis in both animals.

1) Muscle fiber growth and fiber type differentiation do not differ in *dy*, *mdx* and normal muscles[3]

In both *dy* and *mdx* mice, muscle fiber size and fiber type differentiation were similar up to 10-15 days of age when necrotic fibers began to appear. Apart from the necrotic areas, the muscle fiber morphology appeared to be normal, i.e. there was no distinct difference in muscle fiber size, fiber type differentiation and the number of myosatellite cells. Muscle fibers probably undergo necrosis only when they reach a certain stage of maturation. These findings suggest that there is no relationship between muscle fiber growth and subsequent disease progression.

2) The cellular response to muscle fiber necrosis is more striking in *mdx* than *dy* mouse

The necrotic process appears after 15 days of age in both animals. The necrotic fibers tended to be accompanied by very marked cellular response in

mdx muscle, while there were only scattered reactive cells which chanced little phagocytic activity in *dy* muscle (Fig 1). After 1 month of age in *mdx* muscles, most of the muscle apart from the clusters of necrotic fibers were replaced by centronucleated regenerating fibers, whereas in *dy* muscles necrotic and regenerating fibers were scattered throughout the muscle with marked variation in fiber size and interstitial fibrosis. The earlier and more intense cellular response to muscle fiber necrosis is probably the crucial event leading to subsequent more active regenerative process in the *mdx* mouse. It is well-known that factors originating from the macrophage stimulate satellite cell proliferation, especially PDGF (platelet derived growth factor), bFGF (basic fibroblast growth factor) and TGF-β (transforming growth factor-β)[4].

3) MyoD and myogenin are expressed in the early stages of muscle regeneration

To understand how *MyoD* and *myogenin*, genes responsible for initiation of myoblast proliferation and myotube formation are expressed in the regenerative process, we examined these gene products immunohistochemically in an experimentally induced myonecrosis model. Bupivacaine hydrochloride (Marcaine®), a local anesthetic which induces massive fiber necrosis and rapid regeneration, was injected into the adult rat soleus muscle[5]. Almost all the fibers underwent necrosis immediately after the injection. Macrophages became evident at 24 hours and the necrotic muscle was filled with mononuclear cells (mostly acid phosphatase-positive macrophages) by 48 hours after injection, when satellite cells were actively dividing. At 72 hours after fiber necrosis, small basophilic regenerating fibers with central nuclei with the morphologic characteristics of myotubes began to appear among the mononuclear cells. The myotubes increased in number and size and the regenerating fibers had rapidly almost totally replaced the necrotic fibers 5-7 days after the bupivacaine hydrochloride injection[6].

MyoD was maximally expressed 48 hours after muscle fiber necrosis when satellite cells were activated and had started to proliferate. The satellite cell, remnant of the myoblast during myogenesis, plays an important role in muscle fibers regeneration to form the myotube. Myogenin was maximally expressed when the basophilic myotubes appeared among the necrotic fibers, the expression of both gene products was similar to that seen in the regenerative

process during myogenesis, but this occurred over a very short period (Fig 2).

4) MyoD and myogenin are expressed in *dy* and *mdx* muscles

To clarify whether the *MyoD* and *myogenin* are expressed during the regenerative process in progressive muscular dystrophies, and whether gene expression is different between *mdx* and *dy* muscle, we applied immunohistochemical staining to both animal muscles at age 6 weeks when fiber necrosis and regeneration are active.

MyoD and myogenin were expressed in both animals : the MyoD was positive in mononuclear cells in the necrotic areas, and myogenin in the early regenerating fibers with the morphologic characteristics of the myotube (Fig 3). Since the cellular response and regenerative process were more active in the *mdx* muscles, the numbers of MyoD and myogenin positive cells were far more numerous in the *mdx* muscles than *dy* muscles. In conclusion, there was no qualitative difference in MyoD and myogenin expressions, but there were quantitative differences between *mdx* and *dy* muscles which were simply the results of the deferring extent of the cellular response, both necrotic as well as regenerative processes between the two animals. Although we have not applied the immunohistochemical staining to human dystrophic muscles, we believe that it is highly likely that both *MyoD* and *myogenin* play an important role in muscle regeneration in both injured and diseased human muscles.

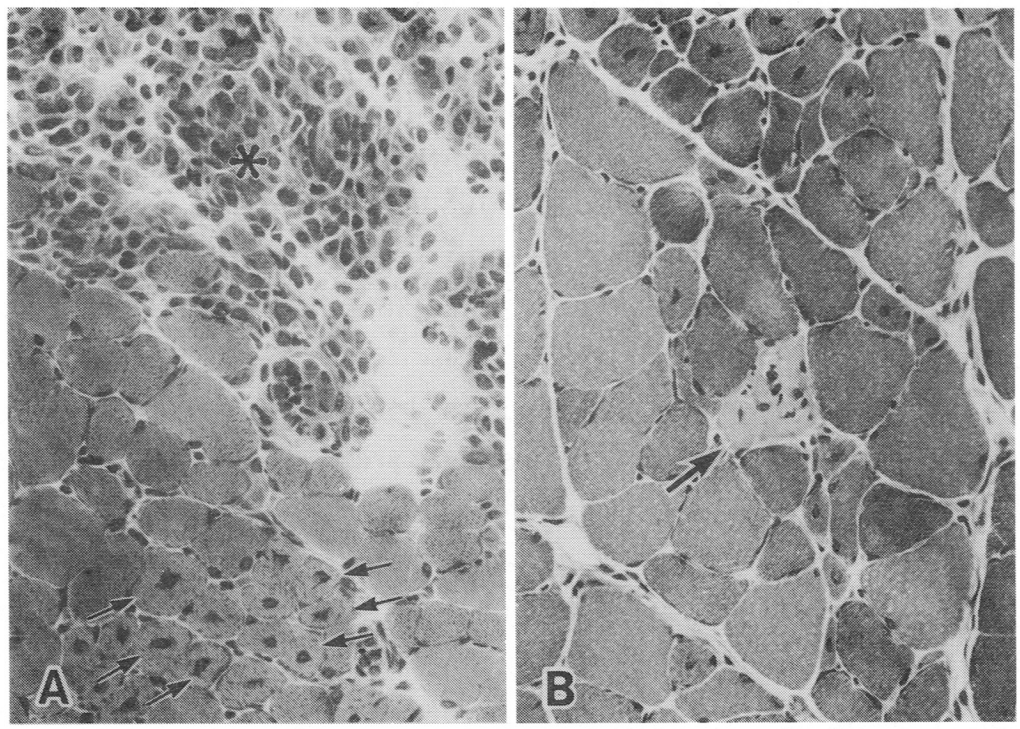

Fig 1. There is massive fiber necrosis with extensive cellular reaction (asterisk) in the *mdx* mouse muscle (A). Note an aggregate of regenerating fibers with centrally placed nuclei (arrows). In *dy* mouse (B), there are only scattered necrotic fibers (arrow) with little cellular reaction. There is mild endomysial fibrosis.
A,B: hemotoxylin and eosin, ×450.

