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Analysis of Mitochondrial DNA Somatic Mutations in OXYS and Wistar Strain Rats

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Abstract—Rats of the OXYS strain are sensitive to oxidative stress and serve as a biological model of premature aging. We have compared spectra of somatic mutations in a control region of mtDNA from the liver of the OXYS rat strain and of Wistar rats as a control. The majority of nucleotide substitutions in the mutation spectra were represented by transitions: 94 and 97% in the OXYS and Wistar rats, respectively. It was shown that 40% of somatic mutations in the control region of mtDNA from Wistar rats were significantly consistent with the model of dislocation mutagenesis. No statistical support for this model was found for mutations in the control region of mtDNA from OXYS rats. The mutation frequency in the ETAS section was higher in the OXYS strain rats than in Wistar rats. These results suggest different mechanisms of mutagenesis in the two rat strains under study.

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Key words: OXYS rats, Wistar rats, somatic mutations, mtDNA, liver

Mitochondria, the cellular organelles responsible for respiration in tissues, are descendants of a bacterial symbiont of ancient eukaryotes whose genome was essentially transferred into the host cells. The residue of this genome in mammals is represented by the contemporary mitochondrial DNA (mtDNA) which is ~16,000 bp in length and encodes 13 proteins, two rRNAs, and 22 tRNAs. The mtDNA has one small regulatory part called D-loop which includes a start of replication and transcription for the heavy chain of mtDNA [1].

Like every DNA, mtDNA is subject to mutations, which can arise as a result of spontaneous errors of DNA polymerase γ (Pol γ) or because of chemical modification of replicated DNA. Mutations in mtDNA are represented not only by base substitutions (point mutations). Deletions of elongated parts of mtDNA are another frequent type of mutations [2, 3]. The mtDNA is located close to the electron transport chain of mitochondria, which are the main source of reactive oxygen species (ROS), which in turn are the main cause of somatic mutations in mtDNA. This situation along with a limited fidelity of Pol γ preferentially determine a high rate of spontaneous mutations in mtDNA [2, 3]. According to the mitochondrial theory of aging, age-associated accumulation of disorders in mtDNA leads to dysfunction of the energy biogenesis system, which is closely related with aging mechanisms [4]. Some evaluations show that the rate of accumulation of somatic mutations in mtDNA is 100-1000 times higher than in nuclear DNA. At least in some tissues, the rate of point mutations in mtDNA is very high. Thus, in the brain each mtDNA molecule contains, on average, about two point mutations [5]. Their number increases with age, and this suggests a relation between age-associated changes in cells and the whole body and accumulation of somatic mutations in mtDNA. Although the mitochondrial theory of aging was formulated more than 30 years ago, it still

Abbreviations: mtDNA, mitochondrial DNA; 8-oxoG, 8-oxoguanine; Pol γ, DNA polymerase γ; ROS, reactive oxygen species.

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needs a strict experimental confirmation and is actively debated [6-8].

Traditionally, humans and mice are the main objects for studies on this problem. Study of regularities in arising of somatic mutations in mtDNA in the rat strain OXYS selected in the Institute of Cytology and Genetics, Siberian Division, Russian Academy of Sciences (ICG SD RAS), seems very promising. This strain is the first Russian model of premature aging and age-related diseases. The OXYS strain rats are characterized by a short life span and an early development of age-related diseases: cataract, macular dystrophy, osteoporosis, hypertension, and also changes in the emotional and cognitive spheres specific for senescent humans and animals [9-11]. The premature aging of OXYS strain rats is supposed to be related with functional disorders in mitochondria, which appear even in young animals and increase in number with age [12].

