

Research Article

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Detection of small discrete 5'-terminal fragments of 18S rRNA in mouse cells

Protein biosynthesis is a critically important cellular process. With age, structural damage to ribosomes may accumulate, potentially leading to the synthesis of misfolded polypeptides and reduced regenerative capacity and cell viability. Therefore, there is a need to develop universal molecular markers of ribosome integrity. A DNA construct was designed to generate DIG-labeled RNA probes for detecting 5'-terminal fragments of mammalian 18S rRNA. RNA isolated from the liver, lungs, kidneys, and skeletal muscles of young and aging mice was analyzed by Northern blotting using these probes. Small, discrete 5'-terminal fragments of 18S rRNA, similar to the stress-induced 5'18SrRF75 and 5'18SrRF135 molecules identified in plants, were detected in mouse tissues. The levels of these fragments in lung and skeletal muscle tissues were significantly higher than those in the liver and kidneys. Notable differences were observed in the kidneys, lungs, and skeletal muscles of young (3-month-old) versus aging (over 2-year-old) animals. These results suggest that the molecular mechanisms underlying the discrete fragmentation of 18S rRNA may be conserved across species. Such mechanisms may play a role in regulating protein biosynthesis under stress. The small fragments 5'18SrRF75 and 5'18SrRF135 could potentially serve as universal markers of translational apparatus integrity.

Keywords: 18S rRNA, discrete fragmentation, stress markers, mRNA translation, aging, ribosome, small RNAs, oxidative stress.

Introduction

Chronic inflammatory diseases that lead to cell destruction significantly harm human health, society, and economies around the world. In clinical medicine, diagnostic methods commonly involve measuring inflammatory mediators—such as cytokines, chemokines, IgE, histamine, and serotonin—as well as specific groups of leukocytes in patients' blood [1]. However, these stress markers primarily assess the overall immunological and inflammatory status of the body; they cannot evaluate the intracellular processes that cause cell dysfunction and death. Furthermore, these markers are typically detectable only in the later stages of disease progression. Additionally, all of the markers mentioned are species-specific, necessitating the development of unique panels for each individual mammalian species.

In modern laboratory diagnostics, there is a steady trend toward integrating new classes of molecular markers—specifically microRNAs, specific mRNAs, and the pool of small non-coding RNAs—to verify stress-induced and pathological states. The use of these analytes provides a comprehensive assessment of the molecular and genetic mechanisms underlying destructive processes at the cellular and tissue levels. Small, discrete fragments of ribosomal RNA (rRNA) are considered promising candidates for new stress markers in humans and mammals. The high concentration of ribosomes in cells results in a significant copy number of rRNA, which constitutes 80–90 % of the total cellular RNA pool [2]. Consequently, diagnostic test systems targeting rRNA demonstrate analytical sensitivity several orders of magnitude higher than primer kits designed for mRNA and microRNA [3]. Furthermore, the structural organization of rRNA genes, characterized by the presence of highly conserved domains alongside variable regions, enables the development of universal detection systems applicable to a wide range of mammalian biological samples [4].

The mechanism of mRNA translation inhibition in both prokaryotes and eukaryotes is well understood and involves the cleavage of the large ribosomal subunit RNA at a region known as the sarcin-ricin loop.

This process results in the detachment of a small 3'-terminal RNA fragment [5]. This is the exact mode of action for several plant ribosome-inactivating proteins, including abrin, modeccin, and ricin, as well as toxins from bacteria, like Shiga toxin, and from fungi, such as α -sarcin [6]. It is hypothesized that a similar mechanism may occur in eukaryotic cells involving the RNA of the small (40S) ribosomal subunits. The 40S subunits play a crucial role in the initiation of translation, which is the rate-limiting step in the entire protein biosynthesis process. As organisms age, destructive changes in ribosome structure may accumulate, leading to a cumulative effect. The loss of integrity in the cell's translational machinery can significantly impact various cell types. When cells are unable to produce structural, protective, and regulatory proteins in a timely manner due to impaired ribosomal integrity, their regenerative potential declines, causing them to struggle under stress or adverse growth conditions. Moreover, ribosomes with internal defects may result in the synthesis of misfolded or "defective" polypeptides. The accumulation of abnormally folded prion-like proteins in brain cells is a primary cause of many neurodegenerative disorders, including Alzheimer's disease [7].

