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FEBS Letters 338 (1994) 137–142

FEBS 13581

FEBS LETTERS

# Existence of nuclear-encoded 5S-rRNA in bovine mitochondria

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Received 2 December 1993

**Abstract**

A number of proteins functioning in mitochondria are synthesized in the cytoplasm and imported into the mitochondria via specific transport systems. In mammals, on the contrary, mitochondrial membranes have generally been considered to be impermeable to nucleic acids. However, here we show that an RNA with 120 nucleotides, the sequence of which is identical to that of the nuclear-encoded 5S RNA, exists in bovine mitochondria, although the mitochondrial genome encodes no 5S RNA gene. This RNA molecule was found to be retained in purified bovine mitochondria as well as in the mitoplasts, even after extensive treatment with an RNase, demonstrating that the 5S RNA is actually located inside the mitochondrial inner membrane. The 5S rRNA molecule was also shown to exist in mitochondria from rabbit and chicken.

*Key words:* 5S-rRNA; Transport; Mitochondria; Bovine

## 1. Introduction

Mitochondria, which play a crucial role in energy production in eukaryotic cells, contain a number of proteins involved in respiration and energy generating systems. Animal mitochondria possess their own genomes, coding for 13 proteins, 22 transfer RNAs and 2 ribosomal RNAs, in which the latter two RNA components participate in the mitochondrial translation system [1]. However, a majority of the proteins functioning in the mitochondria are originally encoded by the nuclear genome, synthesized in the cytoplasm and imported into the organella [2].

Although the mechanisms of protein transport have been well documented, little has been reported about the transport of nucleic acids into mitochondria, except for the facts that nuclear-encoded tRNAs are imported into mitochondria in plants [3–5], *Saccharomyces cerevisiae* [6] and protozoa [7–10]. It has been thought that in higher vertebrates tRNAs are not transported from cytoplasm into mitochondria [11]. However, in rat mitochondria an RNA moiety of RNase MRP which participates in mitochondrial RNA processing has been reported to be present in the organella [12], although other data contradicts this finding [13]. 5.8S rRNA has also been presumed to be imported into rat mitochondria [14]. Thus, the situation regarding the transport of nucleic acids in mammalian mitochondria is generally still ambiguous. Here we present experimental evidence indicating that 5S rRNA encoded by the nuclear genome is abundantly present in bovine mitochondria.

## 2. Materials and methods

### 2.1. Materials

RNase $T_1$ was purchased from Sankyo, Japan. RNase PhyM and RNase A and $T_4$ RNA ligase were from Pharmacia and RNase $U_2$ from Sigma. RNase CL3 was purchased from Boehringer. *E. coli* alkaline phosphatase and $T_4$ polynucleotide kinase were from Takara Shuzo. [$\gamma$-$^{32}$P]ATP and [$^{32}$P]pCp were purchased from Amersham. Other chemicals were obtained from Wako Chemical Industries.

### 2.2. Preparation of 5S RNA transcript

A 5S RNA transcript used as a size marker in polyacrylamide gel electrophoresis was prepared as follows [15]. Cytoplasmic 5S RNA from bovine liver ribosomes was converted to cDNA by using reverse transcriptase activity in Taq DNA polymerase [16,17]. The cDNA was amplified by PCR and joined to the promoter region for T7 RNA polymerase which had been chemically synthesized and inserted into the plasmid pUC19 according to the method already reported [15]. After the 5S RNA gene was amplified by cloning, 5S RNA was transcribed from the gene.

### 2.3. Preparation of mitochondria and mitoplasts

Preparation of mitochondria and mitoplasts from bovine liver was carried out as described previously [18]. Further purification of mitochondria was done by ultracentrifugation through a sucrose density

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*SSDI* 0014-5793(93)E1498-B

gradient [19]. Mitoplasts from chicken or rabbit liver were prepared by the same procedure as that used for bovine mitoplasts.