Earlier we showed that activities of repair enzymes responsible for elimination of uracil, hypoxanthine, and 8-oxoguanine (8-oxoG) from DNA increase with age in nuclei and mitochondria of liver cells in the OXYS and Wistar strain rats. Activities of these enzymes in the liver cells of the OXYS rats are higher than in the Wistar rats [13]. Nevertheless, oxidized bases are accumulated in nuclear DNA of different organs of the OXYS rats faster than in the Wistar rats [14, 15]. Moreover, content of oxidized proteins in the liver and especially in the brain of OXYS rats increases with age faster than in Wistar rats [16-20].

Antioxidants prevent and/or decrease manifestations of premature aging in the OXYS strain rats, and this suggests the involvement of ROS in its pathogenesis [16-18, 21]. However, the efficiency of antioxidants was more associated with their ability to prevent disorders in functions of mitochondria than with a decrease in the level of oxidative modifications of proteins and lipids [17, 18]. In particular, an increased rate of somatic mutations caused by damage to mtDNA by ROS can provoke disorders in functions of mitochondria. To test this hypothesis, in the present work we have analyzed somatic mutations in the control region of mtDNA in the Wistar and OXYS strain rats.

The control region of mtDNA located between the genes of proline and phenylalanine tRNAs is the most variable region of mammalian mtDNA [1, 22-27]. In rats the control region of mtDNA is about 900 bp in size, and in other animals it can vary from 800 to 5000 bp [28]. The control region of mtDNA in different strains of laboratory rats, including the outbred strain Wistar, is the most variable part of the mitochondrial genome [29]. But for the OXYS strain rats there are no data on arising and accumulation of somatic mutations in this region. Moreover, it is not known if the OXYS and Wistar strain rats are different in intensities of accumulation of mutations in different parts of the control regions depending on their functional roles. Thus, this work was designed to compare somatic mutation spectra in the control region of mtDNA from the livers of the OXYS and Wistar strain rats.

MATERIALS AND METHODS

Sequencing of mtDNA. The work was performed on three-month-old male rats of the OXYS and Wistar strains from the Laboratory of Animal Breeding (ICG SD RAS). Total liver DNA was isolated from frozen tissues stored at –70°C. Primers for amplification of rat mtDNA with the control region were chosen using the GeneRuner program. The direct primer (5′-ATGAAATTAATGTCCCGATAG-3′) corresponds to the mtDNA 15269-15291 region, and the inversed primer (5′-TTACCAACCCTGAGAGGTAC-3′) corresponds to the mtDNA 312-292 region. The fragment obtained as a result of combining these primers consists of 1342 nucleotides and contains genes encoding $tRNA^{Thr}$, $tRNA^{Pro}$, D-loop, $tRNA^{Phe}$, and 12S rRNA. PCR was performed using a high fidelity DNA polymerase HF2 (Clontech, USA) with a correcting subunit (Advantage^{TM} – HF2 PCR kit). Amplified fragments of mtDNA into the T-vector were cloned with a pGem® T-easy Vector kit (Promega, USA). Then the T-vector was transformed to *E. coli* cells of the strain *XL1-Blue MRF′* (Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi*-1 *recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*Z* ΔM15 Tn10(Tet^{R})]). Individual clones containing the vector with insertions of mtDNA fragments were taken by white-blue selection. The selected clones were grown overnight at 37°C in LB medium [30]. Plasmid DNA was isolated by boiling, and the presence of insertions and mutations in them was confirmed by sequencing according to Sanger. Sequences of 112 and 101 fragments of the liver mtDNA from Wistar and OXYS strain rats, respectively, were determined. The resulting sequences of the insertions were compared with sequences in mtDNA from *Rattus norvegicus* rats annotated in GenBank (accession code X14848). The resulting sequences were compared and substitutions were detected using the MultAline program (http://prodes.toulouse.inra.fr/multalin/multalin.html) [31]. Based on the findings, mutation spectra were designed for the control region of mtDNA from the Wistar and OXYS strain rats. To design the spectra, we analyzed a fragment of 1260 nucleotides in length – from nucleotide 15,271 to 229 of the mtDNA. The mutation rate was determined as the ratio of number of detected mutations to the length of the DNA fragment analyzed.

