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Research Article

Nuclear Expression of a Mitochondrial DNA Gene: Mitochondrial Targeting of Allotopically Expressed Mutant ATP6 in Transgenic Mice

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Nuclear encoding of mitochondrial DNA transgenes followed by mitochondrial targeting of the expressed proteins (allotopic expression; AE) represents a potentially powerful strategy for creating animal models of mtDNA disease. Mice were created that allotopically express either a mutant (A6M) or wildtype (A6W) *mt-Atp6* transgene. Compared to non-transgenic controls, A6M mice displayed neuromuscular and motor deficiencies (wire hang, pole, and balance beam analyses; $P < 0.05$), no locomotor differences (gait analysis; $P < 0.05$) and enhanced endurance in Rota-Rod evaluations ($P < 0.05$). A6W mice exhibited inferior muscle strength (wire hang test; $P < 0.05$), no difference in balance beam footsteps, accelerating Rota-Rod, pole test and gait analyses; ($P < 0.05$) and superior performance in balance beam time-to-cross and constant velocity Rota-Rod analyses ($P < 0.05$) in comparison to non-transgenic control mice. Mice of both transgenic lines did not differ from non-transgenic controls in a number of bioenergetic and biochemical tests including measurements of serum lactate and mitochondrial MnSOD protein levels, ATP synthesis rate, and oxygen consumption ($P > 0.05$). This study illustrates a mouse model capable of circumventing *in vivo* mitochondrial mutations. Moreover, it provides evidence supporting AE as a tool for mtDNA disease research with implications in development of DNA-based therapeutics.

1. Introduction

Throughout mitochondrial evolution, gene transfer from the mitochondrial compartment to the nucleus has been an ongoing process [1]. Through AE, this phenomenon is replicated in the laboratory. AE gene therapy was postulated as a strategy for correcting diseases involving mitochondrial DNA (mtDNA) mutation [2, 3] and as a means to overcome the scarcity of animal models for diseases caused by mitochondrial DNA mutations [4, 5]. AE was shown to occur in cultured cells [6–9] and in somatic tissues following delivery via viral vector [10, 11]. Here, we report the first germline competent transgenic model of AE.

Transversion of T to G at position 8993 of the human mitochondrial genome causes substitution of arginine for a conserved leucine in residue 156 (L156R) of the mitochondrial encoded MT-ATP6 gene [12]. This gives rise to Neuropathy, Ataxia and Retinitis Pigmentosa (NARP) [13], and Maternally Inherited Leigh Syndrome (MILS) [14] disease states. Expression of a recoded mutated *Atp6* in mice was postulated to bring about mitochondrial and functional deficits similar to those seen in human patients. To test this hypothesis, two versions of a nuclear-coded mouse *Atp6* gene element were expressed in transgenic mice. Our initial transgenic mouse model coded for the protein sequence of the wild-type murine *Atp6*. The second model encoded the same sequence with the exception of amino acid 156 where leucine was replaced with an arginine residue. Mitochondrial physiology and motor function were assessed in resultant transgenic lineages.

2. Materials and Methods

2.1. Gene Synthesis. Two forms of the nuclear-coded mitochondrial *Atp6* gene were synthesized *de novo*. Gene

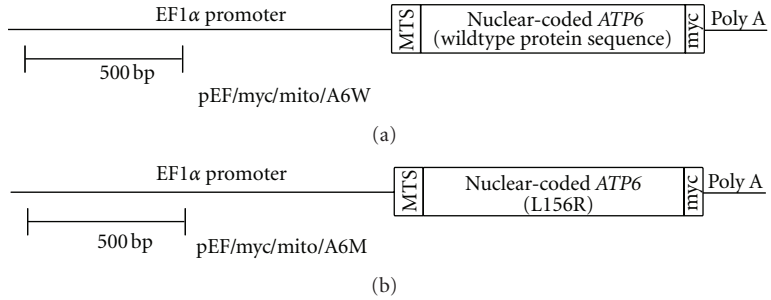

Figure 1: Schematic representation of AE DNA constructs. High-level transcription is driven by the human EF1α promoter. Protein-coding elements include the N-terminal mitochondrial transport signal of human cytochrome oxidase VIII, nuclear-coded wild-type (a) or mutant (b) mouse Atp6 gene sequence and a C-terminal myc epitope tag.

synthesis was performed using a three-step polymerase chain reaction (PCR)-based technique [15]. *Atp6* genes were synthesized coding for murine wild-type and mutated amino acid sequences (L156R) using nuclear codons. Oligonucleotides/primers (25nt) spanned forward and reverse sequences to be synthesized.