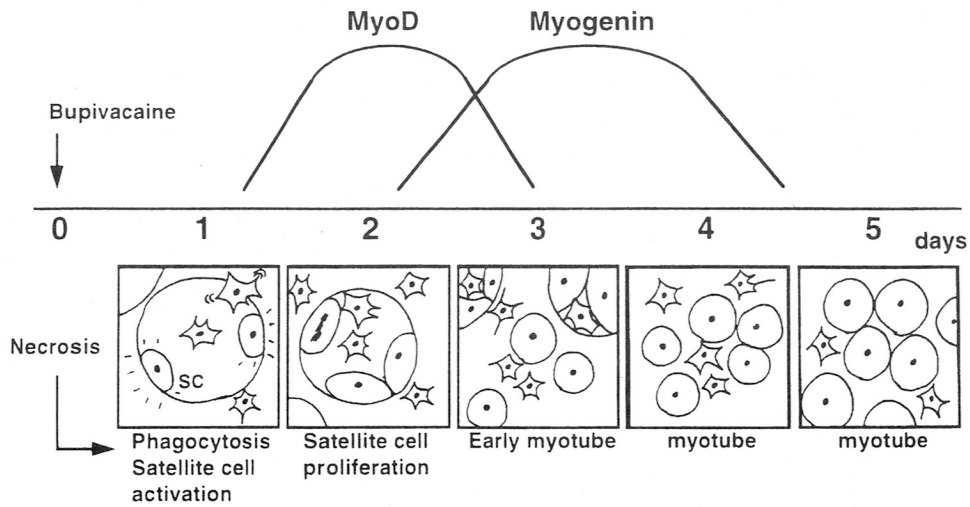

Fig 2: MyoD and myogenin expression in experimentally induced muscle fiber necrosis followed by regeneration. MyoD is most intensively expressed 2 days after myonecrosis when the satellite cells start to proliferate, and myogenin at 3-4 days when early myotube formation occurs.

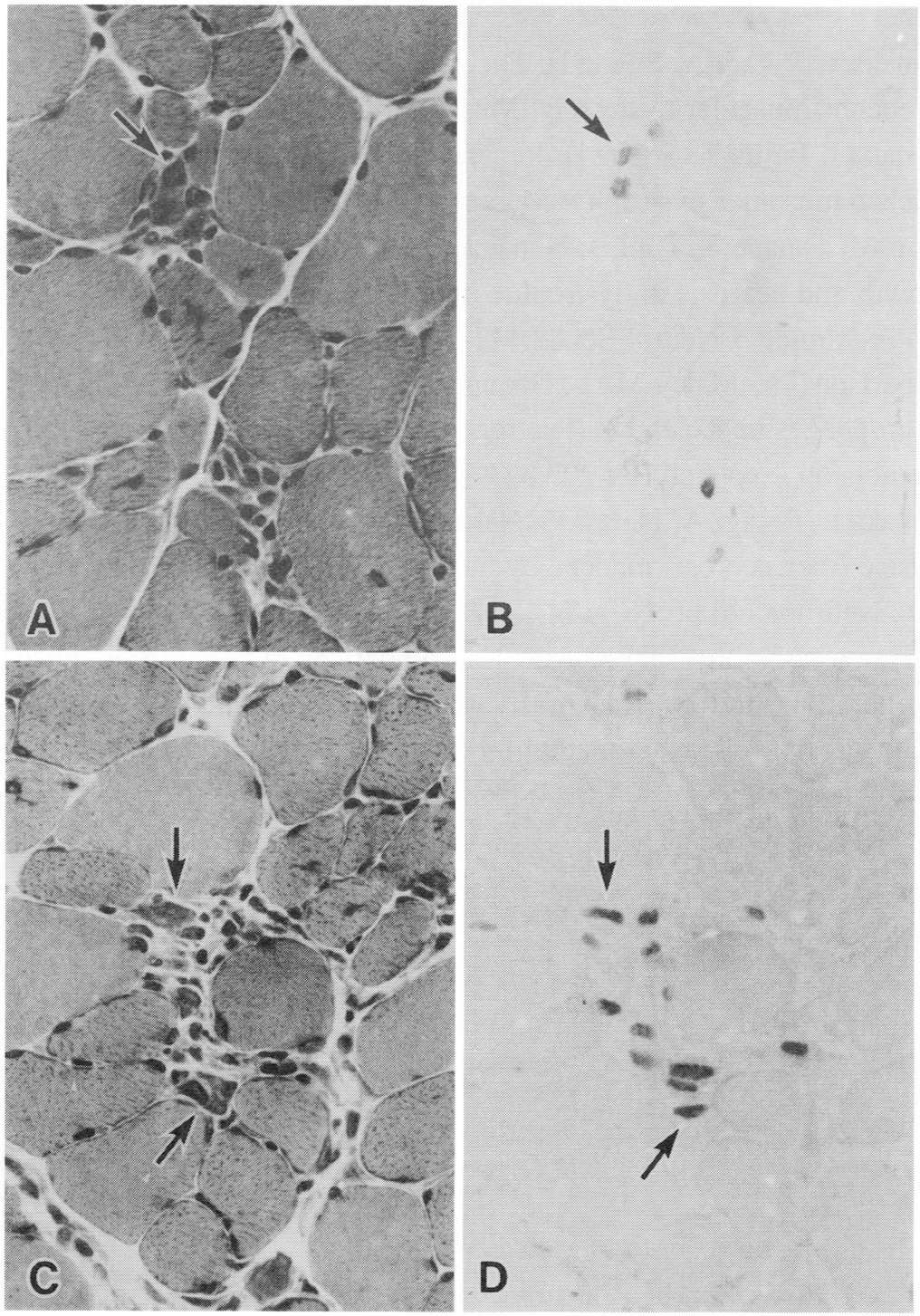

Fig 3: MyoD is strongly expressed in both *dy* (A,B) and *mdx* (C,D) mouse muscles in necrotic fibers filled with macrophages and proliferating satellite cells. There is no qualitative but there is quantitative difference between the two animals.
A,C: hematoxylin and eosin, B,D: immunostaining with anti-MyoD antibody.
A-D: ×600.

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Local Production of IL-12p40 Prevents Allogeneic Myoblast Rejection

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ABSTRACT

The p40 subunit of interleukin12 (IL-12p40) has been known to act as an IL-12 antagonist *in vitro*. We here describe *in vivo* immunosuppressive effect of IL-12p40 in allogeneic myoblast transplantation. A murine myoblast cell line, C2C12, was transduced with retrovirus vectors carrying the lacZ gene as a marker and the IL-12p40 gene. IL-12p40 secreted from the transfectant inhibited the IL-12-induced interferon γ (IFNγ) production by splenocytes *in vitro*. Survival of C2C12 transplanted into allogeneic recipients was substantially prolonged when transduced with IL-12p40. Cytokine (IL-2 and IFNγ) production and cytotoxic T lymphocyte (CTL) induction against allogeneic C2C12 were impaired in the recipients transplanted with the IL-12p40 transfectant. Delayed-type hypersensitivity (DTH) response was also significantly diminished in the IL-12p40 recipient. Furthermore, serum antibodies against β-galactosidase of the T-helper 1 (Th1)-dependent isotypes (IgG2 and IgG3) were decreased in the IL-12p40 recipients. These results indicate that locally produced IL-12p40 exerts a potent immunosuppressive effect on Th1-mediated immune responses that lead to allograft rejection. Therefore, IL-12p40 gene transduction would be useful for preventing the rejection of allografts such as myoblast and genetically modified own cells that are transduced with potentially antigenic molecules in gene therapy including dystrophin transduction.

INTRODUCTION

Th1 cells that produce IFNγ and mediate cellular immune responses have been implicated in the pathogenesis of allograft rejection[1-2]. Recent studies revealed a crucial role of IL-12 in the Th1 development[3]. IL-12 is a heterodimeric cytokine composed of 35 kD (p35) and 40 kD (p40) subunits, the latter of which is responsible for receptor binding[4-5]. It has been demonstrated that the expression of p35 and p40 is differentially regulated and that p40 can be produced as monomer or homodimer in the absence of p35, which acts as an IL-12 antagonist *in vitro*[6-8]. However, the physiological function of IL-12p40 *in vivo* remains unclear. In this study, we examined the *in vivo* effect of IL-12p40 by transducing the IL-12p40 gene into myoblast allograft and found a potent immunosuppressive effect. A possible utility of this strategy for preventing rejection in allogeneic myoblast transplantation for muscle dystrophy and in gene therapy introducing potentially antigenic molecules is discussed.