Internal primers were used to confirm the presence of mutations in the second chain. Two direct and two inverse primers were used: IF#1 361 – (5′-TCCATATGACTATCCCTGTCCC-3′) – 382, IF#2 898 – (5′-GGCTTCTCCATTCTAGTAGACC-3′) – 919, IR#1 512 – (5′-AGTAAGAACCAGATGCCTGG-3′) – 493,

IR#2 917 – (5′-TCTACTAGAATGGAGAAGCC-3′) – 898.

Fidelity of the DNA polymerase HF2 was monitored by re-amplification of an individual DNA clone with an established sequence of the insertion without point substitutions. The resulting PCR-product was cloned into the T-vector, transformed into *E. coli* cells of the *XL1-Blue MRF′* strain, and individual clones were selected. The selected clones were grown in LB medium, plasmid DNA was isolated, and the insertion was sequenced.

Analysis of mutational hotspots. Mutation spectra are characterized by hotspots, which are specific regions of DNA with increased mutation frequencies as compared to the class of positions with the lowest mutation frequency. Hotspots of mutations were analyzed using the special CLUSTERM program [32, 33]. Differences in the mutation frequency for each class positions were accidental (mutation rates were homogenous), whereas they were statistically significant for positions of different classes (homogeneity was absent). Thus, the mutation spectrum can be presented as a combination of classes of positions with different mutational probabilities [32, 33].

RESULTS

We have designed and analyzed somatic mutation spectra for the control region of mtDNA from the Wistar and OXYS strain rats. To design the mutation spectra, we analyzed segregated copies of a mtDNA fragment of 1260 nucleotides in length – from nucleotide 15,271 to 229 of mtDNA, which included the control region of rat mtDNA. The total mutation rate in this fragment of mtDNA was determined as the ratio of detected mutation number to the length of the DNA fragment analyzed.

Altogether, we analyzed 112 clones containing a plasmid with mtDNA fragments from the liver of the OXYS strain rats and 101 clones containing a plasmid with mtDNA from the liver of the Wistar strain rats. In all cases, the insertions were sequenced. Mosaic mutations were detected in 16 and 3 fragments of mtDNA from the OXYS and Wistar strain rats, respectively. Therefore, to design mutation spectra, we chose 96 mtDNA-containing clones from the liver of the OXYS strain rats and 98 clones with mtDNA fragments from the liver of the Wistar strain rats. Note that not all cloned PCR-copies contained somatic mutations of the mtDNA fragment under analysis. The incidences of mutant fragments (i.e. plasmids with mtDNA fragments containing one and more somatic mutations) were 50 and 43%, respectively, in the OXYS and Wistar strains.

The total mutation rates in the spectra of somatic mutations in liver mtDNA from the two rat strains were high and virtually the same, $2.7 \cdot 10^{-4}$ on average. Such a high mutation rate cannot be associated with errors of DNA polymerase HF2 used for amplification of the selected region of mtDNA. In additional experiments, we monitored the fidelity of DNA polymerase HF2 in replication of the same sequences under similar conditions. This was realized by a re-amplification of DNAs from individual clones containing insertions with established sequences. Twenty clones were analyzed, which corresponded to 25,200 nucleotides. No new mutations which could arise as a result of DNA polymerase HF2 errors were found.