2.2. Plasmid Cloning/Transgenic Mouse Production. Synthesized *Atp6* DNAs were cloned in frame into the pEF/ *myc*/ mito plasmid (Invitrogen). Elements in this expression system include the promoter for human EF-1α [16], the mitochondrial targeting sequence from the human cytochrome c oxidase subunit VIII gene[17], and an in-frame 3′ myc epitope tag [18] (Figure 1). This gave rise to two plasmids, pEF/myc/mito/A6W and pEF/myc/mito/A6M procedures for generating transgenic mice were as described [19]. A6M and A6W transgenic mice were produced on C57BL/6 and B6(B6SJLF1) genetic backgrounds, respectively. Mice were genotyped by PCR analysis; forward and reverse primers were 5′tggccattccactatggg3′and 5′gatggctggcaactagaagg3′; expected amplification product size was 473 bp. All mouse procedures were approved by the Auburn University Institutional Animal Care and Use Committee.

2.3. Transmission Electron Microscopy. Electron microscopy was performed to analyze mitochondrial localization of allotopically expressed ATP6. Anaesthetized mice were perfused with 3% paraformaldehyde and 0.2% glutaraldehyde. Striatum was diced into 1-2 mm pieces and fixed in the same perfusion solution for 30–40 min. Tissue was rinsed, dehydrated in a graded ethanol series, and embedded in LR White Medium Grade Resin (Electron Microscopy Sciences). Immune labeling of sections utilized 1 : 4000 rabbit polyclonal anti-myc antibody (Abcam) with 1 : 100 donkey anti-rabbit Ultrasmall gold (Aurion) (diameter of average gold cluster <0.8 nm) as secondary label. R-Gent SE-EM (Aurion) was utilized for silver enhancement of samples.

2.4. Motor Assays. Forty mice were subjected to all motor tests. Groups of 5 males and 5 females were hemizygous A6M and nontransgenic (C57BL/6), hemizygous A6W and nontransgenic (B6(B6SJL)). All neuromuscular and motor tests followed a paradigm outlined earlier [20] and as detailed below. Motor-testing analyses were initiated at weaning (21 days of age) and progressed until completion in the order described below. All neuromuscular and motor tests were performed blinded as to the genotype of the mouse being tested. Motor testing commenced with the wire hang test, followed by the balance beam test, constant velocity Rota-Rod, accelerating Rota-Rod, pole test, and gait test.

2.5. Wire Hang Test. Neuromuscular strength was measured using a wire hang test [20]. Latency to fall was recorded with a maximum time of 240 sec. Experimental data collection began immediately without mouse training sessions. Twelve trials were completed for each mouse consisting of four trials per day over a three-day period; each trial was separated by at least 20 min.

2.6. Balance Beam Test. Motor coordination was tested using a balance beam [21]. Six beams of different sizes included three square beams with 28, 12, and 5 mm widths and three round beams with 27, 17, and 11 mm diameters. Data were collected in two trials for each of the six beams. Latency to cross the beam into the escape box and footslips was recorded.

2.7. Rota-Rod Test. Two analyses were performed using a Rota-Rod apparatus [20]. In the first analysis, mice ran on the Rota-Rod at a constant rotational velocity of 36 RPM for a maximum of 240 sec. In the second, rotational velocity gradually increased from 5 to 40 RPM over a period of 300 sec. In both analyses, latency to fall was measured. Data collection proceeded over a three-day period with four trials performed each day separated by 30 min intervals.

2.8. Pole Test. Motor coordination was evaluated with a pole test apparatus [20]. Mice were placed head-up near the top of the pole. Latency to turn 180 degrees and reach the base of the 50 cm pole was measured with a maximum time of 240 sec. Data collection continued over the course of three days with four trials performed each day separated by 15 min intervals.

2.9. Gait Analysis. Gait was assessed as described [21]. Nontoxic paint was applied to paws, and the mice were allowed to run the length of a 50 cm chute at a 30° incline into an escape box. Distances between footfalls were measured.

