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**Research Article**

Defects of the mitochondrial genome are important causes of disease. Despite major advances in our investigation of patients, there is no effective therapy. Progress in this area is limited by the absence of any animal models in which we can evaluate treatment. To develop such a model we have injected human myoblasts into the tibialis anterior of SCID mice after inducing necrosis. After injection of normal human myoblasts, regenerating fibers expressed human beta-spectrin, confirming they were derived from fusion of human myoblasts. The stability of the muscle fibers was inferred by demonstrating the formation of motor end plates on the regenerating fibers. In addition, we show the presence of human cytochrome c oxidase subunit II, which is encoded by the mitochondrial genome, in the regenerated fibers. After injection of human myoblasts containing either the A8344G or the T8993C heteroplasmic mitochondrial DNA mutations, human beta-spectrin positive fibers were found to contain the mutation at a similar level to the injected myoblasts. These studies highlight the potential value of this model for the study of mitochondrial DNA defects.



# SCID Mice Containing Muscle with Human Mitochondrial DNA Mutations

## An Animal Model for Mitochondrial DNA Defects

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## Abstract

**Defects of the mitochondrial genome are important causes of disease. Despite major advances in our investigation of patients, there is no effective therapy. Progress in this area is limited by the absence of any animal models in which we can evaluate treatment. To develop such a model we have injected human myoblasts into the tibialis anterior of SCID mice after inducing necrosis. After injection of normal human myoblasts, regenerating fibers expressed human β-spectrin, confirming they were derived from fusion of human myoblasts. The stability of the muscle fibers was inferred by demonstrating the formation of motor end plates on the regenerating fibers. In addition, we show the presence of human cytochrome *c* oxidase subunit II, which is encoded by the mitochondrial genome, in the regenerated fibers. After injection of human myoblasts containing either the A8344G or the T8993C heteroplasmic mitochondrial DNA mutations, human β-spectrin positive fibers were found to contain the mutation at a similar level to the injected myoblasts. These studies highlight the potential value of this model for the study of mitochondrial DNA defects. (*J. Clin. Invest*. 1998. 102:2090–2095.) Key words: muscle fibers • mitochondria • heterologous transplantation • cultured cells • muscular diseases**

## Introduction

Human mitochondrial DNA (mtDNA),[1] the only extrachromosomal DNA, is a small (16.5 kb) genome encoding 13 essential proteins of the respiratory chain as well as 22tRNAs and 2rRNAs (1). Defects of this genome are now recognized as important causes of human disease with many patients presenting with muscle or neurological abnormalities (2, 3). These defects take the form of either point mutations or rearrangements, such as deletions and duplications. Point mutations can occur in either the RNA or protein encoding genes, although the former are much more common (4). Most mtDNA mutations are heteroplasmic (the presence of both mutated and wild-type mtDNA) with a biochemical defect and clinical phenotype only apparent when mutant mtDNA levels are high (5–7).

Despite major advances in our understanding and investigation of patients with mtDNA defects, there is no effective treatment (8). Since any pathological mutation of mtDNA will result in an abnormal respiratory chain, the effect of any such defect is a severe abnormality of energy metabolism. Dietary management and addition of cofactors is largely unsuccessful and the patients progressively deteriorate, resulting in severe disability or death. The gloomy prognosis and the lack of any therapeutic agent have led us, and others, to consider gene therapy as a means of treating mtDNA defects (9). Although strategies have been devised and shown to be effective in vitro (10), a major hurdle for subsequent development is the lack of an animal model which expresses heteroplasmic pathogenic mtDNA.

Previous work has shown that injection of normal human myoblasts into the tibialis anterior of severe combined immunodeficiency (SCID) mice led to the development of muscle fibers which expressed human dystrophin (11). We wished to determine if we could use a similar approach to develop an animal model which would be valuable in the study of mitochondrial abnormalities. Our studies have focused on two questions. First, using control myoblasts, can we obtain human muscle fibers which are stable and express proteins encoded by human mtDNA? Second, if we inject myoblasts containing mutant mtDNA, do the muscle fibers which form also contain the mutation and at what level?

