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—Original—

Nuclear but Not Mitochondrial DNA Involvement in Respiratory Complex I Defects Found in Senescence-Accelerated Mouse Strain, SAMP8

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Abstract: This study determined pathogenicity of an A11181G mtDNA mutation found in a senescence-accelerated mouse strain, SAMP8. The mutation was at a highly conserved site of the *mt-Nd4* gene, which encodes one of the respiratory complex I subunits. The young SAMP8 expressed reduced complex I activity, which is controlled by both nuclear and mitochondrial DNA (mtDNA). To exclude the nuclear effects, we isolated transmitochondrial cybrids that share the same nuclear background, but possess mtDNA with or without the mutation. The cybrids showed normal respiratory function irrespective of whether their mtDNA possessed the mutation or not, suggesting that the A11181G mutation is not responsible for respiration defects found in SAMP8.

Key words: an mtDNA missense mutation, mtDNA transfer technology, respiratory complex I defects, senescence-accelerated mouse (SAM), trans-mtDNA cybrids

Introduction

Pathogenic mtDNA mutations that induce mitochondrial respiration defects have been proposed to be responsible for mitochondrial diseases, aging, and various age-associated disorders [6, 9, 22]. In fact, age-associated accumulation of somatic mutations in mtDNA and age-associated expression of respiration defects have been reported in human subjects [6, 9, 22].

However, our group has previously proposed that age-associated respiration defects found in human skin fibroblasts are caused not by mtDNA mutations [1] but by nuclear-recessive mutations [4]. Moreover, the presence of mitochondrial interaction (i.e., the exchange of mitochondrial contents between mitochondria) in human cells probably prevents individuals from expressing respiration defects induced by mtDNA containing various somatic mutations accumulated with age [5, 17, 19]. Similar mitochondrial interaction has been reported in mouse tissues [14].

On the other hand, mtDNA mutator mice, generated by introduction of a mutation into the proofreading do-

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main of mtDNA polymerase, simultaneously show accelerated accumulation of somatic mutations in mtDNA, accelerated expression of respiration defects, and premature aging phenotypes when compared with wild-type mice [8, 21]. However, no direct evidence has been provided as to whether or not the respiration defects induce the premature aging phenotypes expressed in the mtDNA mutator mice.

Premature aging phenotypes and age-associated respiration defects are also observed in SAMP8, one of the aging-prone mouse strains derived from senescence-accelerated mouse (SAM) strains, but not in SAMR1, one of the aging-resistant strains derived from SAM strains [15, 16]. Moreover, whole mtDNA sequence analysis of SAMP8 and SAMR1 mice has shown that the former strain possesses a unique missense A11181G mutation in the *mt-Nd4* gene and that the mutation is homoplastic (i.e., all mtDNA molecules in a cell possess the mutation) [12]. This missense mutation could induce mitochondrial respiratory complex I defects because the *mt-Nd4* gene encodes NADH dehydrogenase subunit 4 (ND4), one of the subunits of the respiratory complex I, and the mutation site is highly conserved in most animals (Supplementary Table 1). However, if this mtDNA mutation was responsible for reduced complex I activity, then the reduction would not be expressed as an aging-associated phenotype; rather, it would be expressed from a young age in SAMP8 mice. Moreover, because respiratory complex I activity is controlled by both nuclear and mitochondrial DNA [9, 22], the involvement of nuclear abnormalities in the observed respiration defects in SAMP8 cannot be excluded.

We addressed these issues by examining the respiratory function of young SAMP8 mice and by means of mtDNA transfer technology to isolate transmitochondrial cells (cybrids) that shared the same nuclear genome backgrounds, but possessed mtDNA with and without the A11181G mutation from SAMP8 and SAMR1 mice, respectively.

Materials and Methods

Mice, cell lines and cell culture

We obtained 3-month-old male SAMP8/TaSlc (SAMP8) and SAMR1/TaSlc (SAMR1) mice from Japan SLC (Shizuoka, Japan) and C57BL/6JJcl (B6) strain mice from CLEA Japan (Tokyo, Japan). Mito-mice13997 were isolated in our previous study [23]. The P29 cells originated from B6 strain-derived Lewis lung carcinoma, ρ^0 P29 cells, and the transmitochondrial cells (cybrids) were grown in DMEM (Sigma, St. Louis, MO, USA) containing 10% fetal calf serum, uridine (50 mg/ml), and pyruvate (0.1 mg/ml).