Mutations in the spectra were mainly represented by transitions: they were 94 and 97% of all nucleotide substitutions in the mutation spectra of the OXYS and Wistar strains, respectively (Table 1 and Fig. 1). The high frequency of transitions (G > A, C > T, A > G, T > C) as

Table 1. Rates of transitions and transversions in the mutation spectra of mtDNA from the liver of the OXYS and Wistar rat strains in the region under study (nucleotides 15,271-229 of mtDNA)

	OXYS	Wistar		OXYS	Wistar
Transitions	94% of all substitutions	97% of all substitutions	Transversions	6% of all substitutions	3% of all substitutions
A → G	0.342	0.453	A → C	0.014	0.016
T → C	0.357	0.359	T → G	0.028	0.016
G → A	0.071	0.031	A → T	0	0
C → T	0.171	0.125	T → A	0	0
			G → T	0	0
			C → A	0	0
			G → C	0.014	0
			C → G	0	0

Note: No significant difference was found between the rates of transitions and transversions in the mutation spectra of the OXYS and Wistar strain rats (Fisher's test, $P = 0.81$).

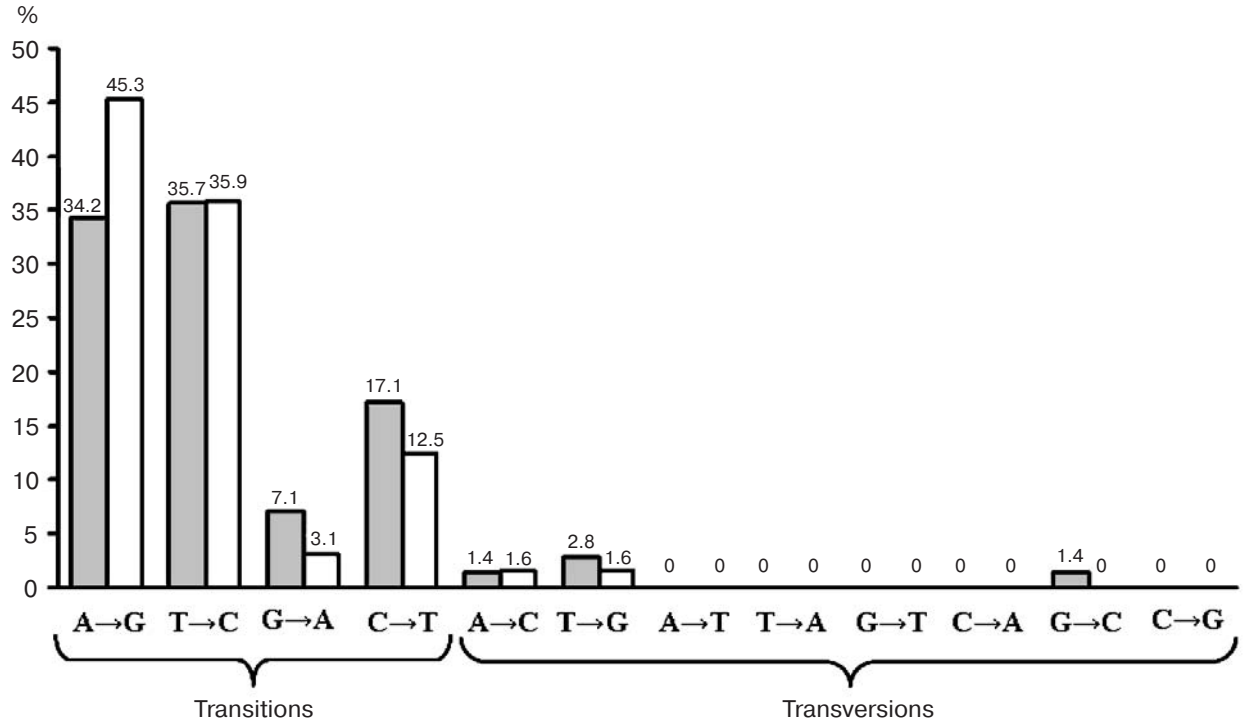

Fig. 1. Percent ratio of somatic mutations in the mutation spectra of liver mtDNA from the OXYS and Wistar strain rats (gray and white columns, respectively) in the region under study (nucleotides 15,271-229 of mtDNA). No significant difference was found between the rates of transitions and transversions in the mutation spectra of the OXYS and Wistar strains (Fisher's test, $P = 0.81$).

compared to that of transversions is in good agreement with earlier data on the population polymorphism in humans [26, 27, 34, 35].