*2.4. RNase treatment of mitochondria and mitoplasts*

After the protein concentrations of mitochondria and mitoplasts were adjusted to 8 mg/ml with a buffer containing 0.25 M sucrose and 2 mM TES-KOH (pH 7.4), RNase A was added to the mitochondria and mitoplast suspensions to a concentration of 10 μg/ml and they were incubated at 30°C. Following the indicated time periods, 1 ml aliquots were withdrawn and the nucleic acid fraction was extracted twice with 1 ml of phenol saturated with 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. Nucleic acids in the aqueous phase were recovered by ethanol precipitation and analyzed by gel electrophoresis.

*2.5. Northern hybridization*

RNAs obtained from either mitochondria or mitoplasts were subjected to 10% polyacrylamide gel electrophoresis in the presence of 7 M urea. The RNA bands developed on the gel were then transferred to a Biodyne transfer membrane (Pall) by using an electroblotter (Atto). After RNAs were fixed to the membrane by the irradiation of UV light at 254 nm for two min, hybridization was performed using the following DNA probes: 5′-TGCCGAAACCCGGGA-3′ complementary to cytoplasmic $tRNA^{Phe}$, 5′-TGTTTATGGAGTTGGG-3′ complementary to mitochondrial $tRNA^{Phe}$, 5′-CCAGGCCCGACCCTGCTTA-3′ complementary to the internal region of 5S RNA, and 5′-AAGCTTACAGCACCCGGA-3′ complementary to the 3′ region of 5S RNA.

*2.6. Sequence determination of RNA*

RNAs for sequencing were treated with *E. coli* alkaline phosphatase to remove their 5′-phosphate and labeled at the 5′-termini with [$\gamma$-$^{32}$P]ATP and $T_4$ polynucleotide kinase. For the labeling of their 3′-termini, RNAs were ligated with [$^{32}$P]pCp using $T_4$ RNA ligase. The labeled RNAs were sequenced by the method of Donis-Keller [20] using RNases $T_1$, $U_2$, PhyM and CL3.

## 3. Results and discussion

The nucleic acids extracted from bovine liver mitochondria which had been purified through sucrose density gradient centrifugation were analyzed using polyacrylamide gel electrophoresis. As shown in Fig. 1, a discrete band corresponding to the 5S RNA transcript of bovine liver origin was detected for the preparation of

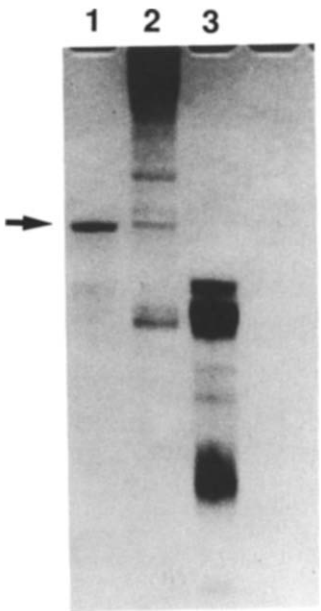


Fig. 1. Analysis of RNA fractions prepared from bovine mitochondria by polyacrylamide gel electrophoresis. Two μg of the 5S RNA transcript (lane 1), 25 μg of the mt RNA fraction (lane 2) and 25 μg of bovine cytoplasmic RNA fraction prepared from the cytoplasmic S-100 fraction (lane 3) were subjected to 10% polyacrylamide gel electrophoresis containing 7 M urea. The arrow indicates the 5S RNA band. The bands were stained with Toluidine blue.