We hypothesize that small 5'- and 3'-terminal fragments of this molecule can serve as a new class of universal stress markers. Detecting these RNA markers could enable earlier and more timely identification of pathological processes that lead to severe chronic diseases. Previous studies have identified universal stress-induced small 5'-terminal discrete fragments of 18S rRNA in plant cells [8–10]. In the present study, we analyzed the profiles of discrete 5'-terminal 18S rRNA fragments in various tissues from mice in two age groups: young and aging animals. To determine whether similar molecules are detectable in mammalian cells, we investigated the profiles of small 5'-terminal 18S rRNA fragments across four tissue types—liver, lung, kidney, and skeletal muscle—in both young and aging mice.

The results of this study generally confirm the universality of the discrete 18S rRNA fragmentation process in eukaryotic organisms, underscoring the need to explore the underlying molecular mechanisms further. Additionally, the potential use of small discrete fragments of ribosomal RNA in diagnosing chronic diseases and destructive processes in humans and animals is of significant interest.

Experimental

Experiments with animals

The Local Bioethics Commission of the RSE on LPP "M. Aitkhozhin Institute of Molecular Biology and Biochemistry", Ministry of Science and Higher Education of the Republic of Kazakhstan, provided a positive decision for experiments involving laboratory animals (Protocol No. 1, dated May 17, 2024). All procedures using laboratory animals were conducted by qualified specialists with extensive experience, ensuring adherence to the principles of humane treatment and rational use of these animals. The study included two groups of black linear C57BL/6 mice (three males per group): one group consisted of young mature animals aged three months, while the other group included aging animals over two years old.

Computer analysis

Nucleotide sequences of 18S rRNA were analyzed using computer software, specifically the MEGA-X, VectorNTI, and RNA-structure v.5.7 programs, with data obtained from the NCBI GenBank database [11]. To assess the degree of discrete fragmentation of the 18S rRNA semi-quantitatively, the blots were evaluated using densitometry in the ImageJ 1.42q program.

Plasmid construction

Molecular cloning procedures, including phosphorylation/dephosphorylation, restriction, and ligation of DNA, as well as transformation of bacterial cells, were performed according to standard protocols [12]. Plasmid DNA was isolated using the High Pure Plasmid Isolation Kit (Roche), following the manufacturer's instructions. *De novo* synthesized oligonucleotides Rat18S_1-35neg_Acc65-F (5'-gtaccgacaagcatatgctactggcaggatcaaccaggta) and Rat18S_1-35neg-Hind-R (5'-agcttacctgggtgatcctgccagtagcatatgctgtcg) were annealed, phosphorylated, and ligated into the pBluescript KSII vector using Acc65I and HindIII restriction sites. The inserts were verified through Sanger DNA sequencing, which was carried out using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the T7 primer (5'-taatacgactcactataggg) according to the manufacturer's protocol. The reaction mixtures were purified using 70 % ethanol and analyzed on a SeqStudio genetic analyzer (Applied Biosystems).

Northern blotting

Total RNA was extracted from 0.1 g of mouse tissues using Trizol (Sigma), following the manufacturer's protocol. RNA electrophoresis was performed on an 8 % polyacrylamide gel containing 8 M urea. Bands were visualized using the Quantum gel documentation system (Vilber). For the synthesis of DIG-labeled RNA probes, *in vitro* transcription was conducted after linearizing the plasmids at the Hind III restriction

site. The DIG RNA Labelling Kit (SP6/T7) (Roche) was utilized according to the manufacturer's instructions. In the Northern blotting procedure, RNA was transferred to a nylon membrane equilibrated in 0.1x Tris-borate buffer using a semi-dry blotting device (Sci-Plas) at a current of 250 mA for 30 minutes. After the transfer, the membrane was dried and subjected to UV light treatment for 2 minutes at 10 mJ/cm² using a crosslinker (UVP). Hybridization with the DIG-labeled probes and the subsequent detection of bands were performed using the DIG Luminescent Detection Kit for Nucleic Acids (Roche), following the manufacturer's protocol. The hybridization temperature was set to 55 °C. For detecting the bound DIG-labeled probes, Anti-Digoxigenin-AP Fab fragment antibodies (Roche) were employed. The detection phase utilized the alkaline phosphatase substrate CSPD (Roche).