The high mutation rate in AT pairs is an interesting feature of somatic mutations in both rat strains.

Highly variable segments of human mtDNA are characterized by the presence of so-called hotspots [22-27]. Mutational hotspots in DNA reflect intrinsic properties of mutagenesis, which manifest themselves at the level of interaction between mutagens, DNA, and repair/replication systems [36]. A hotspot was detected in position 15,448 (four mutations C > T, the TAT<u>C</u>GTA context). This result is significant ($P < 0.001$) and indicates that the region under study is like the control region of human mtDNA in the criterion of hot mutations. These findings allowed us to assign this region to a highly variable segment of mtDNA.

All essential structures responsible for initiation and regulation of transcription and replication of the mitochondrial genome are located in the mtDNA control region [37]. Structure–function organizations of the mtDNA control region are pronouncedly alike in mitochondrial genomes of mammals [38]. As in other mammals, the control mtDNA region in rodents consists of three domains with different variabilities [28, 38]. The central conservative domain (307 bp in length) is flanked by mtDNA regions with higher variabilities—the left domain, which includes the ETAS section, and the right domain, which includes CSB blocks. In the left domain, the conservative block ETAS1 is located which is involved in the termination of 7S RNA synthesis [37]. The right domain contains transcription promoters of both mtDNA chains and also other functional elements including the starting point of the heavy H-chain replication and the CSB block of conservative sequences. This block, in turn, consists of three short parts: CSB1, CSB2, and CSB3 [39]. The CSB1 block in mtDNA from various rodents is the most conservative, which is explained by its important functional role: this block contains RNA/DNA-replacing sites, MRP RNase recognition sites, and 3′-terminal regions of RNA primers [39]. All this is necessary for initiation of mtDNA replication. Functions of CSB2 and CSB3 blocks are not yet clear because they are present not in all animal species [38, 39]. Mutations in the ETAS1 section of mtDNA are found in both rat strains, but their rates are different: there are two mutations in Wistar rats and 11 mutations in OXYS rats. In the CSB1, CSB2, and CSB3 blocks, four and three mutations were recorded, respectively, in the OXYS and Wistar strain rats (Fig. 2).

Thus, 15 mutations have been found in the functional regions under study (ETAS1, CSB1, CSB2, and CSB3) in the OXYS rats, and this is significantly higher than the mutation number in the Wistar rats (five mutations, Fisher's criterion $P < 0.03$). These findings support the hypothesis that the functional elements of mtDNA in the

