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Efficient Mitochondrial Import of Newly Synthesized Ornithine Transcarbamylase (OTC) and Correction of Secondary Metabolic Alterations in spf^{ash} Mice following Gene Therapy of OTC Deficiency

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Abstract

Background: The mouse strain sparse fur with abnormal skin and hair (spf^{ash}) is a model for the human ornithine transcarbamylase (OTC) deficiency, an X-linked inherited urea cycle disorder. The spf^{ash} mouse carries a single base-pair mutation in the OTC gene that leads to the production of OTC enzyme at 10% of the normal level.

Materials and Methods: Recombinant adenoviruses carrying either mouse (Ad.mOTC) or human (Ad.hOTC) OTC cDNA were injected intravenously into the spf^{ash} mice. Expression of OTC enzyme precursor and its translocation to mitochondria in the vector-transduced hepatocytes were analyzed on an ultrastructural level. Liver OTC activity and mitochondrial OTC concentration were significantly increased (300% of normal) in mice treated with Ad.mOTC and were moderately increased in mice receiving Ad.hOTC (34% of normal). The concentration and subcellular location of OTC and associated enzymes were studied by electron microscope immunolocalization and quantitative morphometry.

Results: Cytosolic OTC concentration remained unchanged in Ad.mOTC-injected mice but was significantly increased in mice receiving Ad.hOTC, suggesting a block of mitochondria translocation for the human OTC precursor. Mitochondrial ATPase subunit c [ATPase(c)] was significantly reduced and mitochondrial carbamyΔphosphate synthetase I (CPSI) was significantly elevated in spf^{ash} mice relative to C3H. In Ad.mOTC-treated mice, the hepatic mitochondrial concentration of ATPase(c) was completely normalized and the CPSI concentration was partially corrected.

Conclusions: Taken together, we conclude that newly synthesized mouse OTC enzyme was efficiently imported into mitochondria following vector-mediated gene delivery in spf^{ash} mice, correcting secondary metabolic alterations.

Introduction

Ornithine transcarbamylase (OTC) is a 36 kD protein that catalyzes the second step in the urea cycle, the synthesis of citrulline from ornithine and carbamylphosphate. It is encoded in nuclear DNA, translated in the cytosol on free polyribosomes, and post-translationally imported into the mitochondria with a half-life of about 1 to 2 min (1,2). The enzyme acquires its enzymatic activity after proteolytic cleavage of the leader sequence and assembly into a homotrimer in the mitochondrial matrix (3). OTC deficiency (OTCD) is the most common inborn error of urea synthesis with an estimated prevalence of 1:40,000 to 1:80,000 births. Although the milder forms of OTCD can be effectively treated by stimulating alternative pathways of waste nitrogen excretion and by protein restriction, the severe forms of OTCD represent a therapeutic challenge. Since OTC is mainly expressed in the liver, gene replacement in hepatocytes has the potential to correct the underlying metabolic derangements. The mouse strains sparse fur (spf) and sparse fur with abnormal skin and hair (spf^{ash}) are well-characterized models for the evaluation of gene therapy of OTCD in vivo (4,5). The benefit of gene therapy in OTC-deficient mice has already been elaborated on biochemical and histochemical levels (6), however, it is not clear how efficiently the newly synthesized OTC precursor is translocated by the mitochondrial import mechanism and whether the secondary consequences of OTCD, such as depletion of ATPase (7,8) and alteration of CPSI activity (9–11), are corrected.

The spf^{ash} mouse was chosen in this study because its mutation in the OTC gene results in a reduction of OTC activity and OTC protein to about 7% and 10% of control value, respectively (5). This background is negligible for the immunodetection of OTC on an ultrastructural level.

Spf^{ash} mice were injected with recombinant adenoviruses containing either mouse or human OTC cDNA. Liver sections from the infected mice were harvested 4 days following infusion of vector and were analyzed by electron microscopy for the abundance and distribution (i.e., mitochondria, cytosol and nuclei) of OTC, the subunit c of the ATPase [ATPase(c)], and carbamylphosphate synthetase I (CPSI). Our data demonstrate that newly synthesized mouse OTC precursors following adenovirus infection were efficiently translocated into and correctly processed in the mitochondria of spf^{ash} hepatocytes, while a large fraction of human OTC precursors were retained in cytosol. In addition, secondary alterations, such as mitochondrial ATPase(c) and CPSI concentrations, were completely or partially corrected in mice treated with the vector containing mouse OTC gene.

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Materials and Methods

Animals and Adenoviral Vectors

Spf^{ash} and C3HeB/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in the Wistar animal facility. Spf^{ash}/y and male C3HeB/J mice at 6–10 weeks of age were used in this study. E1-deleted recombinant adenovirus containing wild-type mouse (Ad.mOTC) or human OTC cDNA (Ad.hOTC) driven by a CMV promoter was constructed as described previously (6). The spf^{ash} mice were injected with 2×10^{11} particles of virus through the tail vein and were sacrificed on day 4 post viral injection. Liver tissues were harvested for OTC lysate assay and electron microscopic evaluation.

OTC Lysate Assay

The OTC lysate assay was performed according to the method of Lee and Nussbaum (12) as described previously (6).