Statistical methods

All measurements were conducted in at least three replicates, and the arithmetic mean ($\bar{X}$) along with the standard error of the mean (m) were calculated. The significance of differences between samples was evaluated using the Student's t-test (t_{st}), with differences considered significant at $p < 0.05$.

Results and Discussion

Creation of a DNA construct for the generation of DIG-labeled RNA probes

To select probe sequences for detecting small fragments of 18S rRNA from mammals, as well as other eukaryotic organisms, nucleotide sequences from reference organisms were sourced from the NCBI GenBank database [11]. These sequences were aligned using the MEGA-X bioinformatics program. The results are illustrated in Figure 1.

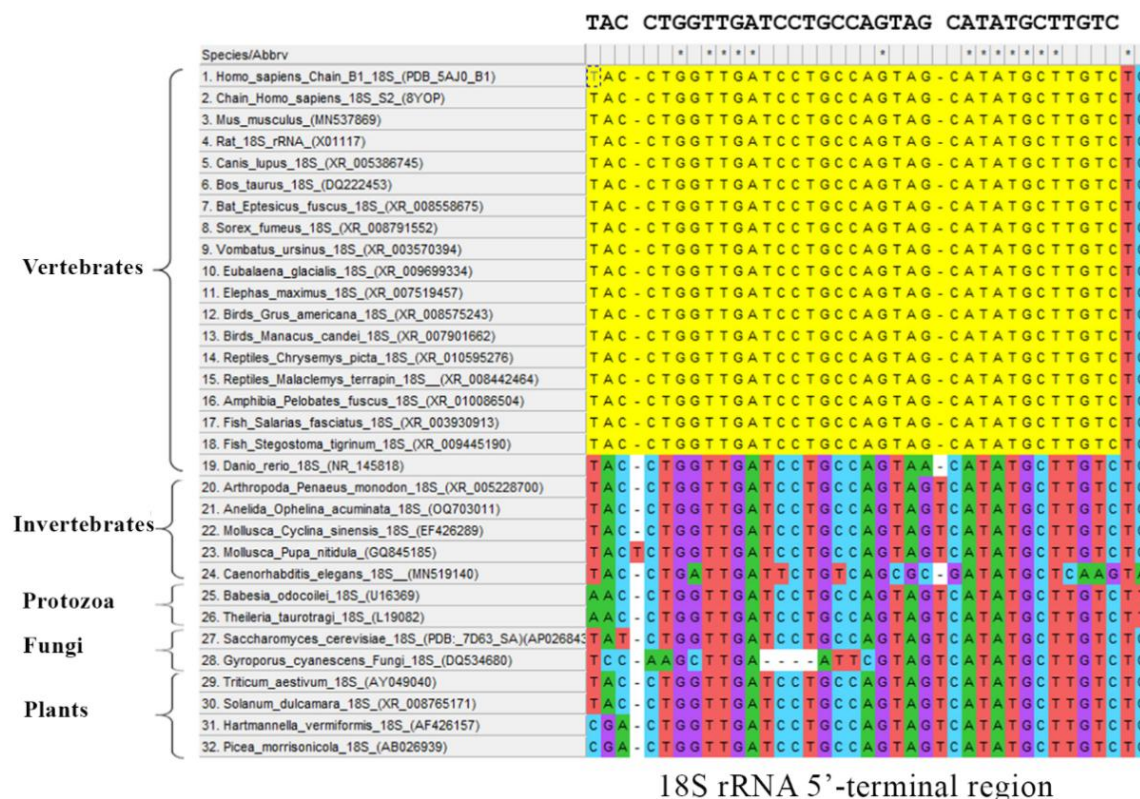
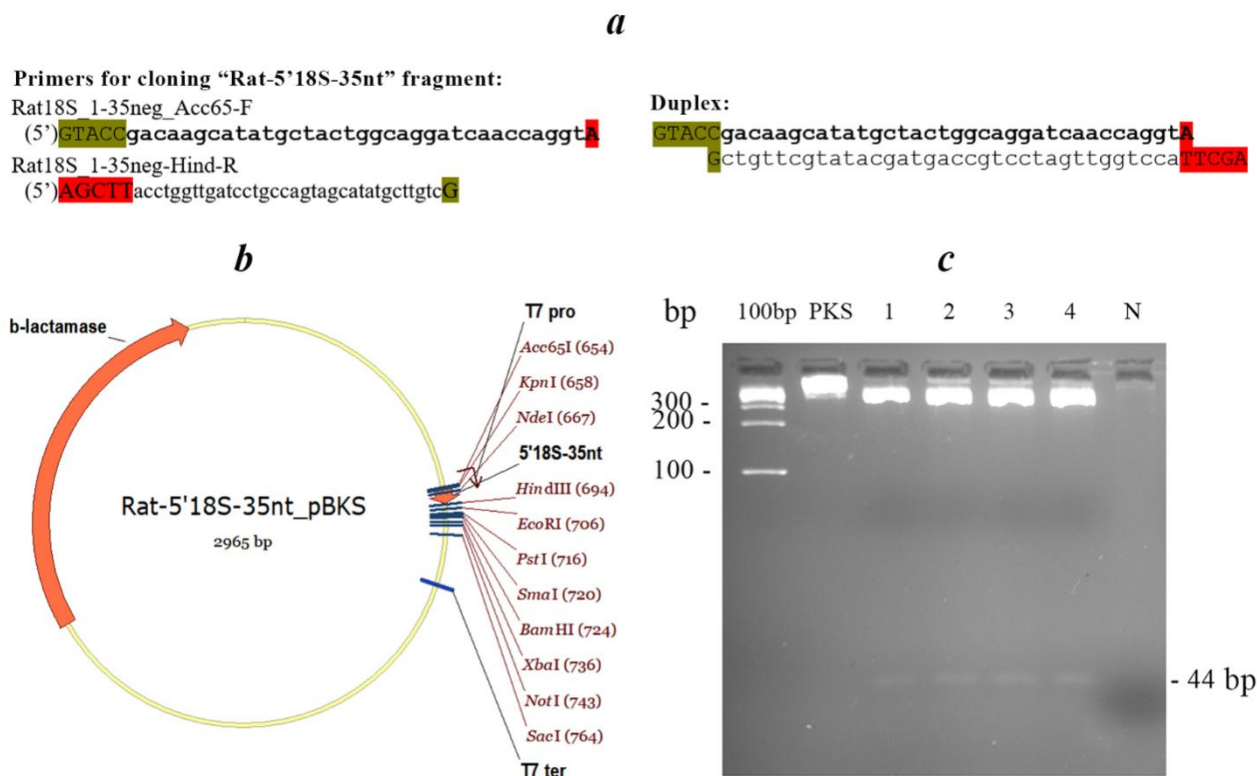