OXYS
sequence
Wistar
nucleotide numeration in studied mtDNA fragment

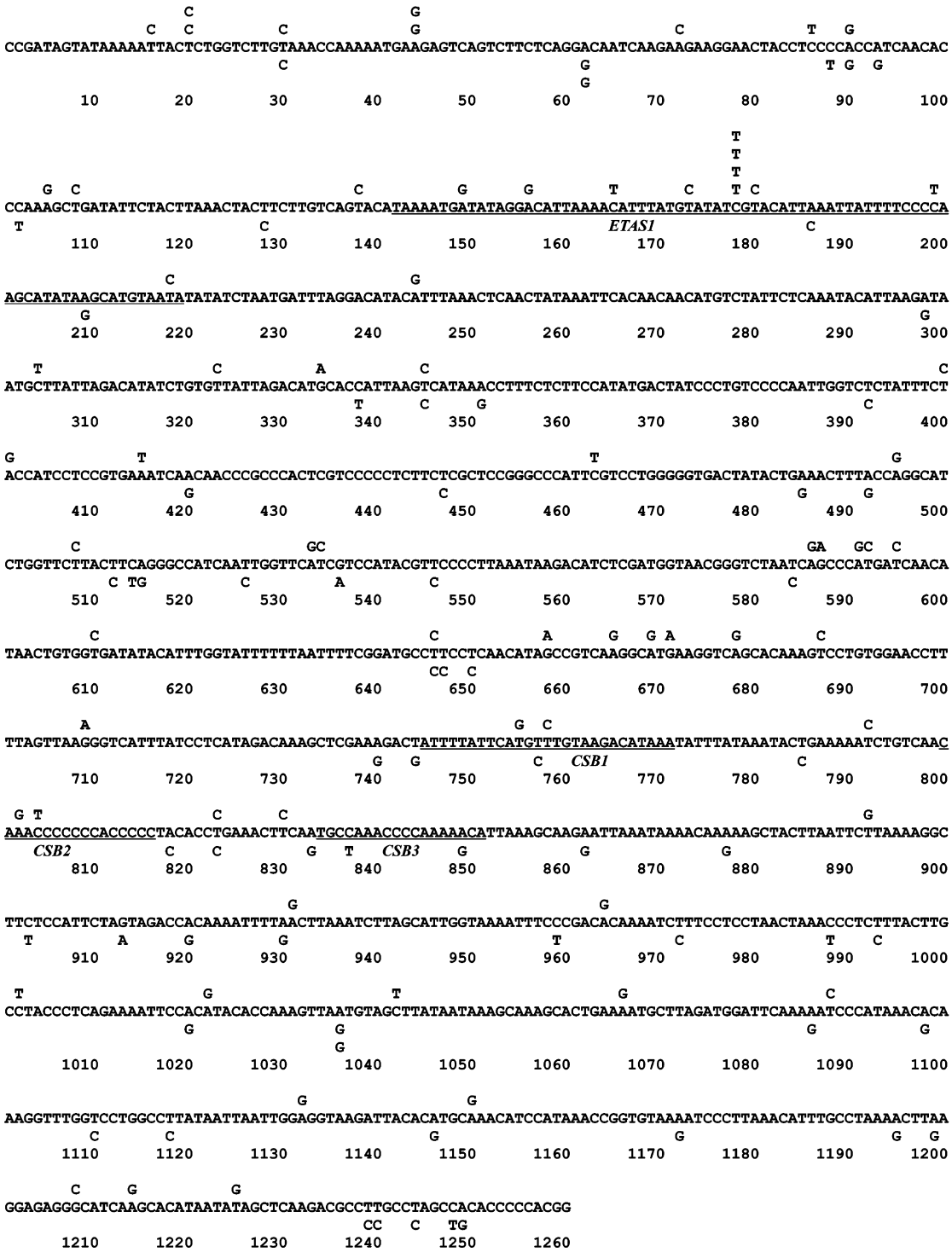

Fig. 2. Somatic mutation spectra in the control region of mtDNA from the OXYS and Wistar strain rats. Substitutions in mtDNA of the OXYS and Wistar rat strains are shown, respectively, above and under the nucleotide sequence. Nucleotide 1 of the sequence corresponds to nucleotide 15,271 of mtDNA, 1-64 nucleotides of the sequence (15,268-15,334 nucleotides of mtDNA) correspond to the $tRNA^{Thr}$ gene region, 66-133 (15,336-15,403) is the $tRNA^{Pro}$ gene, 134-1030 (15,404-16,300) is the D-loop, 1031-1097 (1-67) is the $tRNA^{Phe}$ gene, and 1099-2055 (69-1025) is the region of the 12S rRNA gene. Nucleotides of the functional regions ETAS1, CSB1, CSB2, and CSB3 are underlined.