2.10. Biochemical Assays. Following motor testing, all mice were subjected to a series of biochemical tests. ATP synthesis and respirometry assays were performed the same day as mitochondrial isolation; remaining mitochondria and sera were frozen for lactate and SOD measurements. Mitochondrial isolation and ATP synthesis and respirometry tests were performed on 10 groups of four mice each. Each group contained one A6M hemizygous, one A6M nontransgenic, one A6W hemizygous, and one A6W nontransgenic mouse. A6M transgenic and wild-type mice were euthanized and samples taken at a mean age of 58.7 ± 1.3 and 57.8 ± 1.6 weeks, and at 28.9 ± 5.3 and 46.0 ± 3.3 weeks for A6W transgenic and wild-type mice, respectively.

2.11. Mitochondrial Isolation. Mitochondria were isolated from whole brain, heart, and gastrocnemius muscle as previously reported [22].

2.12. Oxygen Consumption. Aerobic respiration of isolated mitochondria was measured using MitoXpress A65N-1 (Luxcel) [23] in 96 well plates with an Infinite M200 plate reader (Tecan). 100 μg mitochondria were incubated with 12.5 mM of glutamate and malate (States II and III) and 1.65 mM ADP (State III only) in a total volume of 100 μL. State II values were obtained by measuring oxygen consumption in the absence of ADP; State III was measured with ADP present. Respiratory control ratio (RCR) values were calculated by dividing State III values by State II values.

2.13. ATP Synthesis Assay. ATP production rate was measured using the chemiluminescent ATP-consuming reaction of Luciferase-Luciferin [24]. ATP consumption values were calculated by comparing luminescence of a standard ATP concentration curve to luminescent kinetics in the experimental reaction.

2.14. MnSOD Protein Levels. Protein measurements of manganese superoxide dismutase (MnSOD, SOD2) were generated with a SOD2 Protein Quantity Microplate Assay Kit (MitoSciences) ELISA.

2.15. Serum Lactate Assay. Lactate was measured in serum of all experimental mice using the Lactate Colorimetric Assay Kit (Abcam). All samples were measured in duplicate.

2.16. Statistical Analyses. All statistical analyses were performed using SAS software (SAS Institute). Hazard ratios (HRs) with 95% confidence intervals were generated for all measurements that produced censored values (wire hang, balance beam time, Rota-Rod and pole measurements) by proportional hazards regression analysis using PROC PHREG. Analysis of gait measurements was performed with repeated measures modeling using PROC MIXED. *P* values of gait measurements were manually corrected using the Bonferroni method. Odds ratios (OR) were calculated with 95% confidence intervals for balance beam slips analyses with logistic regression using PROC LOGISTIC. *t*-test analyses of oxygen consumption, ATP synthesis, MnSOD and lactate measurements were performed using Proc *t*-test. All data subjected to *t*-test analysis were presented as mean ± SE.

3. Results and Discussion

Two synthesized, nuclear-coded ATP6 genes were each cloned into vectors containing a high-level constitutive EF-1α promoter, mitochondrial transport signal, myc epitope tag, and polyadenylation signal (Figure 1).

The A6M expression construct was injected into 263 C57BL/6 and 97 B6(B6SJLF1) embryos from which 3 C57BL/6 and 7 B6(B6SJLF1) transgenic founder mice were derived. The A6W expression construct was injected into 112 C57BL/6 and 78 B6(B6SJLF1) embryos from which 0 C57BL/6 and 5 B6(B6SJLF1) transgenic founder mice resulted. One line of transgenic mice for each construct was selected for further characterization based on transgene expression and fertility in founder transgenic mice.

Allotopically expressed proteins from both A6M and A6W lineages were found to colocalize with mitochondria in electron micrographs of striatum sections (Figure 2). Thus, nuclear expression of a mitochondrial gene can result in mitochondrial localization of the cytoplasmically translated protein.