Immunocytochemistry for Electron Microscopic Evaluation

Liver tissues were fixed in 5% paraformaldehyde, 50 mM Hepes, pH 7 and cryoprotected with polyvinylpyrollidine/sucrose. The samples were then frozen in liquid nitrogen, sectioned (50 nm), and labeled using the technique of Tokuyasu (13,14). Thawed sections were incubated with the primary antibodies at room temperature for 45 min, followed by the secondary antibody for 45 min. The grids were then contrasted, embedded in 2% methylcellulose, and examined using a Philips 400 electron microscope. The primary antibodies used in this study were the following: a polyclonal rabbit antibody against hu-

man OTC (15) (antibody concentration 200 μg/ml), a polyclonal rabbit antibody against human ATPase(c) (16) (antibody concentration 20 μg/ml), and a polyclonal rabbit antibody against rat CPSI (17) (antibody concentration 300 μg/ml). The secondary antibody was a gold-conjugated goat anti-rabbit serum (gold particle diameter of 6 and 12 nm, DIANOVA/D-Hamburg, dilution of 1:10 and 1:50).

Morphometrical Analysis

Liver samples from three untreated C3HeB/J mice, three untreated spf^{ash} mice, three Ad.hOTC-treated spf^{ash} (spf^{ash}/hOTC) mice, and three Ad.mOTC-treated spf^{ash} (spf^{ash}/mOTC) mice were evaluated. Random electron micrographs of hepatocytes were taken from ultrathin frozen sections of three different tissue blocks of control, untreated, and treated spf^{ash} mice. Labeling density of the OTC, CPSI, and ATPase(c) antibodies in mitochondria, cytosol, and nuclei, as well as surface density of mitochondria were determined by point counting as described (18). A total area of at least 50 μm^2 was evaluated for the labeling density in cytosol and nuclei of each mouse. The labeling densities and surface densities of mitochondria were obtained by evaluating at least 150 mitochondria from different tissue blocks. Absolute volumes of nuclei and cytosol, absolute cell volumes of hepatocytes, as well as absolute surface and volume of mitochondria per hepatocyte were determined in 30 hepatocytes per tissue block of C3H, untreated spf^{ash}, spf^{ash}/mOTC, and spf^{ash}/hOTC mice as previously described (18). The percentage of infected hepatocytes in spf^{ash} mice treated with Ad.mOTC was determined by counting the hepatocytes with strong OTC labeling in mitochondria and those with background OTC labeling in three different blocks from each of the three animals in the same group. Values shown are mean ± SD. Statistical analysis was performed by the student's *t*-test.

Results

Mitochondrial OTC Was Significantly Increased in spf^{ash} Hepatocytes Infected with Ad.mOTC

The majority of hepatocytes in spf^{ash} mice receiving Ad.mOTC (spf^{ash}/mOTC, 85.6 ± 11.6%) showed enlarged mitochondria, which were intensely labeled by the OTC antibody (Fig. 1). Upon visual examination, the mitochondrial labeling density in spf^{ash}/mOTC (Fig. 2A) was much higher than that in untreated spf^{ash} mice (Fig. 2C), in spf^{ash} mice receiving Ad.hOTC (spf^{ash}/hOTC, Fig. 2D), and in control C3H mice (Fig. 2B). Quantitative analysis of OTC labeling density (Fig. 3) showed mitochondrial OTC concentrations of 41.1 ± 33.4 gp/μm^2 in control C3H mice and 8.6 ± 9.3 gp/μm^2 (21% of control) in untreated spf^{ash} mice. Mitochondrial OTC concentration in spf^{ash}/mOTC hepatocytes reached 78.6 ± 52.5 gp/μm^2, about twice that observed in C3H control and about nine times more than that observed in untreated spf^{ash} mice. The concentration of OTC protein was also increased moderately in spf^{ash}/hOTC hepatocytes (23.7 ± 15.5 gp/μm^2). Taking the absolute mitochondrial volume into account, the numbers of gold particles within the mitochondria of each hepatocyte were 223,459 in C3H control, 46,516 in untreated spf^{ash} mice, 634,504 in spf^{ash}/mOTC, and 134,901 in spf^{ash}/hOTC mice. This indicated that the amount of mitochondrial OTC per hepatocyte in spf^{ash}/mOTC mice was, in fact, 3-fold higher than that in normal C3H mice. The OTC labeling density in the cytosol, in general, was much lower than that in mitochondria. However, the highest cytosolic OTC concentration was found in spf^{ash}/hOTC hepatocytes (3.7 ± 1.2 gp/μm^2, Fig. 4A), in contrast to that in spf^{ash}/mOTC (2.0 ± 1.5 gp/μm^2, Fig. 4B), C3H (1.6 ± 1.5 gp/μm^2), and untreated spf^{ash} (1.6 ± 1.3 gp/μm^2) hepatocytes.

OTC activity as measured using a cell lysate assay was 58.0 ± 7.5 μmol citrulline/mg protein/hr in C3H control mice and 2.6 ± 0.1 in untreated spf^{ash} mice (Fig. 3). The values were 173.2 ± 119.5 and 20.1 ± 14.9 in spf^{ash}/mOTC and spf^{ash}/hOTC mice, respectively. These activities parallel the densities of OTC immunoreactivity observed in mitochondria.