## Methods

*Myoblast culture.* Muscle was obtained from diagnostic muscle biopsies from a 45-yr-old man with the A8344G mutation and a 23-yr-old woman with the T8993C mutation. Muscle biopsies were also obtained from control subjects. The A8344G mutation is one of the most prevalent and best characterized defects of mtDNA and is responsible for myoclonus epilepsy with ragged red fibers (MERRF) (7, 12, 13). The MERRF patient also had a homoplasmic 9-bp deletion (8270–8278), which is a nonpathogenic polymorphism (14). The T8993C mutation is associated with neurogenic ataxia and retinitis pigmentosa (NARP) and maternally inherited Leigh's syndrome. The patient from which the T8993C myoblasts were obtained had Leigh's syndrome.

Excess fat and fibrous connective tissue was removed from the muscle before it was washed in sterile PBS and chopped finely. The satellite cells were obtained after enzymatic dissociation with 0.25% trypsin, 1 mM EDTA in PBS at 37°C. Cell pellets, obtained after cen-

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1. *Abbreviations used in this paper:* MERRF, myoclonus epilepsy with ragged red fibers; mtDNA, mitochondrial DNA; NARP, neurogenic ataxia and retinitis pigmentosa; SCID, severe combined immunodeficiency.

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trifugation, were resuspended in conditioned growth medium, i.e., Ham's F10 with 20% FCS, 2% chick embryo extract, 50 U/ml penicillin, 50 μg/ml streptomycin, 110 μg/ml sodium pyruvate, and 50 μg/ml uridine (15, 16), and exposed to confluent human muscle cultures for 24 h. Myoblasts were established in 25-$cm^2$ Primaria (Falcon) flasks coated with 1% gelatin. Cells were harvested by trypsinization and washed with PBS before injection.

*Animals and myoblast transfer.* Animals used were immunodeficient BALB/C SCID mice (Fox Chase Suppliers, Charles River, UK). The right hind limb was irradiated (18 grays of X-irradiation) 4 d before transplantation, to render muscle incapable of regeneration. 3 d later, barium chloride ($5 \times 10$ μl, 1.2% vol/vol) was injected into the tibialis anterior of the same leg to induce muscle necrosis. The next day, $2–3 \times 10^6$ human myoblasts in a 12–15-μl volume were injected at three different sites into the muscle through a micropipette. Only single injections were performed using the MERRF myoblasts and some control myoblasts. However, a further injection of $2–3 \times 10^6$ cells was performed 10 d later in experiments with the NARP myoblasts and further control myoblasts. All mice injected with the MERRF and NARP myoblasts were killed after 30 d. For those mice injected with control myoblasts and used to assess stability of regenerated fibers, the muscles were not removed until 8 wk after injection.

*Histological, histochemical, and immunocytochemical analyses.* The tibialis anterior muscles were transversely orientated, frozen in isopentane, and cooled to −150°C with liquid nitrogen. Sections were cut using a Reichert Frigocut 2800N cryostat microtome (Reichert Jung, Slough, UK) every 200 μm throughout the muscle and stained with hematoxylin and eosin, until areas showing signs of regeneration were identified. A human specific β-spectrin primary antibody (Novocastra, Newcastle upon Tyne, UK) was used to detect fibers formed from human myoblasts; this was used in conjunction with a rabbit anti–mouse horseradish peroxidase–conjugated secondary antibody (DAKO A/S, Glostrup, Denmark). Detection was performed by incubating sections with 0.5 mg/ml 3,3′diaminobenzidine in 0.1 M phosphate buffer, pH 7.4, 0.1% hydrogen peroxide. The formation of motor endplates was determined by acetylcholine esterase activity, based on the technique of Karnovsky and Roots (17). Expression of human mitochondrial proteins was analyzed using a human specific primary antibody to subunit II of cytochrome *c* oxidase (18) in conjunction with a rabbit anti–mouse horseradish peroxidase–conjugated secondary antibody (DAKO A/S). Detection was performed as above.

*Quantification of levels of mutant mtDNA.* Individual muscle fibers were isolated using a siliconized microcapillary tube, from 30-μm sections labeled with an antibody for human β-spectrin. Single-fiber PCR, based on a previous method (5), was as follows. Individual β-spectrin positive fibers on cross-sections were isolated and placed in individual microfuge tubes containing 10 μl of water. After centrifugation and removal of the water, each fiber was lysed by addition of 5 μl of buffer containing 200 mM KOH, 50 mM DTT, followed by heating to 65°C for 1 h. Neutralizing buffer (5 μl) containing 900 mM Tris base, 200 mM HCl was added to each tube before PCR.