A6M and A6W mice were compared to nontransgenic control mice in a series of neuromuscular and motor assays (Table 1). In wire hang testing, A6M and A6W mice did not perform as well as nontransgenic control mice (A6M: $P = 0.0008$; A6W: $P = 0.002$). Analysis of the time to cross balance beams into an escape box showed that A6M mice performed inferior to nontransgenic controls ($P = 0.0004$), while A6W mice displayed superior performance compared to control mice $P = 0.0067$. Analysis of foot slips in balance beam testing indicated a greater degree of slips by A6M mice than control mice ($P = 0.0191$), but no differences were seen in A6W mice ($P = 0.6305$). A6M and A6W mice both outperformed their respective controls in Rota-Rod testing at a constant rotational velocity (A6M: $P < 0.0001$; A6W: $P = 0.0002$). A6M animals displayed superior performance in accelerating Rota-Rod analyses ($P < 0.0001$) while A6W mice were not different from their nontransgenic counterparts ($P = 0.72$). In the pole test, A6M mice did not perform as well as controls ($P = 0.022$) and A6W mice were not different than controls ($P = 0.46$). Gait analyses did not detect any gait differences in either transgenic line (Bonferroni-corrected *P* values range from 0.36 to 1.0 over several measures).

Of the parameters measured in this group of tests (wire hang, beam time, beam slips, constant Rota-Rod, accelerating Rota-Rod, pole test, gait), A6M mice displayed performance inferior to control mice in four out of seven tests while A6W mice displayed inferior performance to their controls in a single analysis. Additionally, both A6M and

Table 1: Motor and biochemical analyses of A6M and A6W transgenic mice. Hazard and odds ratios are expressed with 95% confidence intervals (95% CI) and P values. Biochemical data are expressed as mean ± standard error of the mean (SE).

	A6M	A6W
Wirehang hazard ratio (95% CI)	HR: 0.54 (0.376–0.770) $P = 0.0008$	HR: 0.424 (0.246–0.731) $P = 0.002$
BB slips/crossing odds ratio (95% CI)	OR: 0.12 (0.021–0.709) $P = 0.0191$	OR: 0.68 (0.141–3.276) $P = 0.6305$
BB time hazard ratio (95% CI)	HR: 1.72 (1.274–2.309) $P = 0.0004$	HR: 0.67 (0.506–0.896) $P = 0.0067$
Constant Rota-Rod hazard ratio (95% CI)	HR: 2.28 (1.722–3.030) $P < 0.0001$	HR: 2.09 (1.414–3.097) $P = 0.0002$
Accelerating Rota-Rod hazard ratio (95% CI)	HR: 2.37 (1.589–3.523) $P < 0.0001$	HR: 1.09 (0.681–1.739) $P = 0.72$
Poletest hazard ratio (95% CI)	HR: 1.50 (1.156–1.941) $P = 0.0022$	HR: 1.10 (0.860–1.399) $P = 0.46$
Gait		
LF-LF	$P = 1.0$	$P = 1.0$
LF-LR	$P = 1.0$	$P = 1.0$
LF-RF	$P = 1.0$	$P = 1.0$
LR-LR	$P = 1.0$	$P = 1.0$
LR-RR	$P = 1.0$	$P = 1.0$
RF-RF	$P = 1.0$	$P = 1.0$
RF-RR	$P = 1.0$	$P = 1.0$
RR-RR	$P = 0.36$	$P = 1.0$
Brain state II (nmol O_2/min/mg protein ± SE)	Transgenic: 10.1 ± 1.2 Nontransgenic: 8.9 ± 1.9 $P = 0.6094$	Transgenic: 7.5 ± 0.7 Nontransgenic: 8.5 ± 0.8 $P = 0.3149$
Heart state II (nmol O_2/min/mg protein ± SE)	Transgenic: 20.2 ± 1.3 Nontransgenic: 21.5 ± 1.6 $P = 0.5291$	Transgenic: 21.1 ± 1.3 Nontransgenic: 21.1 ± 1.2 $P = 0.9634$
Skeletal muscle state II (nmol O_2/min/mg protein ± SE)	Transgenic: 14.5 ± 1.7 Nontransgenic: 16.3 ± 1.8 $P = 0.4672$	Transgenic: 15.4 ± 1.3 Nontransgenic: 14.6 ± 1.2 $P = 0.6687$
Brain state III (nmol O_2/min/mg protein ± SE)	Transgenic: 24.2 ± 2.7 Nontransgenic: 23.1 ± 1.2 $P = 0.7128$	Transgenic: 20.3 ± 1.1 Nontransgenic: 24.0 ± 2.2 $P = 0.1508$
Heart state III (nmol O_2/min/mg protein ± SE)	Transgenic: 31.4 ± 2.0 Nontransgenic: 33.1 ± 3.0 $P = 0.5965$	Transgenic: 31.6 ± 2.1 Nontransgenic: 32.8 ± 1.9 $P = 0.6910$
Skeletal muscle state III (nmol O_2/min/mg protein ± SE)	Transgenic: 35.1 ± 3.5 Nontransgenic: 34.7 ± 4.0 $P = 0.9438$	Transgenic: 34.4 ± 3.7 Nontransgenic: 31.0 ± 2.6 $P = 0.5882$
Brain respiratory control ratio	Transgenic: 2.48 ± 0.18 Nontransgenic: 3.19 ± 0.41 $P = 0.1458$	Transgenic: 2.84 ± 0.21 Nontransgenic: 3.01 ± 0.41 $P = 0.7192$
Heart respiratory control ratio	Transgenic: 1.57 ± 0.08 Nontransgenic: 1.53 ± 0.05 $P = 0.6707$	Transgenic: 1.51 ± 0.06 Nontransgenic: 1.58 ± 0.11 $P = 0.5530$
Skeletal muscle respiratory control ratio	Transgenic: 2.59 ± 0.26 Nontransgenic: 2.16 ± 0.12 $P = 0.1595$	Transgenic: 2.24 ± 0.17 Nontransgenic: 2.20 ± 0.11 $P = 0.8709$