Mitochondria of spf^{ash} Hepatocytes Were Enlarged Following Adenovirus-Mediated Gene Transfer

Electron microscopic examination of liver sections revealed that hepatic mitochondria of untreated spf^{ash} mice were smaller in size than those of C3H control mice. Spf^{ash} hepatocytes infected with Ad.mOTC, but not Ad.hOTC, showed enlarged mitochondria (Figs. 2, 6, and 7). A morphometrical evaluation of mitochondria was conducted by determining the surface densities of 150 hepatic mitochondria in each of the four groups of mice. Mitochondrial surface density is defined as the surface area of a mitochondrion divided by its volume. The

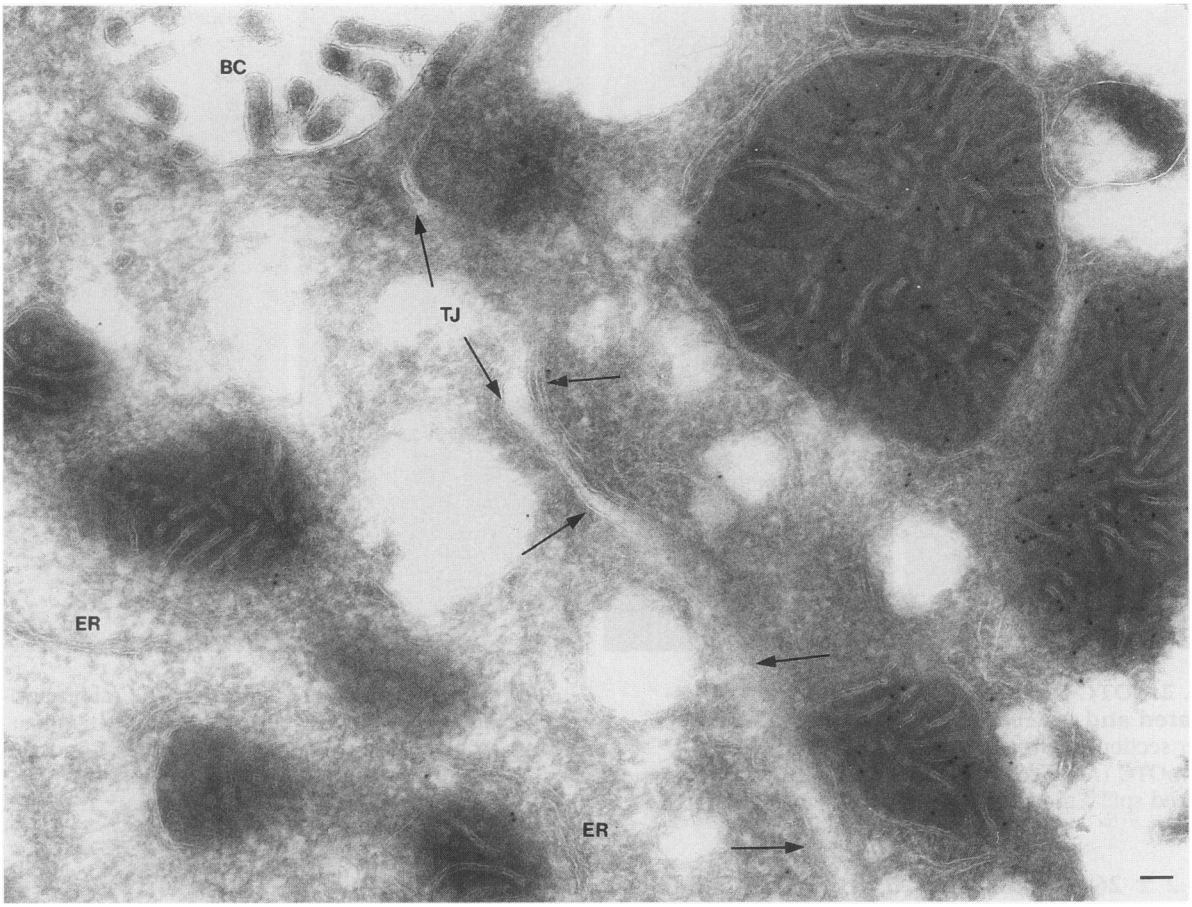

Fig. 1. OTC labeling in infected and uninfected hepatocytes. The ultrathin frozen liver section from a spf^{ash} mouse infected with Ad.mOTC was labeled by the rabbit anti-OTC antibody followed with gold-conjugated goat anti-rabbit antisera. The hepatocyte infected with the OTC virus is characterized by enlarged mitochondria and a much higher mitochondrial OTC labeling density (upper right corner) than in the uninfected hepatocyte (lower left corner). TJ, tight junction complex; BC, bile canaliculus; ER, endoplasmic reticulum. Arrows indicate the lateral membrane of the hepatocytes. Bar, 0.1 μm.

larger a mitochondrion is, the smaller its surface density will be. The average value of mitochondrial surface density was 11.6 ± 3.6 μm^{-1} in spf^{ash}, 10.9 ± 4.2 μm^{-1} in C3H control, 9.8 ± 2.9 μm^{-1} in spf^{ash}/mOTC, and 12.3 ± 4.9 um^{-1} in spf^{ash}/hOTC hepatocytes. As shown in Figure 5, the mitochondrial surface density profile in spf^{ash}/mOTC hepatocytes shifted from that of untreated spf^{ash} mice toward that of the normal C3H mice, whereas the mitochondrial surface density profile in spf^{ash}/hOTC mice remained unshifted. The difference in average surface densities between mitochondria of untreated spf^{ash} and that of spf^{ash}/mOTC hepatocytes was highly significant ($p < 0.001$). Taken together, results from the quantitative analysis supported our visual observation that the mitochondria of spf^{ash}/mOTC mice were larger than those in untreated spf^{ash} or spf^{ash}/hOTC mice.