For quantification of the MERRF mutation, a mismatch reverse primer 5′ TTT CAC TGT AAA GAG GTG TG*G 3′ (nucleotide position 8346–8366) and forward primer 5′ GAT GCA ATT CCC GGA CGT C 3′ (nucleotide position 8102–8120) were used to generate a 265/256-bp fragment (depending on whether 9-bp deletion was present or absent). Conditions were 94°C 5 min (1 cycle), 94°C 1 min, 58°C 1 min, 72°C 1 min (30 cycles), followed by 72°C 8 min (1 cycle). After addition of 30 pmol primer, 1 U *Taq* polymerase (Boehringer

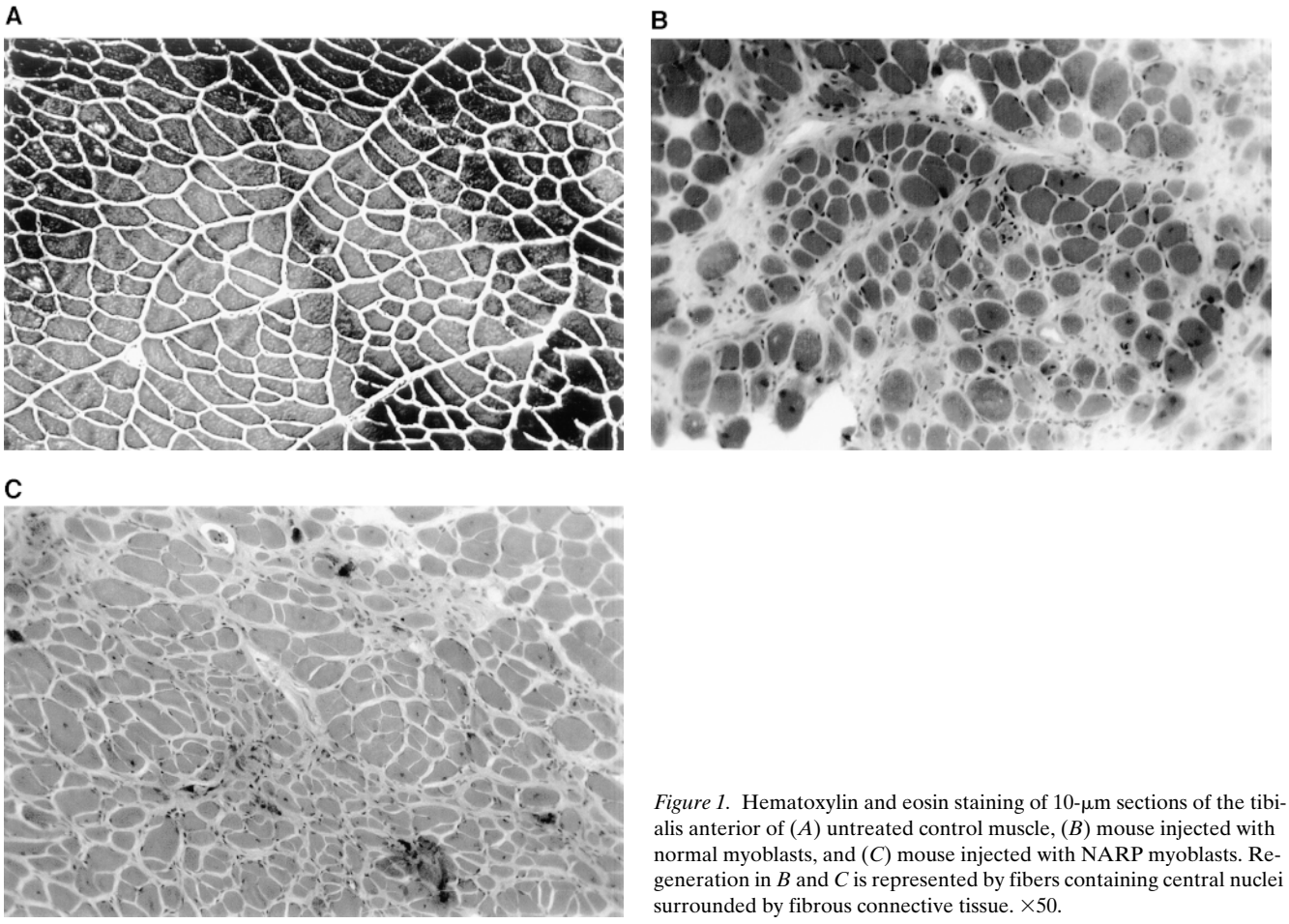


*Figure 1.* Hematoxylin and eosin staining of 10-μm sections of the tibialis anterior of (*A*) untreated control muscle, (*B*) mouse injected with normal myoblasts, and (*C*) mouse injected with NARP myoblasts. Regeneration in *B* and *C* is represented by fibers containing central nuclei surrounded by fibrous connective tissue. ×50.

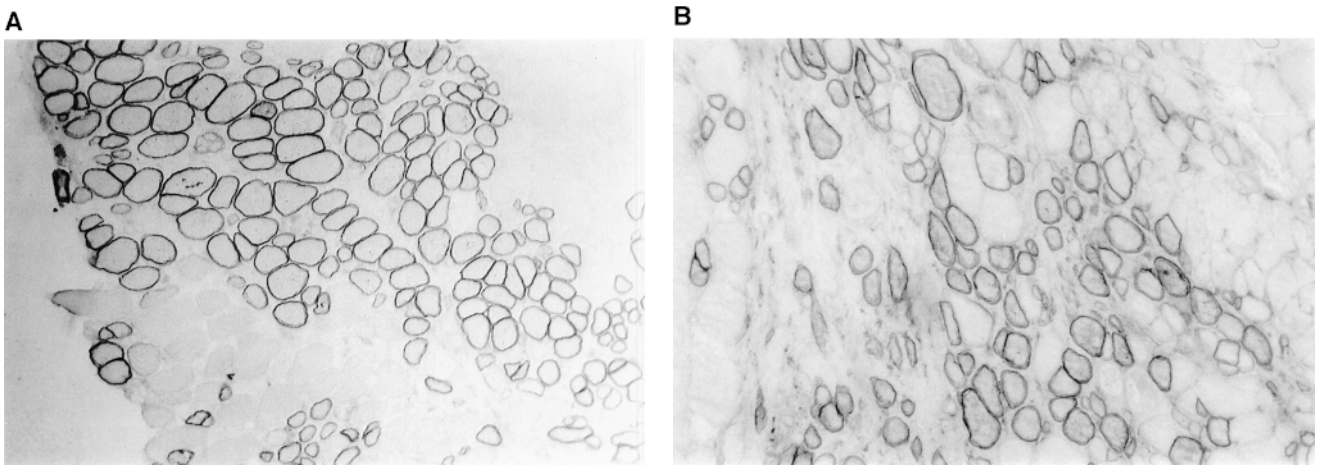


*Figure 2.* 8-μm sections of tibialis anterior labeled with human β-spectrin antibody from (*A*) mouse injected with control myoblasts and (*B*) mouse injected with NARP myoblasts. The fibers expressing human β-spectrin are clearly shown by the staining around the periphery of the fiber. ×50.

Mannheim, Mannheim, Germany) and 2.5 μCi [α-$^{32}$P]dCTP (3,000 Ci/mmol) (Amersham International, Buckinghamshire, UK), the products were submitted to a single "hot" last cycle; 94°C 8 min, 58°C 2 min, and 72°C 12 min. All PCRs were performed using a Hybaid Touchdown thermal cycler and reagents from Advanced Biotechnologies (Surrey, UK). Samples were subjected to phenol/chloroform extraction and diluted to 100 cpm/μl. 1,000 cpm were incubated with 10 U BanII (Boehringer Mannheim) at 37°C overnight. The samples were electrophoresed through a 12% polyacrylamide gel and analyzed using a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA). On restriction enzyme digest, wild-type DNA yields fragments of 152, 72, and 41 bp, whereas if the MERRF mutation is present, the 72-bp fragment is cut into 52 and 20 bp. The injected MERRF myoblasts carry a nonpathogenic, homoplasmic mtDNA, 9-bp deletion resulting in a 32-bp fragment instead of the 41-bp fragment when cut with BanII.