Table 1: Continued.

	A6M	A6W
Brain ATP synthesis (nmol/min/mg protein)	Transgenic: 60 ± 2.0 Nontransgenic: 64 ± 6.7 $P = 0.5991$	Transgenic: 65 ± 7.9 Nontransgenic: 62 ± 4.9 $P = 0.7143$
Heart ATP synthesis (nmol/min/mg protein)	Transgenic: 64 ± 10.8 Nontransgenic: 58 ± 5.7 $P = 0.6006$	Transgenic: 68 ± 8.3 Nontransgenic: 65 ± 4.7 $P = 0.7549$
Skeletal muscle ATP synthesis (nmol/min/mg protein)	Transgenic: 36 ± 2.9 Nontransgenic: 40 ± 3.6 $P = 0.4167$	Transgenic: 38 ± 2.1 Nontransgenic: 37 ± 2.3 $P = 0.6902$
Brain MnSOD protein levels (arbitrary colorimetric values)	Transgenic: 4.6 ± 0.66 Nontransgenic: 5.2 ± 0.47 $P = 0.4098$	Transgenic: 5.4 ± 1.11 Nontransgenic: 4.2 ± 0.39 $P = 0.3514$
Heart MnSOD protein levels arbitrary colorimetric values)	Transgenic: 3.7 ± 0.11 Nontransgenic: 4.0 ± 0.15 $P = 0.2691$	Transgenic: 4.6 ± 0.33 Nontransgenic: 4.2 ± 0.13 $P = 0.2569$
Skeletal muscle MnSOD protein levels (arbitrary colorimetric values)	Transgenic: 5.1 ± 0.93 Nontransgenic: 5.0 ± 0.57 $P = 0.9361$	Transgenic: 3.9 ± 0.35 Nontransgenic: 3.9 ± 0.46 $P = 0.9590$
Serum lactate (mM)	Transgenic: 11.2 ± 0.83 Nontransgenic: 12.5 ± 0.83 $P = 0.2073$	Transgenic: 9.6 ± 0.51 Nontransgenic: 11.6 ± 0.73 $P = 0.0370$

LF: left front; LR: left rear; RF: right front; RR: right rear.