RESULTS

Biochemical and Biological Characterization of lacZ and IL-12p40 Transfectants.

In order to ease identification of the transplanted myoblasts in the recipient skeletal muscle, we first introduced the lacZ gene encoding *E. coli* β-galactosidase (β-gal) as a marker into the myoblast cell line, C_2C_{12} (C2), originated from a C3H ($H\text{-}2^k$) mouse. A stable transfectant expressing a high level of β-gal (C2Z) was established by FACS sorting after FDG staining (Fig. 1A). Subsequently, C2Z was further introduced with the IL-12p40 gene and a stable transfectant producing a high level of IL-12p40 (C2Zp40) was selected (Fig. 1B). Moreover, IL-12p40 proteins with apparent molecular weight of 40 kD were immunoprecipitated by anti-mouse IL-12p40 mAb from the supernatant of C2Zp40 cells under both nonreducing and reducing conditions (Fig. 1C). The triplet most likely represents differently glycosylated forms of IL-12p40.

We next verified the *in vitro* effect of IL-12p40 secreted by C2Zp40. Culture supernatant of C2Zp40, which contained IL-12p40 at 100 ng/ml,

exhibited an antagonistic effect on rIL-12-induced IFNγ production by splenocytes (data not shown).

Preservation of C2Zp40 in Allogeneic Mice.

To estimate the *in vivo* effect of IL-12p40, we transplanted C2Z and C2Zp40 into the quadriceps of fully allogeneic C57BL/6 (H-2^b) mice. Survival of the transplanted myocytes was periodically estimated by histochemical staining of the tissue sections with a β-gal substrate (X-gal) and HE. As represented in Fig. 2A at day 10 after the transplantation, intact C2Z cells expressing β-gal disappeared within 8 days and a massive infiltration of mononuclear cells was observed, indicating the occurrence of a typical cellular rejection. In contrast, intact C2Zp40 cells expressing β-gal were well preserved in spite of a massive mononuclear infiltration (Fig. 2B). Some C2Zp40 cells appeared to be incorporated into the muscle and were preserved even after 4 weeks after the transplantation. These results indicate that locally produced IL-12p40 is effective in preventing the allograft rejection.

Cytokine Production and CTL Induction in C2Zp40-transplanted Mice.

To analyze the mechanisms for the prolonged allograft survival, we estimated cytokine production and CTL induction from the C2Z or C2Zp40 recipients. When re-stimulated with irradiated C2Z *in vitro*, IL-2 and IFNγ production by splenocytes was impaired in the C2Zp40 recipient compared to the C2Z recipient (Fig. 3A). Furthermore, no CTL activity was induced in the splenocytes from the C2Zp40 recipient (Fig. 3B). These results indicate that locally produced IL-12p40 prevented the development of IFNγ-producing Th1 cells and alloreactive CTL precursors *in vivo*.

Inhibition of DTH Response in C2Zp40 Recipient.

To prove that locally produced IL-12p40 suppresses Th1-mediated cellular immune responses *in vivo*, we next investigated whether it also affects DTH. DTH responses were elicited in the C2Z or C2Zp40-transplanted mice by *s.c.* injection of C2Z into ear or footpad, and were significantly diminished in the C2Zp40 recipient (data not shown), indicating that locally produced IL-12p40 inhibited the induction of cellular immune responses represented by DTH.

Isotype Distribution of Serum Anti-β-galactosidase Antibodies.

In order to further verify the effect of locally produced IL-12p40 on the Th1 development, we analyzed the isotype profiles of serum antibodies raised against the b-gal introduced into the allograft. It has been known that the production of IgG1 and IgG2/3 is mainly helped by Th2 and Th1, respectively[9, 10]. As compared with the C2Z recipients, serum antibodies of IgG2 and IgG3 isotypes were significantly decreased in the C2Zp40 recipients (Fig. 4). This further indicates that the Th1 development in response to the allografts was suppressed by the locally produced IL-12p40.

DISCUSSION

In the present study, we demonstrated that the transduction of IL-12p40 gene is an effective way for preventing allogeneic myoblast rejection. Transplantation of allogeneic myocytes has been tried to improve the muscle functions in patients with severe muscular dystrophy but so far achieved little success due to rapid rejection[11-12]. Our present strategy would be immediately applicable to such a case.

Duchenne muscular dystrophy (DMD) is caused by defective expression of dystrophin[13]. Since it has been demonstrated that introduction of normal dystrophin transgene into the *mdx* mice, an animal model for DMD, corrected dystrophic symptoms, a similar gene therapy for DMD is expected[14-15]. However, the introduction of dystrophin gene into immunocompetent hosts innately lacking dystrophin might lead to rejection of the modified cells due to immune responses against the dystrophin. Consistent with this notion, we recently found that C57BL/10 myoblasts transplanted into the *mdx* mice are acutely rejected in spite of the same genetic background except for dystrophin and that dystrophin itself serves as the CTL target antigen (manuscript in preparation). Such a compromising host immune response to the introduced gene products is also expected in gene therapies for other genetic deficiencies, such as hemophilias, where myoblasts may serve as vehicles for gene transfer [16-17]. Therefore, cotransduction of the IL-12p40 gene along with the target gene would be an effective strategy for preventing the rejection of genetically modified cells.