Isolation of cybrids

As nuclear donors and mtDNA recipients for isolation of the cybrids, we used ρ^0 P29 cells, which were obtained by treatment of P29 cells with ditercalinium (1.5 μg/ml) to eliminate their own mtDNA [2]. Platelets from SAMP8 and SAMR1 mice were fused with ρ^0 P29 cells by polyethylene glycol, respectively. Fusion mixture was grown in a selection medium without uridine and pyruvate (UP-) that allows exclusive growth of the mtDNA-repopulated ρ^0 P29 cells (cybrids). Unfused ρ^0 P29 cells failed to grow in the selection medium due to significant respiration defects caused by the absence of mtDNA.

Genotyping of mtDNAs in the cybrids

To confirm transfer of mtDNAs from SAMP8 and SAMR1 mice into the P29mtSAMP8 and P29mtSAMR1 cybrids, respectively, RFLP analysis of the PCR products was carried out. For recognition of the A11181G mutation, a 194-bp fragment containing the 11181 site was amplified by PCR. The nucleotide sequences from nucleotide position (np) 11,102 to 11,125 (AAC AAT ACT AAT AAT CGC ACA TGG) and np 11,295 to 11,272 (CTA TTA GAT TGA TTG AAG GGG GTA) were used as oligonucleotide primers. Combination of the PCR-generated mutation with the A11181G mutation creates a restriction site for *Eag* I and generates 115-bp and 79-bp fragments on *Eag* I digestion. The restriction fragments were separated in 3% agarose gel.

Biochemical measurement of respiratory enzyme activities

Mitochondrial respiratory complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome *c* reductase), and complex IV (cytochrome *c* oxidase) are components of the electron

transport chain and are located in the mitochondrial inner membrane. Brain, kidney, and muscle from 3-month-old mice and cells in log-phase growth were used, and the respiratory complexes were assayed as described before [11]. Briefly, for estimation of complex I + III activity, NADH and cytochrome *c* (oxidized form) were used as substrates, and the reduction of cytochrome *c* was monitored by measuring absorbance at 550 nm. For estimation of complexes II + III activity, sodium succinate and cytochrome *c* (oxidized form) were used as substrates, and the reduction of cytochrome *c* was monitored as described above. For estimation of complex IV activity, cytochrome *c* (reduced form) was used as a substrate, and the oxidation of cytochrome *c* was measured at 550 nm.

Measurement of ROS production

Reactive oxygen species (ROS) generation was detected with 2'-,7'-dichlorofluorescein diacetate (DCFH-DA; Invitrogen, Carlsbad, CA, USA). Cells were incubated with DCFH-DA, washed twice, and then immediately analyzed by using a FACScan flow cytometer (Becton, Dickinson and Company, Mountain View, CA, USA).

Measurement of mtDNA copy number

The mtDNA copy number was measured by real-time PCR using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and QuantiTect SYBR Green PCR Kit (QIAGEN). Total cellular DNA was used as a template and was amplified with specific primer pairs for mtDNA and GAPDH. They were tested functionally in quantitative analysis, and the results were optimal. Melting curve analysis showed that there was no primer dimer formation. The mtDNA copy numbers per cell were calculated using GAPDH amplification as a reference for nuclear DNA content.

Statistical analysis

All analyses and experiments were repeated at least three times. Statistical analysis was performed to test whether there was a significant difference, using a 2-tailed unpaired Student's *t*-test, which follows a Student's *t*-distribution. All values are means ± SD, and values with $P<0.05$ were considered to differ significantly.

Results

Examination of respiratory function in tissues of young SAMP8 mice

If the A11181G mutation in the *mt-Nd4* gene of SAMP8 mice [12] is pathogenic, complex I defects would be expected to appear before the onset of aging. Therefore, we first examined mitochondrial respiratory complex activities in tissues from young (3-month-old) SAMP8 mice. As positive controls expressing normal complex I activity, we used tissues from age-matched SAMR1 mice and B6 strain mice, which do not possess the mutation. SAMR1 mice are derived from SAM strains, but they do not express aging-accelerated phenotypes [20] or age-associated respiration defects [15, 16]. As a negative control expressing respiratory complex I defects, we used tissues from mito-mice13997 [23], a mouse model that we recently generated by the introduction of pathogenic mtDNA with a G13997A mutation in the *mt-Nd6* gene [3].