Figure 1. Results of alignment of nucleotide sequences of 5'-3'-terminal fragments of 18S rRNA of eukaryotic organisms in the MEGA-X program

The 35-nucleotide region at the 5'-end of mammalian 18S rRNA was selected for the detection of 5'-terminal small ribosomal RNAs. This region of the 18S rRNA is highly conserved and remains unchanged across nearly all vertebrates (Fig. 1). Although there may be minor discrepancies in the sequence (one to two letters), probes that are complementary to this 18S rRNA locus are generally effective for detecting small 5'-terminal 18S rRNA fragments from other eukaryotic organisms, with a few exceptions. Notably, the 5'-terminal fragments of the roundworm *Caenorhabditis elegans* and the basidiomycete *Gyroporus cyanescens* do not follow this pattern.

To create DNA constructs for synthesizing DIG-labeled probes using the *in vitro* transcription method, we designed, synthesized, and purified the primers Rat18S_1-35neg_Acc65-F and Rat18S_1-35neg-Hind-R. When these primers were annealed, they formed a 35-nucleotide DNA fragment that corresponds to the 5'-terminal region of eukaryotic 18S rRNA. This fragment is flanked by the restriction sites *Hind*III and *Acc*65I (Fig. 2a).



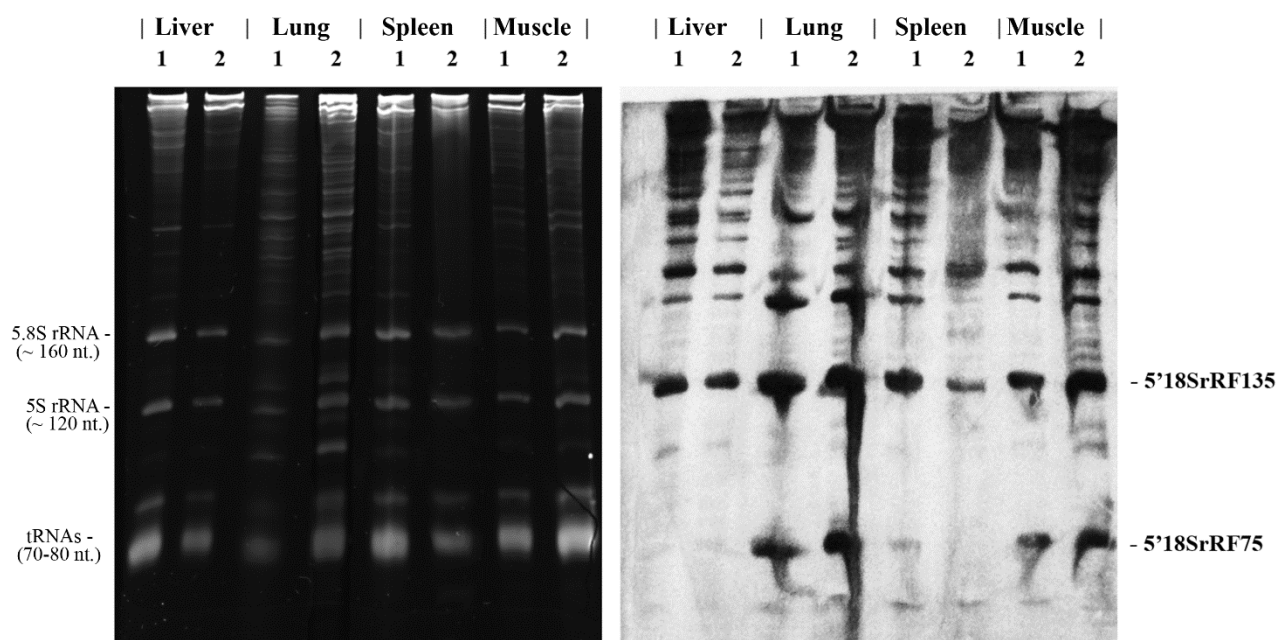
(a) — Formation of oligoDNA duplex for cloning. (b) — Rat-5’18S-35nt_pBKS plasmid map. (c) — Results of restriction analysis (*Acc*65I/*Hind*III) of DNA clones after ligation. A 1.5 % agarose gel electrophoregram is shown. 100 bp — GeneRuler 100 bp DNA Ladder (Thermo Fisher Sci.), PKS — pBluescript KSII vector, 1-4 — DNA clones, N — negative control.

Figure 2. Stages of creating the DNA construct Rat-5’18S-35nt_pBKS for producing an RNA probe to the 5'-end of mammalian 18S rRNA

After phosphorylation, the duplexes were cloned into the pBluescript KS(II) vector at identical restriction sites. The resulting plasmid, designated Rat-5’18S-35nt-pBKS, is illustrated in Figure 2b. We confirmed the presence of inserts of the appropriate size in the DNA clones through restriction analysis using the endonucleases *Hind*III and *Acc*65I, with the results shown in Figure 2c. To verify the accuracy of the cloned nucleotide sequences in these constructs, we performed DNA sequencing using the T7 primer. This resulted in the successful creation of the DNA construct Rat-5’18S-35nt_pBKS, which allows for the synthesis of DIG-labeled probes that target the 5'-terminal region of eukaryotic 18S rRNA from the T7 promoter.