Mitochondrial ATPase(c) and CPSI Concentration Were Corrected in spf^{ash} Mice following Adenovirus-Mediated Gene Transfer

Labeling densities for ATPase(c) and CPSI within mitochondria, cytosol, and nuclei were also evaluated in hepatocytes of spf^{ash}/mOTC, spf^{ash}/hOTC, untreated spf^{ash}, and C3H control mice. The purpose of this analysis was to examine whether the intracellular concentrations of these proteins were altered by correction of the OTC deficiency by gene therapy. Electron microscopy revealed that concentration of ATPase(c) was lower in mitochondria of untreated spf^{ash} hepatocytes (15.8 ± 14.2 gp/μm^2) in comparison to that in C3H control mice

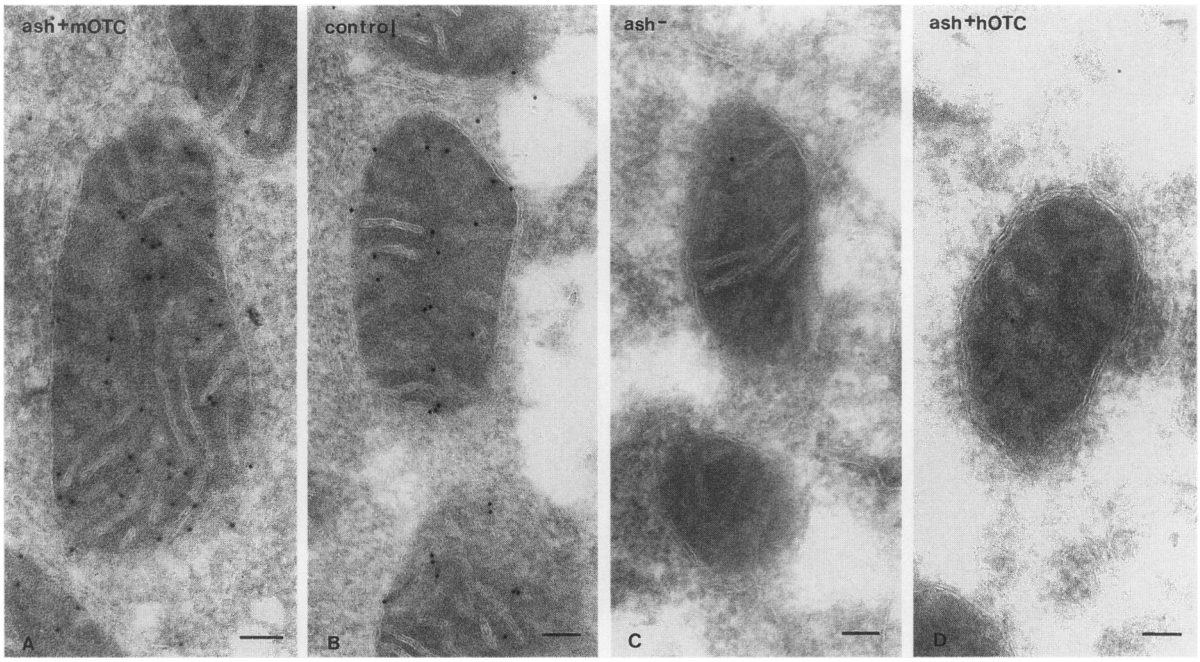

Fig. 2. OTC labeling in mitochondria of virus-treated and untreated mice. Ultrathin frozen liver sections from a spf^{ash} mouse treated with Ad.mOTC (A), a control C3H mouse (B), an untreated spf^{ash} mouse (C), and a spf^{ash} mouse treated with Ad.hOTC (D) were labeled by the rabbit anti-OTC antibody followed with gold-conjugated goat anti-rabbit antisera. Representative mitochondria are shown from each mouse. Bars, 0.1 μm.

(23.2 ± 26.2 gp/μm^2, $p < 0.001$; Figs. 6 and 8). Mitochondrial ATPase(c) labeling densities were significantly increased in spf^{ash}/mOTC hepatocytes (23.6 ± 16.7 gp/μm^2, $p < 0.001$), but remained unchanged in spf^{ash}/hOTC hepatocytes (17.0 ± 13.8 gp/μm^2; Figs. 6 and 8). The numbers of gold particles labeling ATPase(c) within the whole mitochondrial compartment of hepatocytes were 85,459 in untreated spf^{ash} mice, 126,042 in C3H control, 190,828 in spf^{ash}/mOTC, and 96,764 in spf^{ash}/hOTC mice. No significant differences were observed in cytosol and nuclei ATPase(c) densities among the four groups of animals.

Visualization of CPSI labeling in the electron microscope revealed a moderately increased number of gold particles in mitochondria of untreated spf^{ash} hepatocytes compared to those in C3H control hepatocytes (Fig. 7). Quantitative evaluation showed that mitochondrial CPSI labeling density was 75.6 ± 44.9 gp/μm^2 in untreated spf^{ash} hepatocytes, 56.9 ± 32.9 gp/μm^2 in control hepatocytes, 70.6 ± 40.8 gp/μm^2 in spf^{ash}/mOTC, and 48.8 ± 38.8 gp/μm^2 in spf^{ash}/hOTC hepatocytes (Fig. 8). The differences in mitochondrial labeling between control and untreated spf^{ash} mice ($p < 0.001$) and control and Ad.mOTC treated mice ($p < 0.01$) were both significant.