The T8993C mutation creates an additional HpaII site at position 8992. PCR was performed using the forward primer 5′ GTG ATT ATA GGC TTT CGC 3′ (nucleotide position 8863–8880) and the reverse primer 5′ CAG ATA GTG AGG AAA GTT G 3′ (nucleotide position 9841–9859) to generate a 997-bp fragment. Conditions were 94°C 5 min (1 cycle), 94°C 1 min, 50°C 1 min, 72°C 1 min (30 cycles), followed by 72°C 8 min (1 cycle). Hot last cycle was performed as described above (with the conditions, 94°C 8 min, 50°C 2 min, and 72°C 12 min). 1,000 cpm were incubated with 10 U HpaII (Boehringer Mannheim) at 37°C overnight. The samples were electrophoresed through a 5% polyacrylamide gel and analyzed using a PhosphorImager system (Molecular Dynamics). On restriction enzyme digest, wild-type DNA yields fragments of 567 and 430 bp, whereas if the NARP mutation is present, the 430-bp fragment is cut into 130- and 300-bp fragments.

## Results

*Muscle regeneration in SCID mice.* The left tibialis anterior muscles, which had not been irradiated or treated with barium chloride, were normal with no evidence of degeneration (Fig. 1 *A*). However, all muscles irradiated and then injected with barium chloride showed small fibers with internal nuclei, indicating that regeneration had occurred (19) (Fig. 1, *B* and *C*). In muscles injected on day 1 only, regeneration varied from 5 to 50% of the cross-sectional area. The same degree of regeneration was observed for muscles injected with either normal myoblasts or those containing the MERRF mutation. However, in

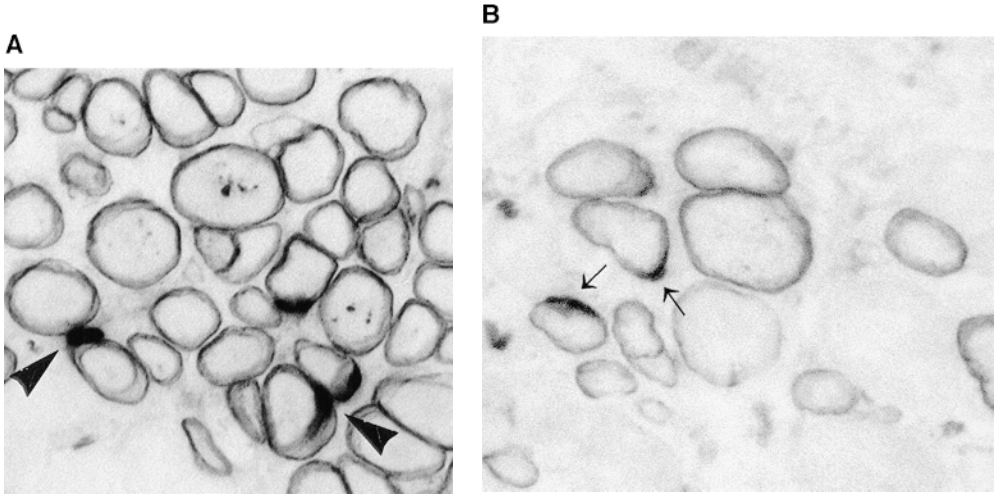


*Figure 3.* 8-μm sections of tibialis anterior reacted for acetylcholine esterase activity and labeled with human β-spectrin antibody from (*A*) mouse injected with normal myoblasts and (*B*) from mouse injected with NARP myoblasts. The intensely stained areas at the edge of the fibers represent motor end plates (*arrows*), suggesting that the regenerated human fibers are innervated. ×115.