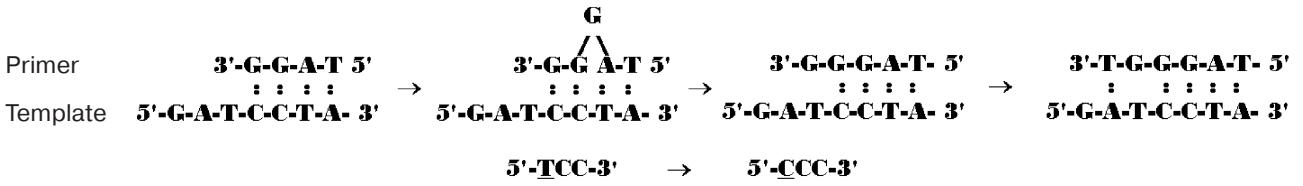

Fig. 3. Model of dislocation mutagenesis in the primer chain. Dislocation in the primer chain during the H-chain replication. The resulting nucleotide substitution is shown under a schematic picture of the dislocation model.

OXYS rats are more easily subjected to somatic modifications, which is manifested by the increased mutation rate in these regions relative to the Wistar rats.

Dislocation mutagenesis has been observed *in vitro* for some polymerases including DNA polymerase γ, which replicates mtDNA. The presence of monotonously repeated sequences in DNA (e.g. ...AAAA...) can lead to production of nucleotide substitutions by the short-time dislocation mechanism (Fig. 3). This model suggests that a short-time dislocation of DNA strands in the repeated nucleotide sequences in the primer or template chain can lead to insertion of the next complementary nucleotide [40]. Analysis of a reconstructed phylogenetic mutation spectrum (distribution of mutations in the sequence analyzed) in the human control region has shown an important role of dislocation mutagenesis in production of nucleotide substitutions in human mtDNA [26, 27].

To verify the suitability of the dislocation mutagenesis model to mechanisms of arising of the detected mutations, mutation spectra of the OXYS and Wistar strains were analyzed using a Monte-Carlo procedure (KUNKEL program) [41]. A significant fraction of nucleotide substitutions (40%) in the mutation spectrum of the Wistar strain was consistent with the dislocation mutagenesis model (Table 2 presents examples of such mutagenesis). Moreover, the dislocation model for the primer chain (Fig. 2) and for the template chain has high statistical support ($P(W \le W_{random}) = 0.014$) and ($P(W \le W_{random}) = 0.028$), respectively. Such a significant confirmation of the dislocation model suggests that the majority of somatic mutations in mtDNA from the Wistar strain rats can arise during the replication as a result of errors of DNA polymerase γ. On analyzing the mutation spectrum of the OXYS strain no statistical confirmation was found for the dislocation mutagenesis model ($P(W \le W_{random}) = 0.18$). Consequently, the molecular mechanisms of mutagenesis in mtDNA in the liver of the OXYS and Wistar can be different and, possibly, many mutations in mtDNA in the OXYS strain can arise as a result of a pre-replication modification of DNA.

Table 2. Examples of probable arising of mutations as a result of dislocation mutagenesis in the mutation spectra of mtDNA from the liver of Wistar rats in the region under study (nucleotides 15,271-229 of mtDNA)

Nucleotide number in mtDNA	Substitution type	Number of substitutions	Mutation context
15332	A → G	2	AGG A CAA
15784	C → T	1	CTT C AGG
15785	A → G	1	TTC A GGG
15816	T → C	1	CGT T CCCC
15916	T → C	1	GCC T TCC
15917	T → C	1	CCT T CCT

DISCUSSION

It has been suggested that the premature aging of the OXYS rats should be associated with functional disorders in mitochondria recorded in the liver even in young (two-three-month-old) animals and increasing with age [12, 17]. We supposed that the dysfunction of mitochondria observed in the OXYS rats could be related with specific features of accumulation of somatic mutations in mtDNA. In particular, our interest was just in point mutations in the control region of mtDNA because this region, located between the genes of proline and phenylalanine tRNAs, is the most variable region of mammalian mtDNA [1, 22-27]. The control region of the rat mtDNA is about 900 bp, but in other animals its size varies from 800 to 5000 bp [28]. Thus, we expected to find a significant number of somatic mutations in a relatively small portion of mtDNA, and this seemed promising for a rather easy detection of the mutation number necessary for designing and comparative study of mutational spectra.