Discussion

Our study provided further evidence that adenovirus-mediated gene therapy is able to correct the metabolic defect in the spf^{ash} mouse model of OTC deficiency. Ultrastructural analysis demonstrated efficient mitochondrial import of newly synthesized OTC protein in hepatocytes infected with recombinant adenovirus carrying the mouse OTC transgene. This supports previous studies demonstrating reconstitution of ureagenesis in the spf^{ash} mouse following gene transfer (5). Mitochondrial OTC concentrations achieved with the mOTC virus were distinctly higher than those observed with the hOTC virus. The low cytosolic concentration of vector-derived OTC within hepatocytes, which is similar to that of C3H control hepatocytes, suggested that OTC was rapidly translocated into mitochondria following gene transfer. The cytosolic OTC density in hepatocytes infected with the hOTC virus, however, was much higher than that in untreated spf^{ash} and spf^{ash} mice treated with the mOTC virus, an indication that a substantial fraction of human OTC precursors may not be transported into mitochondria. The total number of gold particles per spf^{ash}/hOTC hepato-

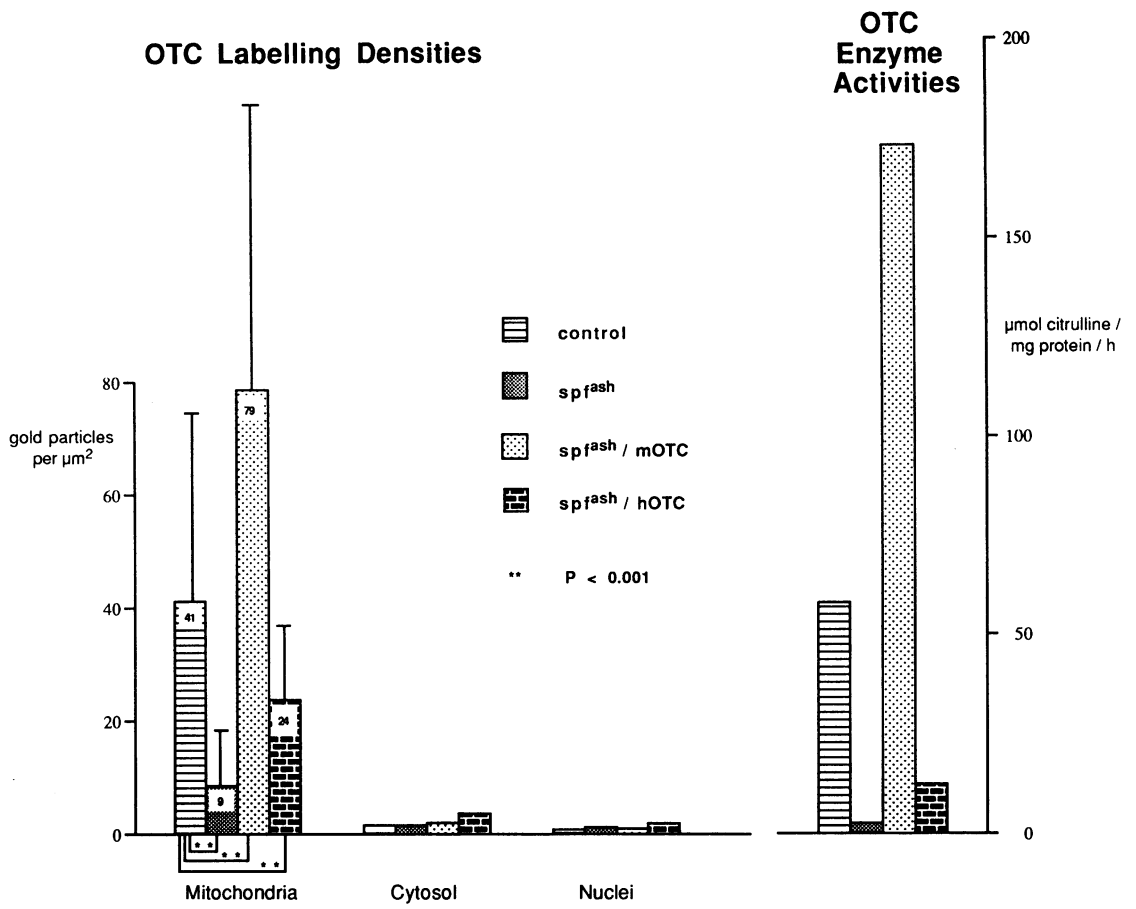

Fig. 3. OTC labeling densities in mitochondria, cytosol, and nuclei. OTC labeling densities were quantified in hepatic mitochondria, cytosol, and nuclei of control C3H mice, untreated spf^{ash} mice, and spf^{ash} mice treated with either Ad.mOTC or Ad.hOTC virus. Values were obtained by evaluating at least 150 mitochondria and 150 μm^2 area of nuclei and cytosol from each group. Liver OTC activities were determined by OTC lysate assay.

cyte was significantly larger than that of untreated spf^{ash} mice, but smaller than those of C3H control and spf^{ash}/mOTC mice. Considering the fact that OTC precursor has a half-life of 1–2 min in cytosol, it was possible that some of the human OTC precursors had been degraded in spf^{ash}/hOTC hepatocytes. It is interesting to note that the mitochondrial concentrations of OTC within hepatocytes in spf^{ash}/mOTC mice exceeded mitochondrial OTC concentrations in normal C3H hepatocytes, in agreement with the enzyme activity of homogenates. We found that the mitochondria in spf^{ash} hepatocytes were smaller than those in C3H mice and were returned to normal size after correction with the mOTC vector, a finding consistent with the mitochondrial uptake of newly synthesized OTC following transfection.