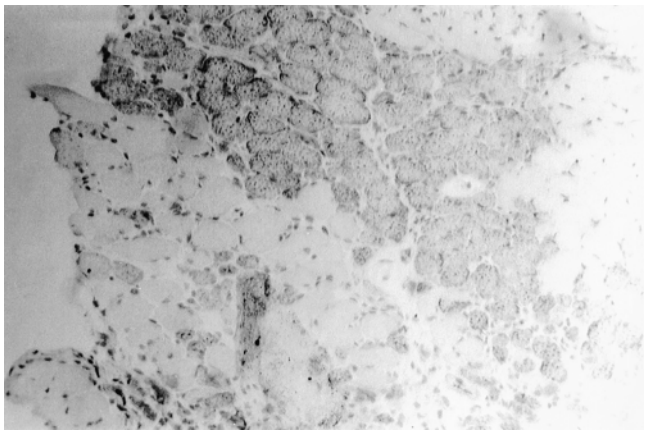

*Figure 4.* Tibialis anterior from a mouse injected with control myoblasts and labeled with an antibody specific for human cytochrome *c* oxidase subunit II. Fibers expressing this mitochondrially encoded subunit have a dark granular appearance. This section is a serial section of Fig. 3 *A*, and on comparison it can be seen that all β-spectrin positive fibers are expressing cytochrome *c* oxidase subunit II. ×50.

subsequent studies when muscles were injected on day 1 and 11, with either myoblasts containing the NARP mutation or control myoblasts, the percentage of regeneration increased to ~ 70% (Fig. 1, *B* and *C*).

*Nature of regenerated fibers.* To establish if the regenerated fibers were of human origin, expression of human β-spectrin was determined by immunocytochemistry. Muscles which had been irradiated, injected with barium chloride, and then injected with PBS as controls showed regenerated fibers, but none expressed human β-spectrin (not shown). However, β-spectrin positive fibers were clearly identified in sections from the muscles injected with either control myoblasts (Fig. 2 *A*) or myoblasts containing mutated mtDNA (Fig. 2 *B*). The results clearly indicate that both the MERRF and NARP myoblasts are able to contribute to the formation of new fibers. To establish if the regenerated muscle fibers were stable, we wished to determine whether motor end plates had formed and thus if innervation had occurred. There was colocalization of acetylcholine esterase activity at the surface of β-spectrin positive fibers, thus indicating the presence of neuromuscular junctions (Fig. 3, *A* and *B*). Finally, we wished to determine if the fibers formed from control myoblasts expressed proteins which were encoded by the human mitochondrial genome and thus synthesized within the mitochondrion. Using an antibody specific to human cytochrome *c* oxidase subunit II, we show that in the fibers expressing human β-spectrin, there is also expression of human cytochrome *c* oxidase subunit II (Fig. 4).

*Analysis of mutant load in regenerated muscle fibers.* Having established that stable human fibers are formed after injection of myoblasts containing either the A8344G MERRF mutation or the T8993C NARP mutation, we determined whether these fibers harbored the respective mutated mtDNAs. Individual β-spectrin positive fibers were isolated from 30-μm sections and a fragment of mtDNA amplified for restriction digest analysis. The regenerated muscle fibers from mice injected with both the MERRF and NARP myoblasts contained a high level of mutated mtDNA, similar to that found in the original myoblasts (Fig. 5, *B* and *C*).

## Discussion

There were two main objectives to ensure that our animal model would be of value to study disease due to mtDNA mutations. The first component was to demonstrate the formation of stable human muscle fibers in mature muscle. We have shown that after injection of human myoblasts, regenerated muscle fibers express human β-spectrin. When control myoblasts are injected, the regenerating fibers also express human cytochrome *c* oxidase subunit II, confirming expression of the human mitochondrial genome. Our approach is based on that described by Huard et al. (11) in which they show that human myoblasts could be injected into the tibialis anterior of SCID mice and form fibers expressing human dystrophin. The studies by Huard et al. (11) also conclude that the fibers obtained were innervated because of the accumulation of dystrophin and desmin observed on some fiber membranes. We have now added to these data by showing that the regenerated fibers expressing human β-spectrin are likely to have formed motor end plates, by demonstrating the presence of acetylcholine esterase activity at the surface of these fibers.

The second objective was to determine whether regenerated human muscle fibers would contain mutated mtDNA after injection of myoblasts from patients with mitochondrial disease. The formation of myotubes in vitro has been shown to be severely impaired in myoblasts which lack mtDNA and are thus respiration deficient (20). Therefore, we wanted to ensure that myoblasts harboring mtDNA mutations, which at high levels cause respiratory chain defects, could contribute to the regeneration process. Both the MERRF and NARP mutations were detected at high levels in β-spectrin positive fibers and thus we have demonstrated that it is possible to create mice that will express heteroplasmic human mtDNA defects.