SAMP8 mice showed significantly less complex I + III activity in the kidney (30% reduction; $P<0.05$) and muscle (20% reduction; $P<0.05$) when compared with the levels in the control SAMR1 mice; the same trend was observed in the brain, but it was not statistically significant. In contrast, the activities of complexes II + III and complex IV in SAMP8 mice were not significantly different from the levels in the SAMR1 mice in any of the tissues tested (Fig. 1). Since the activity of complexes II + III was not reduced in the SAMP8 tissues, we consider that the reduced activity of complexes I + III reflects reduced complex I activity alone. The different extent of the reduction of complex I + III activity among the tissues of SAMP8 mice (Fig. 1) could be explained, at least in part, by assuming variability among the tissues in the activity of the compensatory function, which is able to restore the mtDNA mutation-induced complex I defects [3, 18].

These results suggest that SAMP8 mice not only express respiration defects with age [15, 16] but also express complex I defects before aging. Thus, the A11181G mutation could be responsible for the complex I defects found in 3-month-old SAMP8 mice. However, because complex I consists of subunits encoded by both nuclear DNA and mtDNA [9, 22], it is still possible that muta-

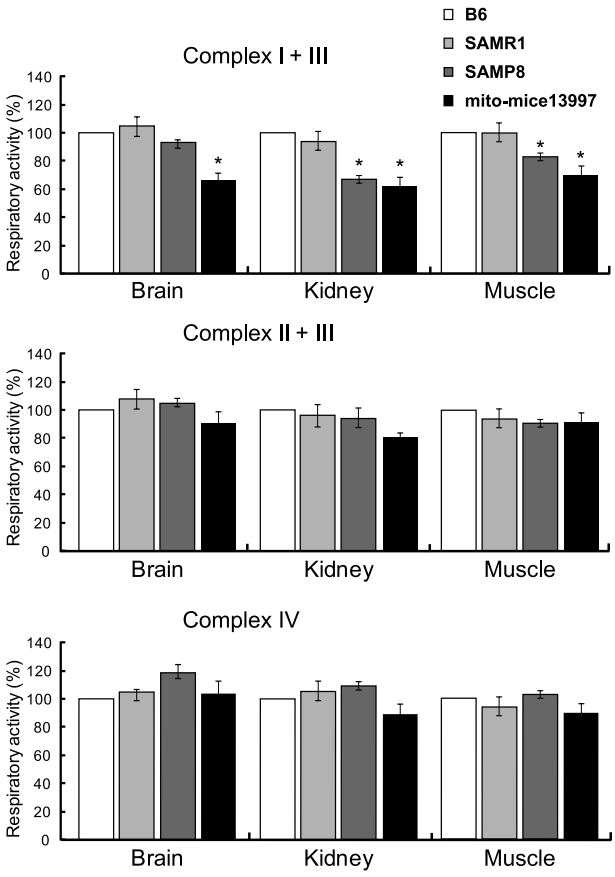

Fig. 1. Biochemical analysis of the respiratory complex activities in the tissues from young SAMR1 and SAMP8 mice. Respiratory complex I+III, II+III, and IV activities were examined using tissues of 3-month-old mice. B6 mice and mito-mice13997 were used as positive and negative controls that express normal and reduced complex I activity, respectively. Data are presented as mean values with the SD (n=3), **P*<0.05 compared with the control group.

tions in the other nuclear-coded subunits of complex I and/or nuclear-coded factors required for the proper assembly and stability of complex I [10] are responsible for the complex I defects.

Transfer of mtDNA from SAM mice into ρ⁰ cells

To address the issue above, we transferred mtDNA from SAMR1 and SAMP8 mice into mouse mtDNA-less (ρ^0) P29 cells, which we had isolated previously [3]. For the cytoplasmic transfer of mtDNA, the ρ^0 P29 cells were fused with platelets from the SAMR1 and SAMP8 mice because platelets do not contain nuclei. Cultivation of the fusion mixture in selection medium without uridine and pyruvate excluded unfused ρ^0 P29 cells and resulted in isolation of the transmitochondrial P29 cells (P29 cybrids), P29mtSAMR1 and P29mtSAMP8 (Table 1).