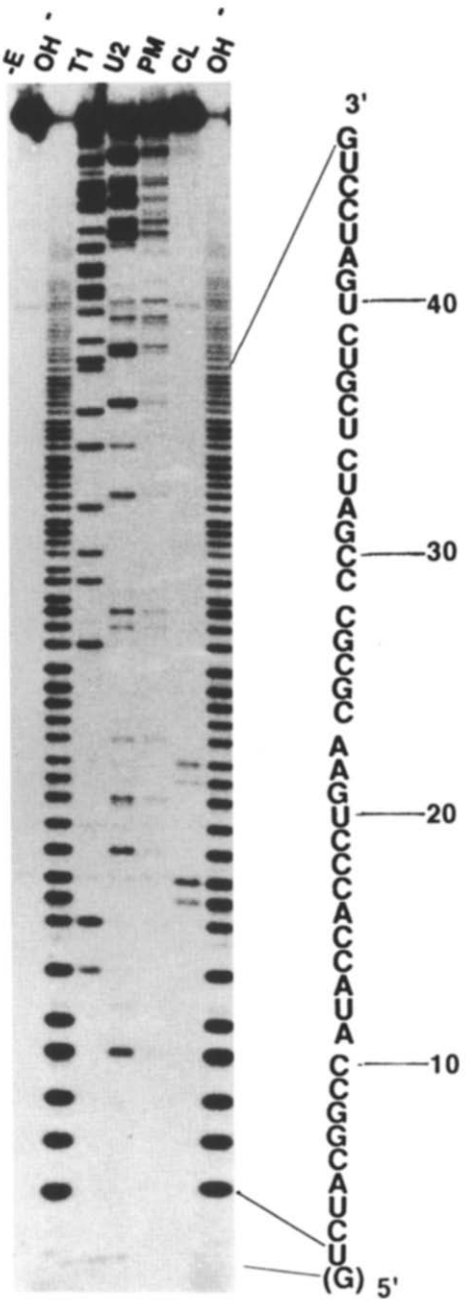


Fig. 2. Nucleotide sequence of 5′-terminal region of bovine liver mt 5S RNA-equivalent. The 5S RNA was purified by polyacrylamide gel electrophoresis containing 7 M urea and the sequence of the 5′ terminal region was determined by the method of Donis-Keller, as described in section 2. -E, OH-, T1, U2, PM and CL indicate no treatment, alkali digestion, RNase T1, RNase U2, RNase PhyM and RNase CL3 treatments, respectively. The sequence of the remaining part of the 5S RNA was also determined by the same procedure. The whole sequence was completely identical to the cytoplasmic 5S rRNA sequence.

mitochondrial nucleic acids. The band seemed to contain RNA because the material extracted from it was sensitive to RNase but resistant to DNase (data not shown). To our surprise, the band density clearly shows that the amount of this RNA in the mitochondria is considerably larger than that of each tRNA species of mitochondrial origin. The band was observed in the mitochondrial fraction but was scarcely discernible in the cytoplasmic S-100 fraction (Fig. 1), indicating that this RNA molecule is accumulated in the mitochondrial fraction. The RNA was also detected in the cytoplasmic ribosome fraction (data not shown).

This RNA of the mitochondrial fraction was further purified by gel electrophoresis and its nucleotide sequence, a part of which is shown in Fig. 2, was determined by Donis-Keller's method [20]. The complete sequence of 120 nucleotides thus determined was identical to that of the 5S RNA in the cytoplasm. No modified nucleotide was detected by the usual detection method using two-dimensional thin-layer chromatography [21].

To ascertain whether the RNA was derived from contamination by cytoplasmic RNA which might have attached nonspecifically to the mitochondrial outer membrane, the following experiments were carried out. First, mitochondria were treated with digitonin to remove their outer membranes, which gave rise to the mitoplasts. RNAs were then extracted with phenol from this mitoplast fraction. A band corresponding to the RNA with 120 nucleotides was also observed on the gel without significant loss in yield during the outer membrane removal process, as shown in Fig. 3b.

Mitochondria and mitoplasts were then treated with RNase A to degrade cytoplasmic RNAs with which they might have been contaminated during the preparation of both fractions. As shown in Fig. 3a, the band of 5S rRNA derived from the mitochondrial fraction purified by sucrose density centrifugation (p-MT) was not degraded, even after treatment with RNase A for 20 min.

On the other hand, the 5S rRNA from the mitoplast fraction (MP), which was also highly resistant against RNase A for up to 15 min (Fig. 3b), was degraded almost completely after 20 min. Judging from the following hybridization experiments, it seems that the degradation of this 5S RNA was caused by the disruption of the mitoplasts during treatment with RNase A for more than 15 min. Since the mitoplasts have only the inner membrane of the mitochondria, it is presumably easier for the mitoplast membrane to be disrupted as compared with the mitochondrial membrane.