We have demonstrated regeneration from myoblasts containing two very different mtDNA mutations. The T8344G mutation involves mt $tRNA^{Lys}$ and has been shown to affect translation of all mitochondrially encoded proteins (7, 21, 22). However, the T8993C mutation is a missense mutation and changes only a single amino acid in the mitochondrially encoded ATPase subunit 6 (23). As regeneration can be obtained from myoblasts containing either of these mutations, there is no reason why the same approach could not be used for any mtDNA mutation which is expressed in cultured myoblasts. Indeed, since it has been shown that skin fibroblasts may be converted into myoblasts (24), it may also be possible to generate similar mouse models using dermal fibroblasts.

Our approach to an animal model is very different from that of Jenuth et al. (25) who created a mouse which was heteroplasmic for a rodent polymorphism by the electrofusion of the cytoplast of one zygote type with a single cell embryo of another type. Using such a model, Jenuth et al. propose that random genetic drift is responsible for the segregation of mtDNA in the female germline, and that this could have important consequences for estimating recurrence risks and predicting fixation rates of new mtDNA mutations. Thus, although this model has been very helpful for addressing issues such as mtDNA segregation, it is currently of limited value for investigation of treatment since no pathological mtDNA mutations have been described in mice. Mouse models for mitochondrial disease caused by nuclear DNA mutations have been created by knockout technology (26–28). Graham et al. (26) disrupted the heart/skeletal muscle isoform of adenine nu-