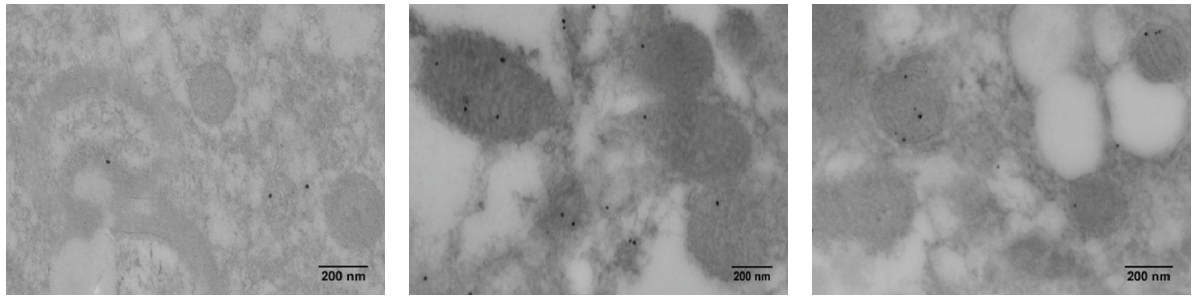

Figure 2: Mitochondrial localization of allotopically expressed ATP6 in brain sections of nontransgenic, A6M and A6W mice. The small amount of cytosolic nonmitochondrial staining is not unexpected as proteins are cytoplasmically translated prior to mitochondrial translocalization.

A6W mice displayed enhanced performance in two measures in comparison to their controls. The superior performance of A6M mice in all Rota-Rod analyses, while surprising, might be explained by differences in what is measured in each test on a physiological or tissue level. This discrepancy of A6M Rota-Rod results versus the other tests suggests that further study and characterization of the functional consequences of allotopic expression of mitochondrial genes is warranted.

A6M transgenic and wild-type mice were euthanized and samples taken at a mean age of 58.7 ± 1.3 and 57.8 ± 1.6 weeks ($P = 0.667$), and at 28.9 ± 5.3 and 46.0 ± 3.3 ($P = 0.014$) weeks for A6W transgenic and wild-type mice. Mitochondria from brain, heart and muscle tissues, and serum from the same mice that underwent motor testing were collected and subjected to a series of biochemical and physiological tests (Table 1). In oxygen utilization studies, State II, III, and RCR measurements showed no difference between mitochondria from A6M and A6W mice and their respective controls ($P > 0.05$). The rate of ATP synthesis in mitochondria isolated from A6M and A6W mice did not differ from that of mitochondria from nontransgenic control mice ($P > 0.05$). Protein levels of MnSOD in mitochondria also did not differ between either transgenic line and its respective control ($P > 0.05$). Serum concentrations of lactic acid were

not different between A6M mice and nontransgenic control mice ($P = 0.297$), but A6W hemizygous mice had lower lactate concentrations than nontransgenic control mice ($P = 0.037$). Dissimilarities in A6W serum lactate concentrations might be due to differences in age at time of euthanasia.

The absence of detectable differences in mitochondrial function between transgenic mice and their nontransgenic counterparts was somewhat surprising in light of the clear differences seen in motor function in A6M mice. These discrepancies may reflect a reduction of stress due to the lag time between the end of motor tests and commencement of mitochondrial isolation. Alternatively, levels of allotopically expressed L156R ATP6 protein in mitochondria might vary during an individual's lifespan such that differences in ATP synthesis that are undetectable in adult mice are of sufficient magnitude in fetal and/or postnatal development to cause a change in developmental trajectory that results in the functional differences observed. Future experiments on mice that undergo motor analysis and functional strain immediately prior to biochemical analysis might yield different results. The experiments reported here using genetically defined mice might yield different results with modified genetic and/or environmental variables.

4. Conclusion

While mtDNA mutations are primary etiologic agents in mitochondrial disease, pathogenic phenotypes are intensified or attenuated by numerous secondary factors including background mtDNA sequence [25], nuclear genetic and environmental influences [26]. The utility of the transgenic approach in allotopic expression of mitochondrial genes is shown here to be successful in delivery of protein derived from a nuclear transcript to mitochondria. Phenotypic characterization of mice carrying a mutant *ATP6* yielded mixed results. In some tests, functional motor deficiencies similar to those seen in human NARP patients were seen, while enhanced performance was observed in others.

The results of these experiments have implications for the potential future use of allotopic expression as a strategy for gene therapy. Nuclear expression of one or more mitochondrial genes in a clinical setting could improve mitochondrial function in the context of mitochondrial disease. This modeling might also provide an effective method for protecting the 13 genes encoded on the mitochondrial genome from the oxidative damage that results from normal aging, age-related neurodegenerative diseases and other pathological states shown to have mitochondrial involvement.

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