A study of the role of mouse age in the accumulation of 5'-terminal fragments of 18S rRNA

In a study examining the role of mouse age in the accumulation of 5'-terminal fragments of 18S rRNA, we aimed to determine whether specific 5'-terminal fragments of 18S rRNA, similar to those found in plant cells, are present in mammalian cells and whether their abundance varies with age. Total RNA from the liver, lung, spleen, and skeletal muscle of both young and aging mice was analyzed using Northern blotting. The results are displayed in Figure 3.



On the left is an 8 % PAA gel, and on the right is a blot of the same gel.
 1 — young animals (3 month); 2 — aging animals (over 2 years old).

Figure 3. Northern blot results for detection of 5'-terminal small discrete fragments of 18S rRNA in different tissues of male mice

To confirm the changes in fragmentation levels associated with aging and the differences between tissues, a semiquantitative densitometric analysis of the blots was performed. Although the same amount of total RNA was loaded onto the gel based on calculated values, the actual RNA load may have varied slightly. Additionally, due to potential lane distortions, relying solely on densitometry measurements could result in biased outcomes. To mitigate this issue, the results were normalized to the density of an internal control present in the original electrophoregram. The control used was 5S rRNA, which is consistently expressed across various cell types. By incorporating this internal control into the calculations, we minimized potential errors related to uneven RNA loading or band distortions. Each group of animals (young and aging) consisted of three individuals, allowing for statistical analysis of the results. The findings from the densitometry analysis are summarized in Figure 4.

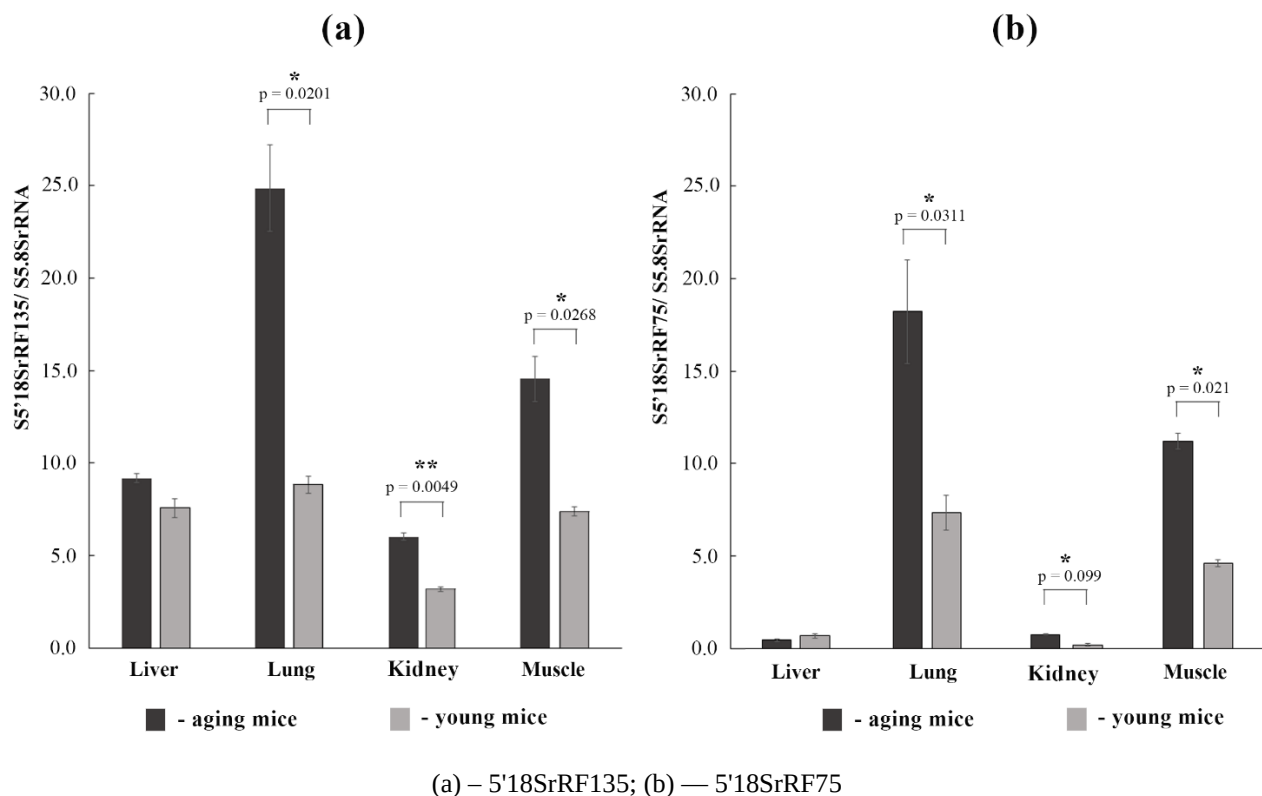


Figure 4. Results of densitometry analysis assessing the effect of aging on the level of discrete fragmentation from the 5'-terminus in mice