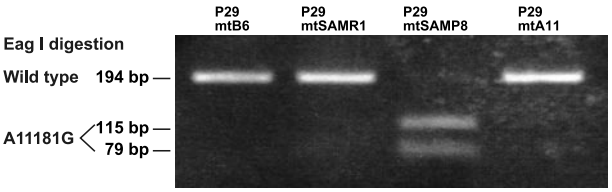

Fig. 2. The mtDNA genotyping of the cybrids. For identification of the A11181G mutation in mtDNA from SAMP8 mice, the PCR products were digested with restriction enzyme *Eag* I. The PCR products of the mtDNA with the A11181G mutation from SAMP8 produce 115-bp and 79-bp fragments due to the gain of an *Eag* I site by an A to G substitution at nucleotide position 11181, whereas those of mtDNA without the mutation produce a 194-bp fragment.

Table 1. Genetic characteristics of parent cells and the cybrids[a)]

Cells	mtDNA genotype	Fusion combination Nuclear donors × mtDNA donors	Selection
Nuclear donors			
ρ^0 P29	mtDNA-less		
mtDNA donors			
Platelets from SAMR1	Wild type		
Platelets from SAMP8	A11181G		
Cybrids			
P29mtSAMR1	Wild type	ρ^0 P29 × platelets from SAMR1	UP-
P29mtSAMP8	A11181G	ρ^0 P29 × platelets from SAMP8	UP-

[a)]Fusion mixture was grown in a selection medium without uridine and pyruvate (UP-) that allows exclusive growth of the mtDNA-repopulated ρ^0 P29 cells, P29mtSAMR1 and P29mtSAMP8. Platelets without nuclei cannot survive, and unfused ρ^0 P29 cells failed to grow in the selection medium due to significant respiration defects caused by the absence of mtDNA.

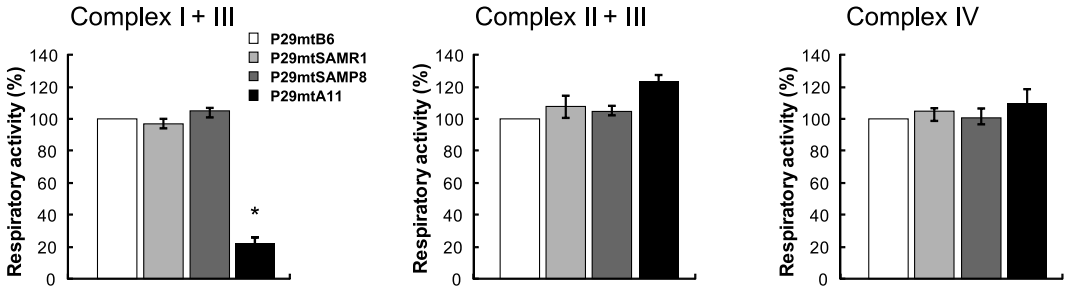

Fig. 3. Biochemical analysis of the respiratory complex activities in the P29mtSAMR1 and P29mtSAMP8 cybrids. Respiratory complex I+III, II+III, and IV activities were examined using the P29mtSAMR1 and P29mtSAMP8 cybrids. P29mtB6 and P29mtA11 cybrids were used as positive and negative controls that express normal and reduced complex I activity, respectively. Data are presented as mean values with the SD (n=3), **P*<0.05 compared with the control group.

To confirm the mtDNA transfer from SAMR1 and SAMP8 mice into the P29 cybrids, we examined the mtDNA genotype of the P29 cybrids by performing restriction enzyme digestion of PCR products amplified using mismatched primers (Fig. 2). The results showed repopulation of the ρ^0 P29 cells by the mtDNA introduced from SAMR1 or SAMP8 mice. Moreover, no fragments without the mutation were detectable in the P29mtSAMP8 cybrids (Fig. 2), suggesting that the mtDNA mutation was homoplasmic in the cybrids. In contrast, no mtDNA in the P29mtSAMR1 cybrids possessed the mutation (Fig. 2). Thus, the P29mtSAMR1 and P29mtSAMP8 cybrids, which shared the same nuclear background as P29 cells, possessed homoplasmic mtDNA without and with the A11181G mutation, respectively.

Respiratory activity in P29mtSAMP8 cybrids harboring the A11181G mutation

We then compared the mitochondrial respiratory complex activities in the P29mtSAMP8 and P29mtSAMR1 cybrids. As positive and negative controls, we used P29mtB6 and P29mtA11 cybrids, respectively. These cybrids share the P29 cell-derived nuclear genome, but P29mtB6 cybrids express normal complex I activity because they possess wild-type mtDNA from B6 mice, and P29mtA11 cybrids express reduced complex I activity because they possess pathogenic mtDNA with the G13997A mutation in the *mt-Nd6* gene [3].