The total mutation rate in the obtained spectra of somatic mutations in the mtDNA control region (15,283-244 bp) in the liver of the OXYS and Wistar rat strains was, on average, $2.7 \cdot 10^{-4}$, thus this region can be considered to be a highly variable segment of mtDNA. In the mutation spectra of the OXYS and Wistar strains, there were 94 and 97% transitions, respectively, and this was consistent with the literature data on the population polymorphism in human mtDNA [26, 27, 42]. Excess transitions in the

mutation spectra of mtDNA could be caused by errors in base-pairing during the mtDNA replication.

We have shown a high rate of somatic mutations in rat liver mtDNA. At first glance, our findings contradict the recently published paper [7] in which somatic mutations in mouse mtDNA were detected using hydrolysis of DNA with the corresponding restriction endonucleases (the RMC method) and the rate of somatic mutations was found to be low. We think that this apparent contradiction can be caused by the study in work [7] only of one restriction site of four nucleotides in length. It is known that the mutation rate can vary several orders in the same sequence and result in appearance of "hot" and "cold" mutation points [36]. On admission that the restriction site studied in work [7] coincides with a "cold" mutation point, this work does not contradict our data.

We revealed a significant difference in the character of mutations in the two rat strains: 40% of mutations in mtDNA of the Wistar strain rats correlated with the dislocation mutagenesis model, whereas mutations in mtDNA of the OXYS rats did not correlate with this model. It seems that dislocation mutagenesis does not play an important role in arising of nucleotide substitutions in mtDNA of the OXYS strain or is masked by other mechanisms of mutagenesis. These differences in the dislocation mutagenesis models suggest that mutagenesis mechanisms can differ in mtDNA of the two studied rat strains. Dislocation mutagenesis is usually believed to be a result of errors in functioning of DNA polymerases [40, 43]. Therefore, some other mechanisms of mutagenesis (e.g. a spontaneous adenine deamination) are also likely to contribute to the somatic mutation spectrum. But in this case the mutation rate in DNA of the OXYS rats would be higher. However, we have not found differences in the mutation rates between the OXYS and Wistar rats. Possibly, the total mutation frequency has an upper limit. Death of mitochondria with a great number of somatic mutations can be such a restrictor of the somatic mutation frequency.

The number of mutations in the ETAS section of the control region in the OXYS rats was significantly higher than in the Wistar rats. The ETAS1 domain is highly conservative, which can be explained by its important functions because it is involved in the termination of synthesis of the primer 7S RNA that is required to initiate the mitochondrial genome replication [39]. Disorders in this process can cause a decrease in the number of DNA copies in mitochondria that, in turn, can seriously disturb functioning of the bioenergetic system of mitochondria [44]. Just this has been shown for liver mitochondria of young OXYS rats [12, 17]. Finally, a significantly increased mutation rate in the mtDNA functional regions in the OXYS rats indicates that rates of functionally important mutations in the OXYS strain can be higher than in Wistar rats. In this case, our findings are quite consistent with the hypothesis that the premature aging of the OXYS rats can be due to disorders in the functional state of mitochondria associated with the high rate of somatic mutations in the functionally important regions of mtDNA.

However, aging is obviously a multifactor process that involves not only changes in the functional state of mitochondria but also accumulation during the human and animal life of oxidative damages to different somatic cell components including oxidative modifications of nuclear and mitochondrial DNAs. We have shown earlier that the level of oxidative damages of nuclear DNA in the liver and especially lung cells in the OXYS rats is higher than in Wistar rats, and this difference increases with age [14, 15, 45]. The accumulation of oxidative modifications of protein and lipids in tissues of the OXYS rats is also markedly higher than in Wistar rats [16-20]. Thus, all these factors can additionally contribute to the accelerated aging of the OXYS rats.

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