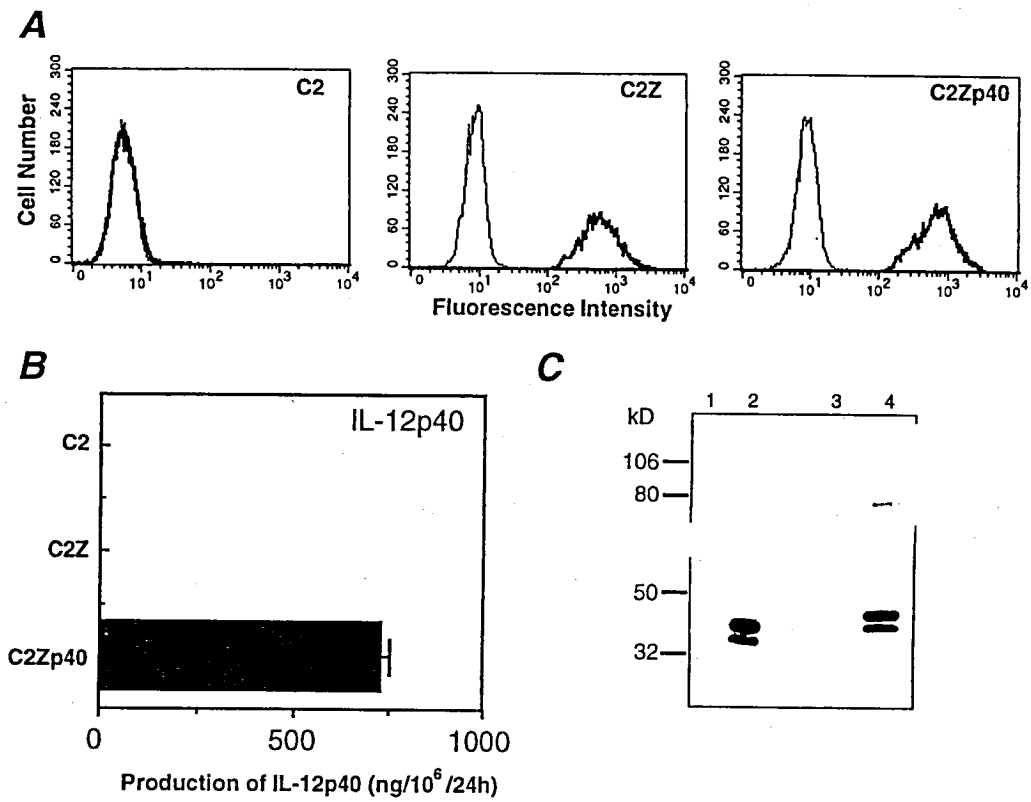

Figure 1. Characterization of C2C12 transfectants. (*A*) Expression of β-galactosidase. Parental C2C12 (C2) and its lacZ transfectants (C2Z and C2Zp40) were stained with a fluorescent β-D-galactosidase substrate, FDG, as described in Materials and Methods, and analyzed on a FACScan. (*B*) Production of IL-12p40. Supernatants were collected after culturing the cells (1 x 10^6/ml) for 24 h, and concentration of IL-12p40 was measured by ELISA. Data represent mean+SD of triplicate wells. (*C*) Immunoprecipitation of IL-12p40 from C2Zp40. C2Z (lanes 1 and 3) and C2Zp40 (lanes 2 and 4) were labeled with [^{35}S]Met and [^{35}S]Cys, and the labeled proteins secreted into culture supernatants were immunoprecipitated with anti-mouse IL-12p40 mAb-conjugated Sepharose. The precipitates were subjected to SDS-PAGE (7.5%) under nonreducing (lanes 1 and 2) or ruducing (lanes 3 and 4) conditions and then to fluorography. The positions of molecular weight standards are indicated at the left in kD.

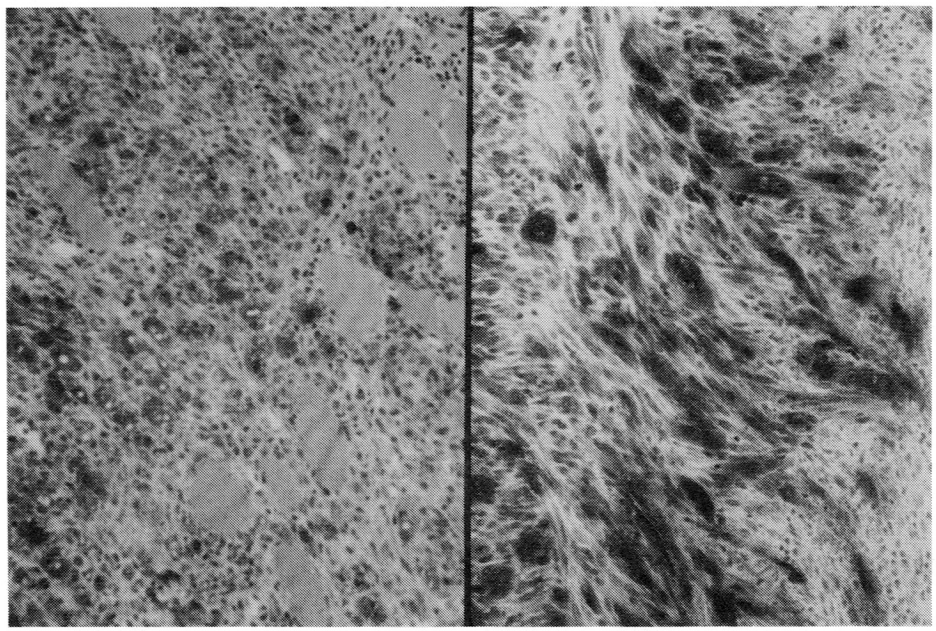

Figure 2. Preservation of C2Zp40 allograft. C2Z (*left*) or C2Zp40 (*right*) cells (1 x10^6) were injected into quadriceps of C57BL/6 mice. Ten days after the transplantation, tissue sections were prepared and stained with X-gal and HE as described in Materials and Methods.

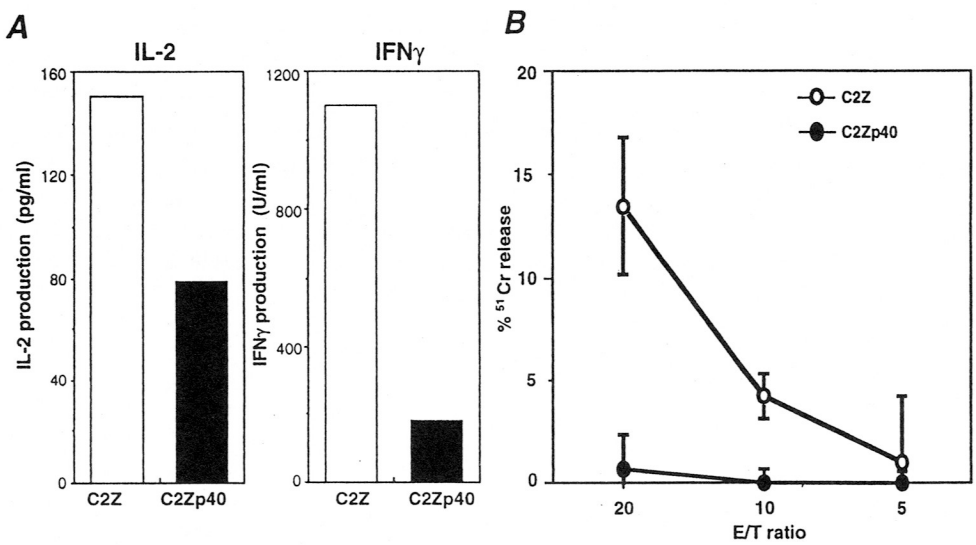

Figure 3. Cellular immune responses of C2Zp40 recipients. *(A)* Cytokine production. Splenocytes from the mice transplanted with C2Z *(open column)* or C2Zp40 *(closed column)* were co-cultured with irradiated C2Z for 2 days. Concentrations of IL-2 and IFNγ in the supernatants were measured by ELISA. *(B)* CTL induction. Splenocytes from the mice transplanted with C2Z (*open circle*) or C2Zp40 (*closed circle*) were co-cultured with irradiated C2Z for 5 days. Cytolytic activity against C2Z was measured by 6 h ^{51}Cr release assay at the indicated E/T ratios. Data represent mean±SD of triplicate wells.

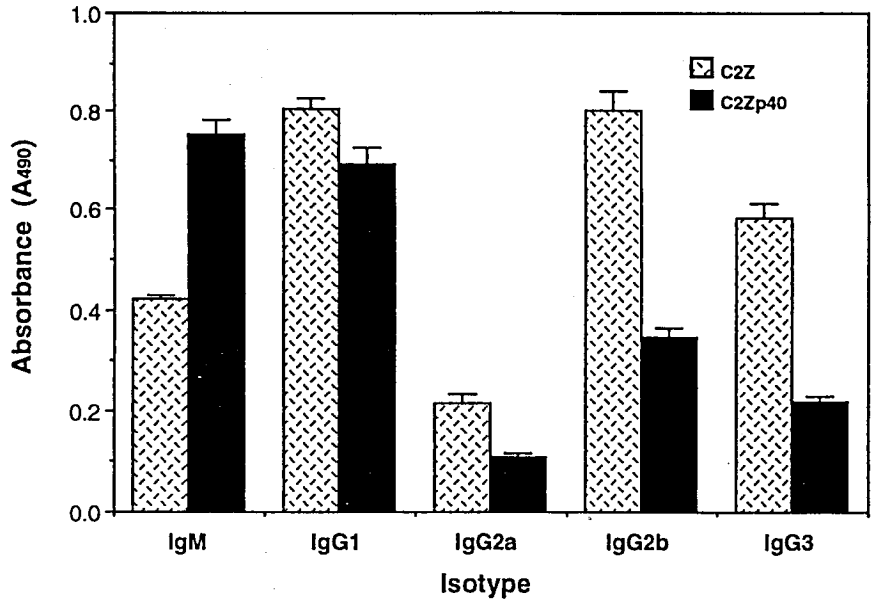

Figure 4. Isotype profile of serum anti-β-galactosidase antibody. Serum IgM, IgG1, IgG2a, IgG2b and IgG3 anti-β-galactosidase Abs from C2Z (*shaded bars*) or C2Zp40 (*closed bars*) transplanted mice were determined by isotype-specific ELISA as described in Materials and Methods. Data represent mean+SD of triplicate samples. Similar results were obtained in three independent experiments.