The levels of all the complex activities in P29mtSAMP8 as well as in P29mtSAMR1 cybrids were not significantly different from those in the P29mtB6 positive controls (Fig. 3), suggesting that a normal level of activity was achieved irrespective of whether the cybrids carried mtDNA with the A11181G mutation or not. In contrast, P29mtA11 cybrids showed significant complex I defects because they carried pathogenic mtDNA with the G13997A mutation. These results suggest that the A11181G mutation would not be responsible for the complex I defects found in the tissues of young SAMP8 mice (Fig. 1).

ROS and mtDNA copy number of P29mtSAMP8 cybrids

A previous study [13] reported that insertion mutations that create a 10A repeat in the *mt-Tr* gene of mtDNA induce mild respiration defects that are not detectable by the conventional procedures used to estimate respiratory function; the presence of mild respiration defects was deduced from the detection of ROS overproduction and the resultant increase in mtDNA copy number, which was presumed to compensate for the mild respiration defects. Although the *mt-Tr* gene in both SAMP8 and SAMR1 mice contains an 8A repeat [12] and thus could not be responsible for the mild respiration defects, we considered that the A11181G mutation observed in the SAMP8 strain mice might induce a slight ROS overproduction and resultant increase in mtDNA copy number.

We examined this possibility by estimating the ROS level and mtDNA copy number in the cybrids (Fig. 4). ROS overproduction was not observed in the P29mtSAMP8 cybrids or in the P29mtSAMR1 cybrids (Fig.

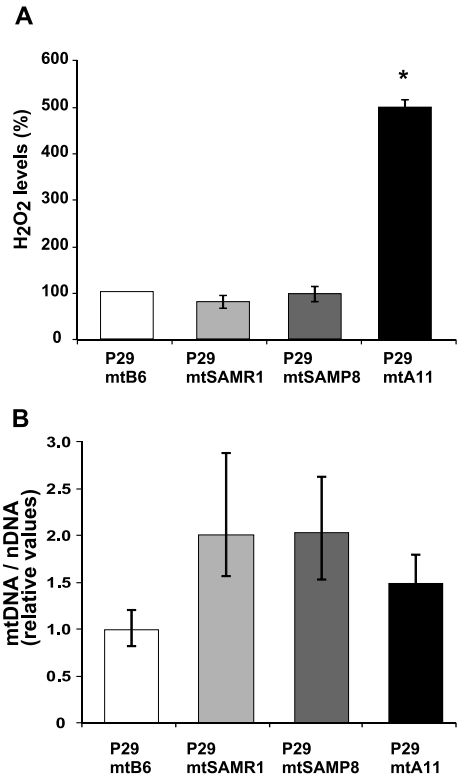

Fig. 4. Estimation of the amounts of ROS and mtDNA copy number in the P29mtSAMR1 and P29mtSAMP8 cybrids. P29mtB6 and P29mtA11 cybrids were used as negative and positive controls that express normal and enhanced ROS production, respectively. (A) Flow cytometric analysis of ROS production. We treated the cybrids with DCFH-DA and carried out flow cytometric analysis for quantitative estimation of H_2O_2. Data are presented as mean values with the SD (n=3). $*P<0.05$ compared with the control group. (B) RT-PCR analysis for estimation of mtDNA copy number.

4A); however, an increase in the mtDNA copy number was observed in both the P29mtSAMP8 and P29mtSAMR1 cybrids (Fig. 4B). Since P29mtSAMR1 cybrids, which do not possess the mutation, also showed a similar increase in mtDNA copy number, this increase could not be due to the A11181G mutation. Taken together, our results suggest that the A11181G mutation corresponds to a nondeleterious polymorphic mutation and that the complex I defects observed in the young SAMP8 mice (Fig. 1) are caused by nuclear abnormalities.