Small ribosomal RNAs similar to the two stress-induced 5'-terminal fragments of plant 18S rRNA (specifically 5'18SrRF75 and 5'18SrRF135) have been identified in mouse cells. The 5'18SrRF135 fragment was detected in all tested tissue types, even in young individuals. In lung cells and skeletal muscle, the levels of this fragment were slightly higher compared to those in liver and kidney cells, although these differences were statistically significant only in aging animals. The 5'18SrRF75 fragment was found in significant amounts only in lung and skeletal muscle cells. These findings may be explained by the fact that mammalian lung and skeletal muscle tissues are subject to elevated oxidative stress. In the lungs, this is due to a high partial pressure of oxygen and constant exposure to external pollutants, which leads to a higher baseline level of reactive oxygen species (ROS) compared to most visceral tissues [13]. Similarly, the increased baseline ROS levels in muscles are attributed to intense metabolism and mitochondrial activity, especially during physical exercise [14].

The data presented in Figure 4 shows that in several tissue types, including the lung, spleen, and skeletal muscle, the levels of both target small fragments of 18S rRNA (5'18SrRF75 and 5'18SrRF135) increase with age. However, this correlation is not observed in the liver, likely due to the low overall levels of these 18S rRNA fragments. Another possible explanation is that liver cells, known as hepatocytes, have an extremely high capacity for proliferation and regeneration. In contrast, lung and kidney tissues are considered stable (expanding) tissues, where cell division occurs infrequently under normal conditions but can be stimulated in response to injury. Generally, the cells in these tissues exhibit moderate to low levels of proliferative activity. Muscle fibers (myofibers), on the other hand, do not divide at all, as they lack centrioles and cannot undergo mitosis.

The detection of small discrete fragments similar to the 5'18SrRF75 and 5'18SrRF135 fragments in mammalian cells, which were previously identified in plants under various stress conditions, suggests that the fragmentation process is universal among eukaryotic organisms. Elevated levels of these fragments have been recorded in cell types that are vulnerable to oxidative stress. Additionally, studies indicate that the levels of both 18S rRNA fragments generally increase in aging animals. The decline in the integrity of the translational machinery with age may have serious consequences, leading to a higher risk of damaging processes

in the body's cells and tissues. Therefore, the 5'18SrRF75 and 5'18SrRF135 molecules can be regarded as universal markers of translational apparatus degradation in mammalian cells.

Conclusion

In conclusion, we have identified small discrete fragments of 5'-terminal 18S rRNA in mouse cells that are similar to the stress-induced small plant rRNAs 5'18SrRF75 and 5'18SrRF135. Quantitatively, these fragments are more abundant in lung and skeletal muscle tissues compared to liver and kidney cells. We hypothesize that this difference is linked to the relatively high ROS in the former two tissue types. Additionally, we found that levels of 5'18SrRF75 and 5'18SrRF135 are elevated in aging animals compared to younger individuals. This age-related decline in the integrity of the cellular translational apparatus may be associated with an increased risk of damaging processes in mammalian cells, potentially leading to chronic diseases. We propose that the small discrete 18S rRNA fragments, 5'18SrRF75 and 5'18SrRF135, should be considered universal markers of translational apparatus damage.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Malysheva A.A.** — investigation, writing — original draft; **Kazanbassov T.S.** — methodology, investigation; **Lopatchenko T.S.** — investigation; **Osikova K.G.** — investigation; **Iskakov B.K.** — conceptualization, writing — review & editing; **Zhigailov A.V.** — formal analysis, investigation, data curation, writing — review & editing.