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Permanent systemic delivery of therapeutic proteins from genetically modified myofibers

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Abstract

In an attempt to use skeletal muscles for the permanent delivery of therapeutic proteins in the serum, we have transduced mouse primary myoblast cultures with retrovirus vectors and engrafted the genetically-modified cells in recipient animals. Repeated exposures to retrovirus vectors allowed gene transfer into near 100% of cultured cells. Injection of cultured myoblasts into regenerating muscles gave rise to up to 60% of genetically-modified myofibers.

Various enhancer-deleted retrovirus have been used, in which the expression of the cDNA of interest was controlled by an internal promoters, including various combinations of skeletal muscle-specific enhancer-promoter elements. Two potentially therapeutic proteins were considered: a lysosomal enzyme (the β-glucuronidase) for which a deficient mouse model is available (the MPS VII mouse) and Epo, which stimulates HbF synthesis and has been proposed for treating human hemoglobinopathies.

In vitro experiments in myoblasts and myotubes indicated that a specific expression in differentiated myotubes can be achieved by using retroviral vectors containing muscle specific control elements such as the MLC enhancer associated with the α-actin promoter or the human desmin enhancer and promoter. High levels of the reporter proteins were detected in culture supernatants. After in vivo implantation, β-glucronidase-secreting myofibers allowed a complete correction of the diseased phenotype in deficient mice which was stable for at least 8 months. Epo-secreting myofibers induced a permanent

increase of the hematocrit which rose over 75%. These data indicated that a permanent delivery of a therapeutic protein can be obtained in mice by implanting genetically-modified myoblasts.

However, in many disorders including Epo-responsive anemias, gene therapy might be proposed only if transgene expression levels can be requested. The tetracycline resistance operon of *E.coli* provides a transcriptional regulatory systems effective in mammalian cells. A chimeric transactivator (tTA) consisting of the tetracycline repressor fused to the transactivation domain of HSV-VP16 stimulates transcription by binding a minimal CMV promoter containing repeats of the tetracycline operator (op7CMV). Binding is abolished by tetracycline, thus impairing promoter activation.

We have constructed two enhancer-deleted retroviral vectors carrying either the Epo reporter gene under the control of the op7CMV promoter or the tTA gene under the control of the muscle-specific human desmin enhancer-promoter, C2.7 myoblasts were transduced with these vectors and the level of Epo secretion was examined in vitro and after engraftement in recipient mice, either in the presence or in the absence of tetracycline. We observed that the transactivation by tTA could be abolished in the presence of tetracycline, both in cell cultures and in animals receiving tetracycline. These experiments showed the feasibility of using tetracycline to modulate the in vivo secretion levels of a therapeutic protein in a polyclonal cell population transduced with retrovirus vectors.

Effective gene transfer into skeletal muscle using adenoviral vector

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Effective gene transfer into skeletal muscle is one of the important steps for gene therapy upon hereditary muscle diseases such as muscular dystrophy. A wide variety of methods have been developed to introduce DNA into skeletal muscle cells, including calcium phosphate precipitation, liposome-mediated transfer, electropolation, retroviral-mediated transfer and adenoviral-mediated transfer. Among them, replication-incompetent adenovirus gene transfer has several advantages; that can introduce transgene into non-dividing cells, and that also allows the promoter dependent transgene expression in the targeted cells.

When recombinant adenovirus was transferred into neonatal mouse skeletal muscle, transfected gene can persist in the nucleus for a longtime, however when transferred into adult skeletal muscle, recombinant adenovirus could not be incorporated into skeletal muscle[1].

In this paper, I describe effective adenoviral-mediated gene transfer into young adult C57BL/10 mouse skeletal muscle.

We have gotten three recombinant adenoviruses, AxCALacZ[2], AxSRαLacZ[2] and AxMHC-IIBLacZ. In AxCALacZ, the *lacZ* gene was driven by β-actin promoter with CMV enhancer and in AxSRαLacZ, the *lacZ* gene was introduced by SV-40 and HTLV-1 dual promoter. Whereas in AxMHC-IIBLacZ, we made using the method "COS-TPC"[3], the *lac Z* gene was driven by the mouse muscle-specific *MHC-IIB* gene promoter[4].

When AxCALacZ was transferred into mouse muscle cell line, C2 under the condition of m.o.i. 10 (multiplicity of infection), almost 100% of the cells revealed β-galactosidase activities. On the other hand, AxMHC-IIBLacZ did not produce apparent activities in the growing C2 cells, but we observed certain numbers of β-galactosidase positive well-differentiated myotubes, when

we evoked differentiation after the infection. To compare the effectiveness of the promoters, we infected the same amounts of recombinant adenoviruses (m.o.i. 10) into C2 cells and examined β-galactosidase activities(Table 1). In this experiment, after the infection with recombinant adenoviruses, we evoked differentiation of the C2 cells by changing the medium. Among three recombinant adenoviruses, AxCALacZ revealed much higher activities than other two, AxSRαLacZ and AxMHC-IIBLacZ. The activities by AxCALacZ at three days after the infection was 290 times more than that of vacant adenovirus Ax1W, that has no insertion instead of E1A, E1B and E3 gene deletion. At seven days after the injection, the activity by AxCALacZ was 167 times more than that by Ax1W. AxMHC-IIBLacZ presented rather low β-galactosidase activity, but the activities were increased at ten days after the infection. This pattern reflected the expression profile of the *MHC-IIB* gene promoter, that was only expressed in highly differentiated fast glycolytic fibers[4].

In order to determine whether adenoviral vector could infect into post-dividing differentiated cells, C2 cells were infected with AxCALacZ under m.o.i.10 at various times after we evoked differentiation. Cells were harvested three days after the infection and the β-galactosidase activity among same amounts of protein have been measured. Cells on the plates were also stained with X-gal to examine whether myotubes really expressed β-galactosidase or not. The highest β-galactosidase activities, we have gotten was from the cells, which we infect the recombinant adenovirus 6 days after we evoked differentiation. Indeed, highly differentiated myotubes revealed strong β-galactosidase activity in the plate under the same interval between the initiation of differentiation and the infection. This data implies that adenoviral vector could infect into post-dividing, even highly differentiated myotubes. When AxMHC-IIBLacZ was infected, β-galactosidase activity was generally low, but the activities tended to increase when the period between changing the medium and infection was becoming longer. This data indicates that the transgene expression in cultured muscle cell is regulated by the promoter in the recombinant adenovirus.

Then, we introduced these recombinant adnoviruses into young adult mouse skeletal muscle in vivo. We injected with AxCALacZ into anterior tibial muscle of 5-6 weeks old C57BL/10 mice(Table 2). After the various

period, we took out the muscle and fixed the muscle specimen to stain with β-galactosidase and hematoxylin-eosin. Seven days after the injection, 66% of muscle fibers in the anterior tibial muscle were stained with β-galactosidase and fourteen days after, around 50% of muscle cells showed positive activities. However, β-galactosidase positive cells became gradually reduced and 10% of the fibers were remained to be positive at twenty-eight days after the injection. We noticed that, however, even in 180 days after the infection, 10% of the fibers still expressed enzyme activities, although activities themselves looked weak. β-galactosidase activities were also observed not only at muscle fibers but also at intrafusal fibers and capsule in muscle spindle, and at endothelial cells in vein.

We also examined injected muscle using immunohistochemical staining against CD4 and CD8. Seven days after the injection, CD4 positive cells have been found in the skeletal muscle specimen. The infiltration of lymphocytes were prominent fourteen days after the injection and CD4 and CD8 positive cells were often detected surrounding β-galactosidase positive cells, among them CD8 positive cells gradually dominated and was evident at twenty-eight days after the injection.