Discussion

The A11181G mutation in the mouse mitochondrial *mt-Nd4* gene is a missense mutation that leads to an amino acid substitution from Ser to Gly at position 339 (S339G) in the ND4 subunit of complex I [12]; the Ser residue is conserved at this position in most animals (Supplementary Table 1). The Arg residue at the next position (i.e., position 340) is also highly conserved, and mutations in human mtDNA that lead to a substitution of Arg to His at this position, and resultant complex I defects, are associated with Leber's hereditary optic neuropathy (LHON) [22]. Even though the young SAMP8 mice expressed mild complex I defects (Fig. 1), no LHON phenotypes have been observed in SAMP8 mice [12]. Our recent study [23] shows that mito-mice13997, which express significant complex I defects in every tissue (Fig. 1), does not express the LHON phenotypes, such as loss of the optic nerve and loss of both the nerve fiber layer and ganglion cell layer in the retina. It has been proposed that a mutation in either the *mt-Nd1*, *mt-Nd4*, or *mt-Nd6* gene of mtDNA is necessary, but not sufficient to express LHON phenotypes and that some additional nuclear gene mutations are required [9, 22]. Hence, even though the A11181G mutation is not responsible for the complex I defects observed in the young SAMP8 mice, we consider that the future addition of specific nuclear abnormalities could induce LHON phenotypes in the SAMP8 mouse model.

We reported previously that mtDNA-induced respiration defects expressed in cultivated P29 cybrids can be restored when the pathogenic mtDNAs are transferred into mouse tissues [7, 23]. This restoration is presumed to be a consequence of some compensatory function that is exclusively present in the tissues but not in cultivated P29 cybrids. For example, P29mtA11 and P29mt6589 cybrids, which possess a G13997A mutation in the *mt-Nd6* gene and a T6589C mutation in the *mt-Co1* gene, show approximately 20% complex I [3] and 40% complex IV activity [7], respectively, when compared with P29mtB6 cybrids. In contrast, tissues from the transmitochondrial mice, mito-mice13997 and mito-mice6589, which in turn possess G13997A and T6589C mtDNA introduced from the P29mtA11 and P29mt6589 cybrids, show approximately 70% complex I [23] and 70% com-

plex IV activity [7], respectively, when compared with age-matched B6 mice. Therefore, we expected that the A11181G mutation might express more profound complex I defects when it was reversely transferred from tissues of SAMP8 mice into cultivated P29 cybrids, in which the compensatory functions present in mouse tissues are not working efficiently. However, the resultant P29mtSAMP8 cybrids did not express complex I defects (Fig. 3); moreover, they did not show overproduction of ROS (Fig. 4), which has been reported to be a marker for the presence of mild respiration defects [13]. All these observations suggest that the A11181G mutation is polymorphic and that the reduction of complex I activity found in the young SAMP8 mice (Fig. 1) is due to nuclear abnormalities.

Another important respiration phenotype of SAMP8 mice is age-associated mitochondrial respiration defects [15, 16]. Our results suggest that this phenotype also would not be caused by the A11181G mutation because this mutation was found to be homoplasmic (Fig. 2) and thus would not accumulate further with age. However, considering the tissue-specific variation of complex I defects (Fig. 1), we cannot completely exclude the possibility that aging of the tissues in SAMP8 mice induces gradual but significant and tissue-specific disruption of the function that compensates the mtDNA-induced respiration defects, resulting in expression of the A11181G mutation-induced complex I defects in the SAMP8 tissues with age. Thus, further studies of SAMP8 mice are required to explain the exact mechanisms of the early onset of complex I defects and the late and age-associated onset of respiration defects.

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Supplementary Table 1. Multiple alignment of amino acid sequences of ND4 subunit

Species	Amino acid number								
	335	336	337	338	339	340	341	342	343
Mouse	E	R	I	H	**S**	**R**	T	M	I
SAMP8	E	R	I	H	**G**	**R**	T	M	I
Human	E	R	T	H	**S**	**R**	I	M	L
Rhesus monkey	E	R	T	H	**S**	**R**	T	M	L
Elephant	E	R	I	H	**S**	**R**	T	M	I
Horse	E	R	T	H	**S**	**R**	T	M	I
Duckbill	E	R	I	H	**S**	**R**	T	M	L
Flog	E	R	T	N	**S**	**R**	T	L	I
Zebrafish	E	R	T	H	**S**	**R**	T	M	I
Ascidian	K	R	Y	H	**S**	**R**	H	I	N
Drosophila	E	R	L	G	**S**	**R**	S	M	L
C. elegans	H	T	S	G	**S**	**R**	M	I	Y

Highly-conserved amino acid residues are shown in **bold**.