We further examined whether gene therapy was able to correct secondary alterations of OTC deficiency. It has been reported that CPSI activity is increased in spf mice (9,10) and either normal (9) or decreased (11) in spf^{ash} mice. In contrast to earlier reports, we found that mitochondrial CPSI concentration was significantly increased in untreated spf^{ash} mice, which is consistent with the findings in spf mice (9). The abnormally high levels of CPSI were partially corrected in mice treated with Ad.mOTC. Because CPSI is degraded with a half-life of 7.7 days (19), complete normalization of mitochondrial CPSI may be expected at a later time point following gene therapy. It was interesting that mitochondrial CPSI density was significantly reduced in spf^{ash}/hOTC mice, while the cytosol and nuclear labeling density were significantly higher in these mice

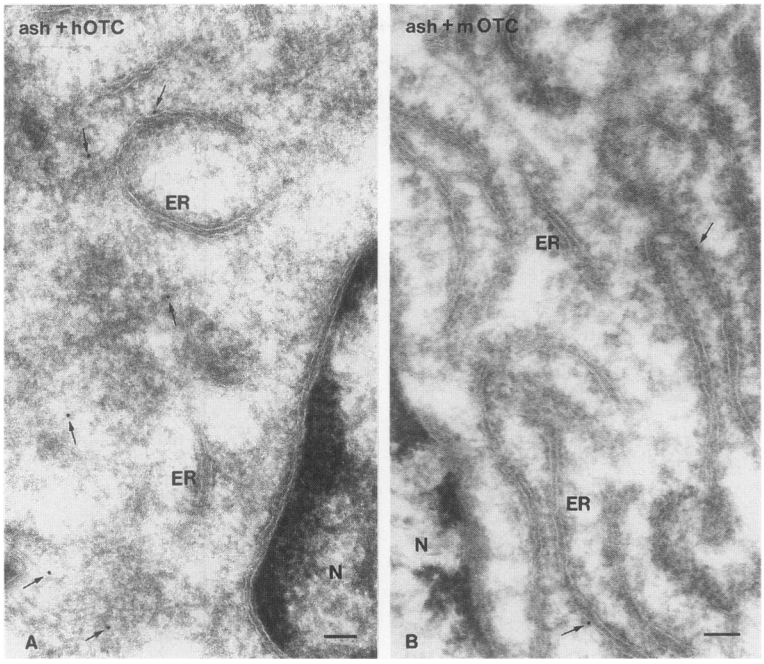

Fig. 4. OTC labeling in the cytosol of spf^{ash}/hOTC and spf^{ash}/mOTC hepatocytes. Ultrathin frozen liver sections from a spf^{ash} mouse treated with Ad.hOTC (A) and a spf^{ash} mouse treated with Ad.mOTC (B) were labeled by the rabbit anti-OTC antibody followed with gold conjugated goat anti-rabbit antisera. Arrows indicate OTC binding sites within the cytosol. ER, endoplasmic reticulum; N, nucleus. Bars, 0.1 μm.

in comparison to those in untreated spf^{ash} mice (Fig. 8). We suggest that human OTC precursors may interfere with the mitochondrial import of CPSI precursor and therefore result in the reduced mitochondrial but increased cytosolic and nuclear concentration of the enzyme.

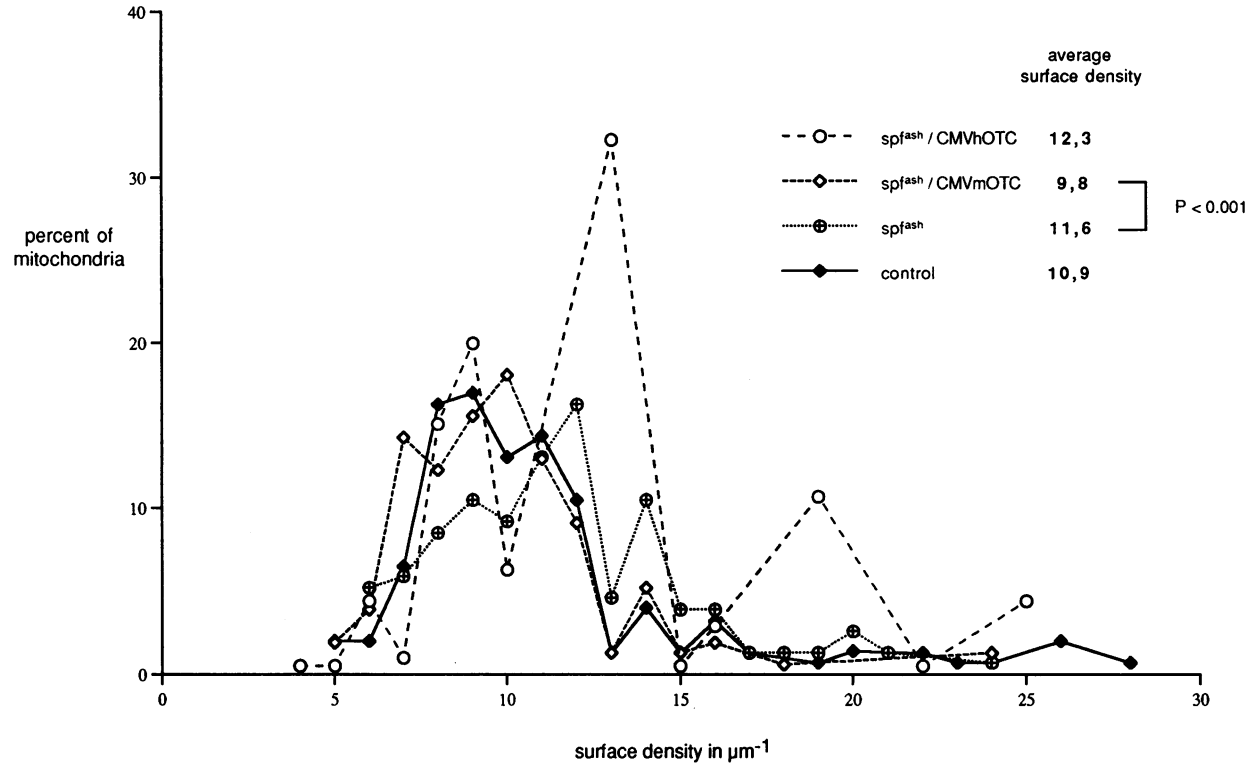

Fig. 5. Distribution of mitochondrial surface density. Surface densities of hepatic mitochondria from C3H control, untreated spf^{ash}, and vector-treated spf^{ash} mice were measured as previously described (18). At least 150 mitochondria were evaluated in each group.