We also compared the efficiency of each recombinant adenovirus in vivo transfer into skeletal muscle(Table 2). In the occasion of AxMHC-IIBLacZ, the same amounts of recombinant adenovirus was injected into anterior tibial muscle of the same age, however the efficiency was drastically diminished from that of AxCALacZ. No muscle cells expressed β-galactosidase activity at seven days after the injection. Infiltration of lymphocytes were recognized regardless of no β-galactosidase positive cells. Moreover, when a vacant adenoviral vector, Ax1W was introduced, lymphocytes infiltration has been also noticed in skeletal muscle specimen. These data indicate that not only the product of inserted gene such as β-galactosidase, but also leaky expressed viral protein induce immunological reaction in infected skeletal muscle with recombinant adenovirus.

The results, high efficiencies of gene transfer using adenoviral vector has never been obtained in previous transfer studies into adult rodent skeletal muscles. The effective gene transfer into young adult rodent skeletal muscle could be explained by 1) the character of backbone replication-incompetent adenovirus 2) highly effective CAG expression unit, chick β-actin promoter

with CMV enhancer and β-globin polyadenylation signal 3) suitable mouse strain for adenoviral gene transfer.

The recombinant adenovirus, we used in this study, are E1/E3 deleted type of vectors and have been made using the COS/TPC method[3], which enables homologous recombination much easier than the previous method. Furthermore, the transcriptional orientation of the transgene is leftward, opposite the deleted E1 gene and the same direction as the E4 gene. This orientation might help the effective transcription of the transgene, because the expression unit with leftward orientation revealed the higher yield of transgene product than those with rightward orientation(personal communication).

We showed the CAG expression unit revealed high expression of transgene in skeletal muscle compared with SRα promoter(SV-40, HTLV-1 dual promoter) and the *MHC-IIB* gene promoter(myosin heavy chain IIB promoter). Since these expression units has the same backbone adenovirus and the same E.Coli *lacZ* gene, the difference of the β-galactosidase expression can be explained by the effectiveness of the promoter. The CAG promoter itself has been proved as strong promoter in various tissues in transgenic mouse studies, however among several tissues this promoter revealed the strongest activities in skeletal muscle[5].

The mouse strain also largely contributes the efficiency of the transgene expression. We used C57BL/10 mice, because this strain has the same background as dystrophin deficient mdx mice. Whereas, other researchers usually preferred BALB/c mice in injection study to skeletal muscle with recombinant adenovirus[1]. There has been no comparison between mouse strain in infection study into skeletal muscle, however in the liver, Barr et. al[6] clearly demonstrated prolonged persistence in C57BL/6 and C57BL/10 mice relative to BALB/c when the common adenoviral vector was infected.

The current problem that we have, is how to prevent elimination process of the transgene-expressed muscle fibers to prolong the expression of transferred gene. We presented the CD4 and CD8 positive cells were participated in the elimination process and not only the transgene product but also viral protein evoke the infiltration of the lymphocytes. To induce immunological tolerance against transgene products such as β-galactosidase and

against viral proteins such as E2a is one of the next steps for the gene transfer into skeletal muscle using adenoviral vector.

Recombinant Adenovirus	Days after infection 1	3	7	10
AxCALacZ	4.9±0.1	34.9±2.9	60.3±8.5	6.0±0.3
AxSRαLacZ	0.0048±0.00064	3.65±0.31	1.98±0.11	1.06±0.036
AxMHC-IIBLacZ	0.0049±0.0035	0.15±0.02	0.44±0.08	1.37±0.32
Ax1W	0.0036±0.0033	0.12±0.026	0.36±0.051	0.51±0.11

Table 1 Comparison of β-galactosidase activities in C2 cells infected with adenovirus vectors

C2 myoblasts were infected with AxCALacZ, AxSRαLacZ, AxMHC-IIBLacZ or Ax1W under 10 m.o.i. After the infection, differentiation has been evoked by the exchange of the medium. Cells were harvested three days later and β-galactosidase assay has been done. The average and standard error of the β-galactosidase activities has been shown by μ units/μg protein.

recombinant adenovirus		AxCALacZ	AxSRαLacZ	AxMHC-IIBLacZ	Ax1W
dose (PFU)		4.1×10^7	6.5×10^7	2.2×10^7	3.4×10^7
days after injection	7	66.6±3.7	1.12±0.07	0	0
	14	48.9±11.8	0.51±0.04	-	0
	28	10.9±7.0	0	0	0
	35	6.3	-	-	-
	47	16.4	-	-	-
	89	11.3	-	-	-
	180	6.3	-	-	-

Table 2 Percentage of β-galactosidase positive fibers after injection with recombinant adenovirus into anterior tibial muscles of C57BL/10

Anterior tibial muscles of C57BL/10 were injected with 50 μl of recombinant adenoviruses AxCALacZ, AxSRαLacZ, AxMHC-IIBLacZ or Ax1W. The dose was indicated as plaque forming unit(PFU). 7, 14, 28, 35. 47, 89, 180 days after the injection, the muscle were removed and β-galactosidase staining has been done. Highest percentage of β-galactosidase positive fibers has been gotten from each frozen muscle specimen and mean with standard error has been calculated from several injection experiments.

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Development of adenovirus vectors for gene therapy of Duchenne muscular dystrophy

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We have inserted into an adenovirus vector a dystrophin cDNA copy(6.3kb) cloned from a 65 year-old patient, presenting a very mild form of Becker muscular dystrophy. This cDNA copy codes for a truncated form of dystrophin, containing a large deletion in the central rod domain of the molecule and named "minidystrophin". The recombinant adenovirus (AdRSVmDys) was intramuscularly injected once into different muscles of newborn *mdx* mice, taking advantage of the relative tolerance of the immune system and very high susceptibility of immature myofibres to adenovirus. The number of minidystrophin-positive fibres could reach two-third of total muscle fibres. Minidystrophin expression was stable at least for one year. A majority of nuclei in these fibres was at the normal submembranous position while dystrophin-deficient mdx myofibres were centronucleated. Expression of minidystrophin can protect efficiently the fibres against the necrosis-regeneration process that affects the dystrophic mouse muscles. These results prompted us to devise experiments meant to see if minidystrophin-expressing fibres have also recovered normal mechanical properties.

We have submitted isolated rapid muscles from adult *mdx* mice to repeated contractions with stretches, since it is known that these eccentric contractions produce a high level of stress. The force drop has been found to be around 60% on average in the *mdx* mouse fast muscle, compared to 20% on average in the normal mouse muscles. This correlates with the important fibre damage that can be observed in *mdx* mouse muscle sections. Intermediate force drops has been found in injected muscles, inversely correlated with the

percentage of fibres expressing dystrophin in each animal and correlated with the number of damaged fibres. These results lead us to the conclusion that muscles with more than 40% of fibres expressing dystrophin would have a force drop almost similar to the normal muscle. These experiments show, for the first time, a functional correction of a muscle after an uneven somatic dystrophin gene transfer in myopathic *mdx* mice model.

To know whether the efficient and long-lasting results obtained in the newborn mice could be extended to adults, we experimented on :
1) the impact of age at injection in normal and *mdx* mutant mice and
2) the duration of expression in adult normal and genetically-immunosuppressed *scid* mice. Adenovirus seems to transduce immature fibres much more efficiently than mature ones. Injection into immnocompetent mice results in a large destruction of infected cells by 3 weeks' time. In contrast, we observed a long term expression of the transgene in *scid* mouse myofibres since they are protected against the specific immune response. Strategies based on genetic manipulation of the adenovirus vector and immunosuppressive treatments were proposed to prevent the host immune response.