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Тышқан жасушаларындағы 18S рРНҚ-ның кіші дискретті 5'-үшты фрагменттерін анықтау

Нәруыз биосинтезі — өте маңызды биосинтетикалық процесс. Жануарлардың жасы ұлғайған сайын рибосомалар құрылымында деструктивті өзгерістер жинақталып, дұрыс жиналмаған (мисфолдингке ұшыраған) полипептидтердің синтезделуіне, сондай-ақ жасушалардың өміршеңдігі мен регенеративті потенциалының төмендеуіне әкелуі мүмкін. Осыған байланысты рибосомалар тұтастығының әмбебап молекулалық маркерлерін жасау қажеттілігі туындап отыр. Сүтқоректілердің 18S рРНҚ-ның 5'-үштық фрагменттерін анықтау мақсатында DIG-таңбаланған РНК-зондтарын алуға арналған ДНҚ-конструкциясы жасалды. Алынған зондтарды қолдана отырып, нозерн-блоттинг әдісімен жас және қартайған тышқандардың бауыр, өкпе, бүйрек және қаңқа бұлшықеттерінен бөлінген РНК-ға талдау жасалды. Тышқан жасушаларында өсімдіктердегі стресс әсерінен түзілетін 5'18SrRF75 және 5'18SrRF135 молекулаларына ұқсас 18S рРНҚ-ның кішігірім дискретті 5'-үштық фрагменттері табылды. Олардың мөлшері өкпе және бұлшықет жасушаларында бауыр мен бүйрекке қарағанда айтарлықтай жоғары болды. Жас (3 айлық) және қартайған (екі жастан асқан) жануарлардың бүйрегі, өкпесі және қаңқа бұлшықеттері арасында айтарлықтай айырмашылықтар анықталды. Нәтижелер рибосома құрамындағы 18S рРНҚ-ның дискретті фрагментациясына қатысатын молекулалық механизмдердің әмбебаптығын дәлелдейді. Бұл механизмдер стресс жағдайында нәруыз биосинтезін реттеуге қатысуы мүмкін. Кішігірім 5'18SrRF75 және 5'18SrRF135 фрагменттері трансляциялық аппарат деструкциясының әмбебап маркерлері бола алады.

Кілт сөздер: 18S рРНҚ, дискретті фрагментация, стресс маркерлері, мРНҚ трансляциясы, картау, рибосома, кіші РНК-лар, тотығу стресі.

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Обнаружение малых дискретных 5'-концевых фрагментов 18S рРНҚ в клетках мышей

Биосинтез белка является критически важным биосинтетическим процессом. С возрастом в структуре рибосом могут накапливаться деструктивные изменения, что приводит к синтезу неправильно свернутых (мисфолдированных) полипептидов, а также к снижению регенеративного потенциала и жизнеспособности клеток. В связи с этим существует необходимость в разработке универсальных молекулярных маркеров целостности рибосом. Для детекции 5'-концевых фрагментов 18S рРНҚ млекопитающих была сконструирована ДНҚ-конструкция для наработки DIG-меченых РНК-зондов. Методом нозерн-блоттинга с использованием полученных зондов была проанализирована РНК, выделенная из клеток печени, легких, почек и скелетных мышц молодых и стареющих мышей. В клетках мышей были обнаружены малые дискретные 5'-концевые фрагменты 18S рРНҚ, подобные индуцируемому стрессом молекулам 5'18SrRF75 и 5'18SrRF135 у растений. Их содержание в клетках легких и мышц было значительно выше, чем в клетках печени и почек. Достоверные различия между молодыми (3 месяца) и стареющими (старше двух лет) животными были продемонстрированы для почек, легких и скелетных мышц. Результаты подтверждают универсальность молекулярных механизмов, участвующих в дискретной фрагментации 18S рРНҚ в составе рибосом. Эти механизмы, вероятно, задействованы в

регуляции биосинтеза белка при стрессе. Малые фрагменты 5'18SrRF75 и 5'18SrRF135 могут служить универсальными маркерами деструкции трансляционного аппарата.

Ключевые слова: 18S рРНК, дискретная фрагментация, маркеры стресса, трансляция мРНК, старение, рибосома, малые РНК, окислительный стресс.

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