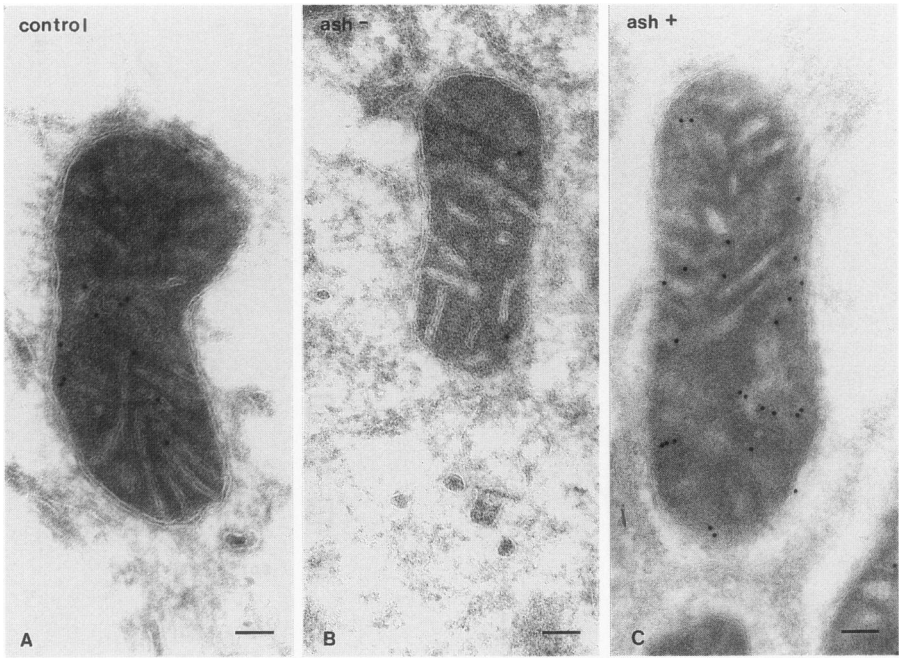

Fig. 6. ATPase(c) labeling in hepatic mitochondria of virus-treated and untreated mice. Ultrathin frozen liver sections from a control C3H mouse (A), an untreated spf^{ash} mouse (B), and a spf^{ash} mouse treated with Ad.mOTC (C) were labeled by rabbit anti-ATPase(c) antibody followed with gold-conjugated goat anti-rabbit antisera. Representative mitochondria are shown from each mouse. Bars, 0.1 μm.

Butterworth et al. (7,8) have reported ATP depletion in liver and brain of spf mice, which is related to dysfunction of the central nervous system in OTC deficiency. We reasoned that a chronic ATP depletion may result in the down-regulation of ATPase in spf^{ash} hepatocytes. We therefore measured the mitochondrial concentration of ATPase(c) in spf^{ash} mice and found that hepatic mitochondrial ATPase(c) was significantly lower in spf^{ash} mice than in normal C3H mice. This is in agreement with the earlier observation that ATP is depleted in the liver of spf mice. The ATPase(c) density was increased to normal levels in spf^{ash} mice treated with mOTC virus. Taking the increase of mitochondrial volume into account, the ATPase(c) content in mOTC-treated spf^{ash} hepatocytes exceeded that in normal C3H hepatocytes. Therefore, it is pos-

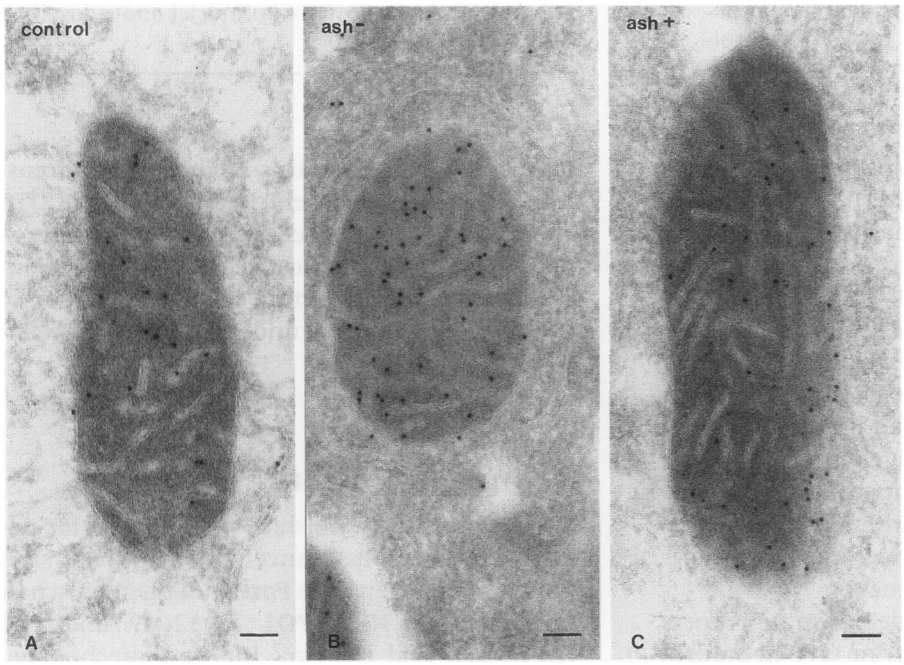

Fig. 7. CPSI labeling in hepatic mitochondria of virus-treated and untreated mice. Ultrathin frozen liver sections from a control C3H mouse (A), an untreated spf^{ash} mouse (B), and a spf^{ash} mouse treated with Ad.mOTC (C) were labeled by rabbit anti-CPSI antibody followed with gold-conjugated goat anti-rabbit antisera. Representative mitochondria are shown from each mouse. Bars, 0.1 μm.