Adenoviral Gene Therapy of Neurodegenerative Diseases

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Introduction

Gene therapy, that is the therapeutic use of genes as drugs, could have two main fields of application in neurodegenerative diseases, hereditary diseases and any disease in which a therapeutic protein, can, in principle, be replaced by its structural gene or cDNA.

Several neurodegenerative diseases could be accessible to gene therapy in the future (for review: 1). Recently, transfer of the tyrosine hydroxylase gene has been reported in an animal model for Parkinson disease. In Alzheimer disease, transfer of genes for neurotrophic factors (i.e. NGF), acetylcholine synthesis enzymes or scavengers of amyloid β protein have to be considered. Our gene therapy approaches for two other neurodegenerative diseases, motor neuronal degeneration like amyotrophic lateral sclerosis, ALS, which is in most cases acquired and Tay-Sachs disease, an inherited lysosomal storage disorder, will be presented below.

Genes can be transferred to the CNS by 2 main strategies, through transplantation of *ex vivo* genetically modified cells or by direct administration *in vivo,* using viral or non-viral vectors. Our group was among the first to demonstrate the feasibility of adenoviral gene transfer into the CNS using a replication deficient adenoviral vector.

Adenoviruses are made up of a linear 36 kb DNA genome flanked by 2 inverted terminal repeats. Their genome can be divided into early genes expressed before onset of viral replication and late genes coding for structural proteins. Adenoviral vectors are derived from serotypes 2 or 5. They are deleted in regions E1 and E3 to accomodate foreign DNA. Second generation vectors are further crippled by a temperature-sensitive mutation in the E2A gene. The recombinant adenoviruses are infectious but replication defective. Adenoviral vectors (DE1/E3) can be propagated in the 293 cell line which transcomplements for the E1A and E1B gene functions.

Wild type adenoviruses only cause benign symptoms mostly in the upper respiratory tract and the eye. They have been used in attenuated form as vaccines in thousands of US-soldiers without reported side-effects. Adenoviral vectors have a broad host and cell type range including non-dividing cells. They can be produced at high titers up to 10^{12} pfu/ml, are relatively stable and drive high level transgene expression with efficient post-translational processing. In the host cell they remain in an episomal position.

On the other hand, their capactiy for foreign DNA is currently limited to 8.5 kb, there is a risk of recombination events with naturally occurring adenoviruses, and the host immune response can result in partial loss of infected cells. These inflammatory phenomena are substantially reduced in second generation adenoviral vectors.

Potential and limitations of adenoviral gene transfer into the CNS

The adenoviral vector used in our initial expeiments encodes the E.coli enzyme beta-galactosidase under the transcriptional control of the RSV LTR promoter and is deleted in gene regions E1 and E3. This vector was used to infect embryonic rat neural cells in primary culture (2). Neurons, labeled by an anti γ-enolase antiserum, and astroglia, labeled by an anti-GFAP-antiserum, could be infected very efficiently. The expression level was stable for at least one month.

Administration into various regions of the CNS was performed by stereotactic injection (3). When the vector was injected into the nucleus of the hypoglossus, virtually all neurons within this structure were infected and

X-Gal-staining extended to axons. Interestingly, the adenovirus itself diffuses poorly from the injection site respecting the anatomical limits of the nucleus.

The adenovirus was also injected into the ventral horn of the spinal cord. This provokes such a high expression of the beta-galactosidase that the enzyme diffuses into the cytoplasm, dendrites and axons of neurons despite its nuclear localisation signal. This provides a "Golgi like" cell staining.

In these experiments no cytopathic effect has been observed for adenoviral doses inferior to $5x10^7$ pfu and no gross adverse effects on the animals health and behavior were noted.

Administration into the ventricular system leads to expression of the transgene in numerous ependymal cells as visualized by immunofluorescence. The virus does not pass the glia limitans membrane. Quantitative measurements showed that the number of infected ependymal cells was stable from 8 to 30 days after injection with a mean of 1 infected cell per hundred injected pfu.

The spread of the adenovirus through the whole ventricular system is illustrated as ependymal cells in the central channel of the lumbar spinal cord are infected at distance of the injection site which was in the suboccipital region.

To summarize: adenoviral gene transfer into a limited CNS structure through stereotactic injection is feasible and efficient. However, most neurodegenerative diseases are characterized by a multifocal or disseminated pathology. In order to reach more widespread neuron or glia populations other strategies are therefore required: Osmotic disruption of the blood-brain barrier followed by intraarterial injection represents one promising method. Alternatively, ependymal cells, infected after intracerebroventricular administration of adenovirus, might serve as cellular platforms for the secretion of therapeutic proteins. A prerequisite to this approach would be the efficient diffusion of such proteins from the LCR into the brain parenchyme and therein.

There is another possibility to target precise regions in the CNS with adenoviral vectors: axonal transport. When the adenoviral vector is injected into the striatum, labeling can not be observed only in the striatum but also in neurons of the substantia nigra, indicating that the viral vectors have been transported retrogradely from axon terminals (3,4). In contrast, no

histochemical staining was observed in neurons that have axons crossing - but not ending - in the injection site, suggesting that viral particles cannot be endocytosed by axons of passage.

Another example of viral transport at distance is illustrated in the olfactory system (5). Infection of mitral cells is shown 4 days after nasal instillation of adeno β-galactosidase vector. In fact, the transport was anterograde in this case, and associated with passage of the vector from axonal teminals of olfactory neurons to mitral cells of the olfactory bulb.

From the olfactory bulb the vector is able to reach locus coeruleus cells which project to the olfactory bulb. Such virus transport is also seen in the neuromuscular system. Intramuscular injection of adenoviral vectors can lead to their retrograde axonal transport in motor neurons of spinal cord and brainstem.

Tay-Sachs disease as a candidate disorder for gene therapy

We are interested in developing a strategy of gene therapy for Tay Sachs disease, a lysosomal storage disorder due to deficiency of hexosaminidase A, an enzyme composed of 2 subunits, α and β. The disease is caused by mutations in the gene coding for the α-subunit. The loss of hexosaminidase A activity leads to accumulation of undegraded GM-2 ganglioside, particularly in neurons, and results in progressive motor and mental retardation. A quantitative correlation exists beween residual enzyme activity and severity of the disease (6).

An adenoviral vector carrying the Hex α cDNA under direction of the RSV-LTR was used to infect cultured fibroblasts from Tay-Sachs patients devoid of Hex α mRNA as shown by Northern-blot analysis. Infected cells accumulate more specific mRNA than control cells. Hexosaminidase A activity of Tay-Sachs fibroblasts assayed with specific substrates is restored by the vector, totally on the artificial substrate and partially on the natural substrate. Furthermore a very high secreted HexA activity is found in the culture medium of infected cells.

The partial restoration observed with the natural substrate contrasts with the high level of transgene expression. The Western blot indicates that this phenomenon is probably due to overexpression of α-chain precursors in

infected fibroblasts, leading to abnormal precursor maturation and abnormal accumulation of these precursors.

Infection of Tay Sachs fibroblasts with the hexosaminidase alpha vector allowed to normalize ganglioside metabolism using the following experimental protocol. Infected cells were loaded with radiolabelled GM1 ganglioside and subjected to a 7 day chase. Extracted Gangliosides were then separated by thin layer chromatography. In normal cells, GM1 is converted into GM2 by lysosomal beta-galactosidase, then into GM3 by hexosaminidase A. In Tay-Sachs fibroblasts, GM2 cannot be hydrolyzed into GM3 and accumulates. Infection of these deficient fibroblasts with increasing titers of the vector progressively normalizes the ganglioside profile (7).

We are now working on a knock out model of Hex A deficient mice which, although not suffering the Tay Sachs neurological symptoms, accumulate GM2 in various cells as soon as the second month of life, and we are testing the efficiency of our vector on both enzyme deficiency in various organs and abnormal ganglioside accumulation.