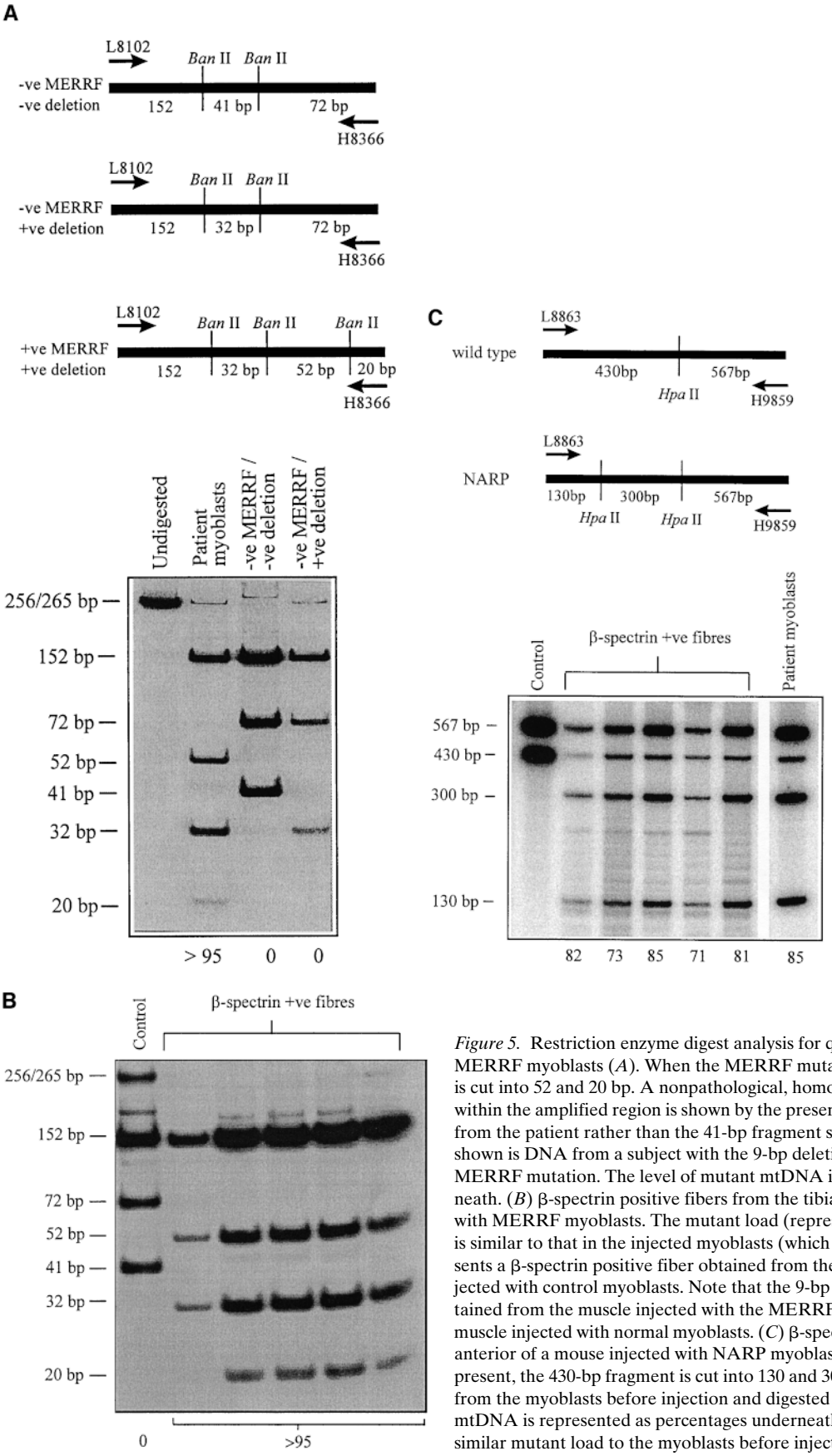


*Figure 5.* Restriction enzyme digest analysis for quantification of mutant mtDNA in MERRF myoblasts (*A*). When the MERRF mutation is present, the 72-bp fragment is cut into 52 and 20 bp. A nonpathological, homoplasmic mtDNA, 9-bp deletion within the amplified region is shown by the presence of a 32-bp fragment in the DNA from the patient rather than the 41-bp fragment seen in the control DNA. Also shown is DNA from a subject with the 9-bp deletion who does not possess the MERRF mutation. The level of mutant mtDNA is represented as percentages underneath. (*B*) β-spectrin positive fibers from the tibialis anterior of a mouse injected with MERRF myoblasts. The mutant load (represented as percentages underneath) is similar to that in the injected myoblasts (which is illustrated in *A*). Control represents a β-spectrin positive fiber obtained from the tibialis anterior of a mouse injected with control myoblasts. Note that the 9-bp deletion is present in the fibers obtained from the muscle injected with the MERRF myoblasts, but not in that from muscle injected with normal myoblasts. (*C*) β-spectrin positive fibers from the tibialis anterior of a mouse injected with NARP myoblasts. When the NARP mutation is present, the 430-bp fragment is cut into 130 and 300 bp. Also shown is digested DNA from the myoblasts before injection and digested control DNA. The level of mutant mtDNA is represented as percentages underneath. The regenerated fibers harbor a similar mutant load to the myoblasts before injection.

cleotide translocator (*Ant 1*). The *Ant 1* mutants showed striking similarities to patients with mitochondrial myopathy and cardiomyopathy, demonstrating that mitochondrial ATP deficiency can cause the clinical phenotype. Larsson et al. (27) disrupted the T*fam* locus, which encodes mitochondrial transcription factor A and demonstrated the necessity of this transcription factor for embryonic development and regulation of mtDNA copy number. Again, although these studies promote our understanding of mitochondrial disease, they do not provide a model in which to assess treatments for disease caused by mtDNA mutations.

Our studies have concentrated on the feasibility of creating an animal model for mtDNA diseases. Treatment of heteroplasmic mtDNA mutations will involve changing the proportion of mutant to wild-type mtDNA and any agent developed may be specifically designed for an individual mutation (10). We have followed mice injected with control myoblasts for up to 8 wk and the fibers remain stable for this period of time; therefore, such therapeutic agents could be assessed on a mouse model before in vivo human studies. Now that we have demonstrated that both MERRF and NARP myoblasts can form muscle fibers in the tibialis anterior, we aim to characterize the pathogenicity of the mutations. The animal model will also be valuable in correlating biochemical and genetic abnormalities. Histochemical analysis of cytochrome *c* oxidase activity and immunocytochemical analysis of mitochondrially encoded proteins in muscles injected with myoblasts containing mutant mtDNA will enable us to study the expression of the genetic defect. There is also potential for assessing temporal fluctuation of mutant mtDNA under different physiological conditions (i.e., rest and exercise). Finally, injection of mutant myoblasts containing different mtDNA mutations may allow us to address key issues such as mtDNA complementation and recombination.

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