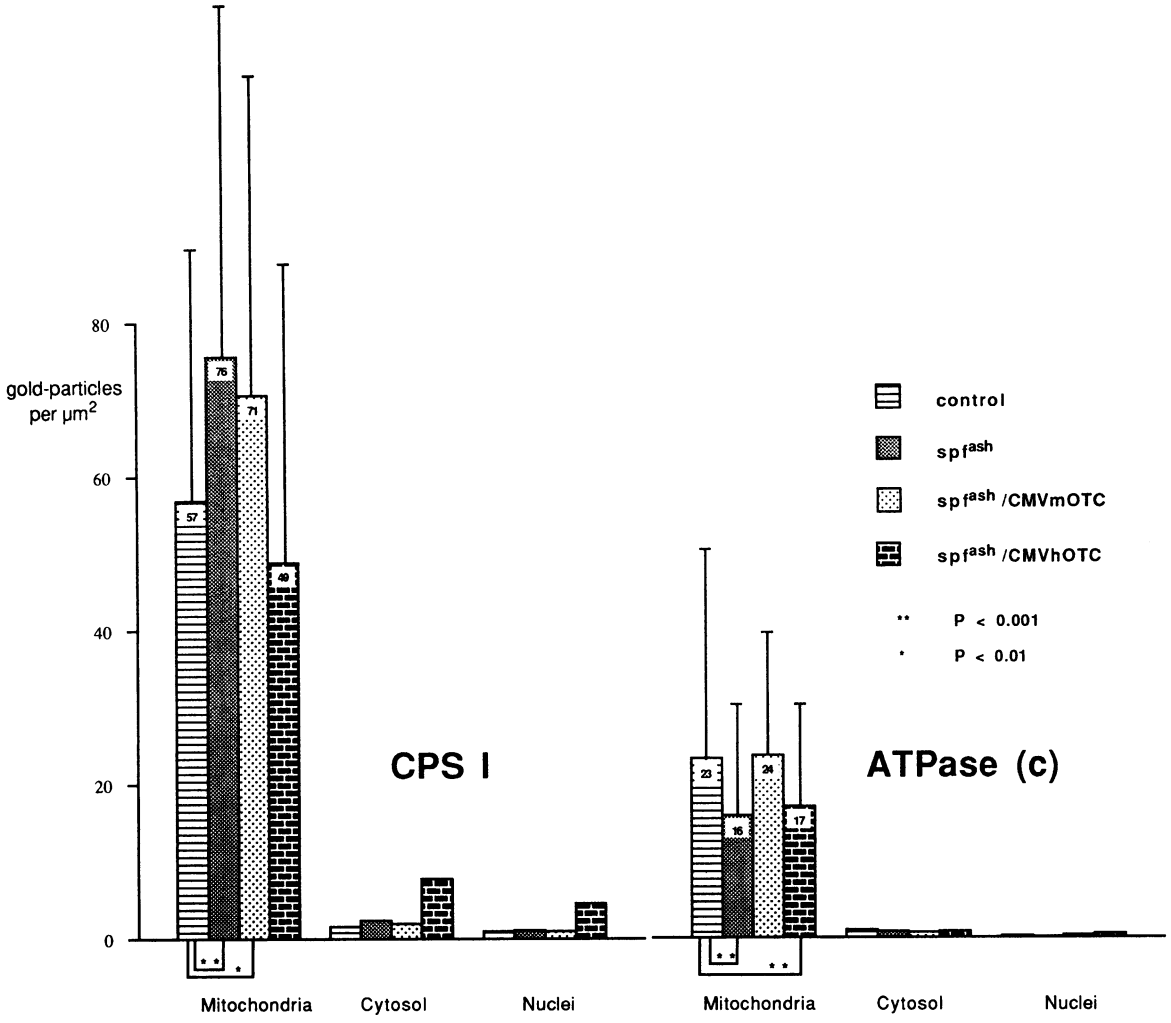

Fig. 8. Mitochondria, cytosol, and nuclei labeling densities of ATPase(c) and CPSI. ATPase(c) and CPSI labeling densities were quantified in hepatic mitochondria, cytosol, and nuclei of control C3H mice, untreated spf^{ash} mice, and spf^{ash} mice treated with either Ad.mOTC or Ad.hOTC virus. Values were obtained by evaluating at least 150 mitochondria and 150 μm^2 area of nuclei and cytosol from each group.

sible that gene therapy may correct the ATP depletion observed in spf^{ash} mice. It is conceivable that the enhanced mitochondrial synthesis of ATPase(c) may also contribute to the increased size of mitochondria in spf^{ash}/mOTC hepatocytes. Taken together, our data indicate that the benefit of gene therapy may include the correction of the hepatic (and possibly the cerebral) energy metabolism. Because an impaired energy metabolism seems to be involved in the irreversible neurotoxic defects following hyperammonemia (7,8), the ability of gene therapy to improve energy metabolism further underlines its efficacy in the therapy of OTC deficiency.

In summary, our studies demonstrate proper biogenesis of recombinant murine OTC in mouse liver following adenovirus-mediated gene transfer. This process is less efficient with human OTC expressed in mouse liver, presumably because of differences in amino acid sequence. The potential of adenovirus vectors in the treatment of urea cycle disorders is under evaluation in clinical trials (20).

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