Administration of neurotrophic factors via gene transfer

Neurotrophic factors play an important role in the normal development and maintenance of neuronal subpopulations and may be required under pathological conditions (8,9). Neurotrophic factors like NGF, BDNF and NT-3, CNTF, GDNF and IGF-1 are now investigated in a variety of neurodegenerative diseases, either in preclinical or clinical studies. The clinical potential of the recombinant factors however is limited by their pharmacological properties.

Ciliary neurotrophic factor, CNTF, for example, has a very short half life in serum of less than 3 minutes, severe cytokine-like side effets such as fever, cachexia, hepatic dysfunction and is not able to cross the blood-brain barrier. For instance after iv injection only 0.04% of CNTF are found in the spinal cord (10). That is why gene transfer might be an advantageous alternative to deliver CNTF and other neurotrophic factors in a more stable fashion and to a precise localisation.

We constructed an adenoviral vector coding for CNTF. On Northern blot of total RNAs from infected fibroblasts two transcripts of expected size

that use different poly A signals of transcription termination are visualized. Secretion and biological activity of the adenoviral CNTF were demonstrated in a chicken ciliary ganglion cell survival assay upon addition of conditioned media from adenovirus infected fibroblasts. The conditioned media show a rise of CNTF activity with increasing adenoviral dose comparable to the control with recombinant CNTF protein. There is no CNTF secretion after infection with a control adenovirus.

In vivo administration of the CNTF vector by intracerebroventricular injection leads to the accumulation of CNTF messenger RNA in numerous ependymal cells, as detected by in situ hybridization in the third ventricle or by immunohistochemistry in the lateral ventricles.

Another well documented action of CNTF is its differentiating effect on glial cells, O2A precursor cells and astrocytes. Stereotactic administration of the adeno CNTF vector into the striatum results in a major increase in GFAP immunostaining, diffusing outside the area of transgene expression detected by CNTF immunostaining. This is probably due to secretion of both exogenous and endogenous CNTF by infected cells, leading to activation of surrounding astrocytes (11).

Neurotrophic factors and motor neuron diseases

Various neurotrophic factors have been shown to exert neurotrophic effects on motor neurons, *in vitro* (12) and *in vivo* (13,14). In our laboratory we are most interested in CNTF, ciliary neurotrophic factor, BDNF, brain-derived neurotrophic factor, NT-3, neurotrophin-3 and GDNF, glial cell-derived neurotrophic factor.

The neuroprotective effect of our CNTF adenovirus has been demonstrated using motor neurons (11). Embryonic chicken motor neurons, purified by immunopanning, do not survive in culture for more than 4 days. Adeno-CNTF infection increases maximal survival to 8 days comparable to a muscle extract. There is an additive effect of both treatments suggesting cooperation of CNTF with other trophic factors.

We next tested neurotrophic factor adenoviruses in the mouse model *pmn* (15). *pmn*-mice are suffering a progressive motor neuronal degeneration beginning in the hindlimbs. A *pmn* mouse aged 4 weeks does

not spread the toes of their hindlimbs and shows a marked atrophy of the pelvic girdle. Later on the disease extends to the forelimbs and other muscles and leads to death before day 52. Death is probably caused by respiratory failure due to degeneration of motor axons innervating the respiratory muscles.

Progressive motor neuronopathy is well characterized by histological and electrophysiological studies. It is caused by an autosomal recessive mutation which has been mapped to chromosome 13 by J.L.Guénet's group who also introduced the adjacent marker Extratoe (16). *Xt/Xt* homozygotes die in utero, *Xt/pmn* heterozygotes, two thirds of the progeny, have a clearly visible extra digit and can be easily identified at birth. This allows for the treatment of homozygeous *pmn/pmn* mice before the onset of first clinical signs of neuronal degeneration.

The RT-PCR experiment demonstrates long-term expression of the CNTF adenovirus after intramuscular vector administration into newborn mice. We detect the adenoviral CNTF RT-PCR product in all injected gastrocnemius muscles but in none of the control muscles. Analogous results were obtained with the adenoviral vector that encode BDNF and NT-3.

Mean survival of non-treated *pmn* mice is about 40 days. Adeno β-galactosidase administered by intramuscular injection does not modify the survival. A combination of adenoviral vectors coding for different neurotrophic factors improved survival to a mean of 64 days and a maximum of 105 days. This represents an increase in survival of more than 50% (G. Haase et al., in preparation). At day 50 a non-treated surviving *pmn* mouse was characterized by severe hind limb atrophy and paralysis while the treated animal had much less pronounced atrophy and a better motor performance.

We counted the number of myelinated axons in the phrenic nerve. At day 25, untreated pmn mice already showed massive axonal loss as compared to control mice of the same litters. This axonal degeneration was in part prevented by intramuscular vector administration.

We are currently investigating 4 different hypotheses to explain the benefit of neurotrophic factors administered by adenoviral gene transfer into muscle. Adenoviral infection of muscle is known to be very efficient in the neonatal period and can result in sustained secretion of recombinant proteins. According to a 1st mechanism the neurotrophic factors could be taken up by motor neurons at their nerve endings and transported to the cell bodies as has

been shown for CNTF, BDNF and NT-3. Otherwise they could have a trophic effect on muscle as was suggested in the case of CNTF. Next they could gain access to the circulation and act systemically. Finally the adenoviral vector itself could be transported to the spinal cord via retrograde axonal transport as has been recently demonstrated in motor neurons (17).

Combinations of neurotrophic factor adenoviruses appear very promising because the factors act on different membrane receptors, via different intracellular signalling mecanisms and might be necessary at different moments of neurodegeneration or for distinct motor neuron subpopulations. Adeno CNTF, NT-3, BDNF and GDNF are administered-alone or in combination-by intramuscular, intravenous, intraperitoneal or intracerebro-ventricular injections.

We also study the impact of the antiadenoviral immune response which might be attenuated by the use of future generation adenoviral vectors or after tolerization by intrathymic injection in the neonate (18).

Of course, much more investigations will be needed to determine if such strategies are also useful in human motor neuron diseases, Spinal Muscular Atrophies and Amyotrophic Lateral Sclerosis.

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Plasmid DNA transfer into skeletal muscle: Implications for immune reactions to expressed proteins

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Abstract

Pure recombinant plasmid DNA can be used for direct gene transfer of various cell types but it is apparently more efficient for transducing skeletal muscle fibers. Comparison of direct gene transfer in normal and regenerating muscle by intramuscular injection of recombinant plasmids, adenovirus or retrovirus shows naked DNA to be equal to or better than the viral vectors. In addition, plasmid DNA is not intrinsically immunogenic or inflammatory, where the viral vectors induce immune responses, probably against the viral coat proteins as well as against expressed viral proteins.

We have attempted to assess the nature of the problems associated with vector transfer and also due to expression of a foreign protein in muscle fibers. This situation is analogous to the expression of a normal cellular protein in an organism in which a mutation has extinguished the expression of the protein. For these studies we have used vectors expressing the bacterial enzyme beta-galactosidase and the envelope protein of the hepatitis B virus. The advantage of the viral protein, which carries the surface antigen, is that the immune response to this immunogen after normal immunization is well characterized and can thus can be compared to the immune response after DNA transfer into skeletal muscle.

One striking feature of these experimental models is that a single injection of DNA leads to a strong, sustained and broad-based immune response to the proteins. Antibodies, T cell proliferation as well as cytotoxic lymphocytes are strongly induced after a single DNA injection. The muscle fibers that become transfected and express the foreign protein degenerate,

apparently due to an immunologically mediated mechanism.

These issues will be discussed within the context of current immunological concepts. The immunological problems associated with gene transfer, and which have been largely ignored until now, may compromise gene therapy protocols.

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