A band corresponding to a part of the most abundant RNAs of the S 100 fraction (cytoplasmic tRNAs) appears in both mitochondria and mitoplasts (Fig. 3). It presumably consists of mitochondrial tRNAs with chain-lengths of around 70 nucleotides, and, perhaps in part of cytoplasmic tRNAs which came from the cytoplasm by import or by contamination. The possibility of tRNA import from the cytoplasm is not excluded because we have previousaly detected cytoplasmic $tRNA^{Met}$ in mitoplasts prepared by the same procedures as described here [22], although cytoplasmic $tRNA^{Phe}$ is not detected in either mitochondria or mitoplasts, as shown in Fig. 4a. Neither can we exclude the possibility

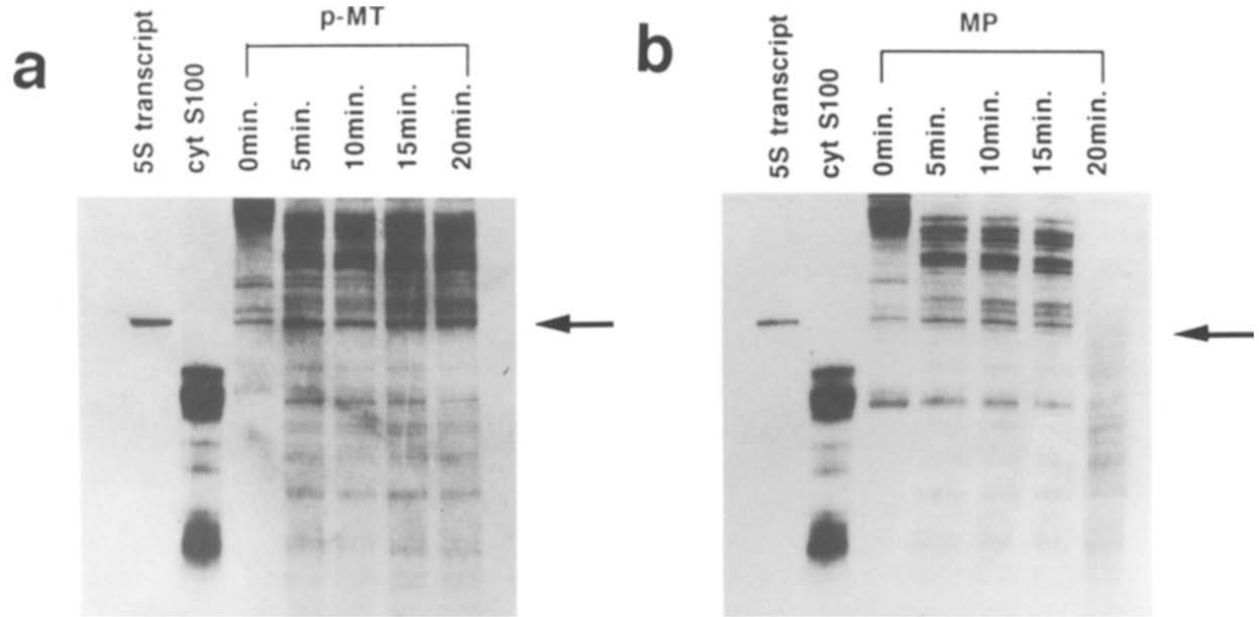


Fig. 3. RNase treatment of mitochondria and mitoplasts. Mitochondria purified by sucrose density gradient (p-MT) (a) and mitoplasts (MP) (b) were treated with 10 μg/ml of RNase A for 0, 5, 10, 15, 20 min as described in section 2. A 5S RNA transcript (5S transcript) was used as a size marker. The cytoplasmic S100 fraction (cyt S100) was also applied onto the gels as a control to estimate the position of the cytoplasmic tRNA fractions. Arrows indicate 5S RNA bands.

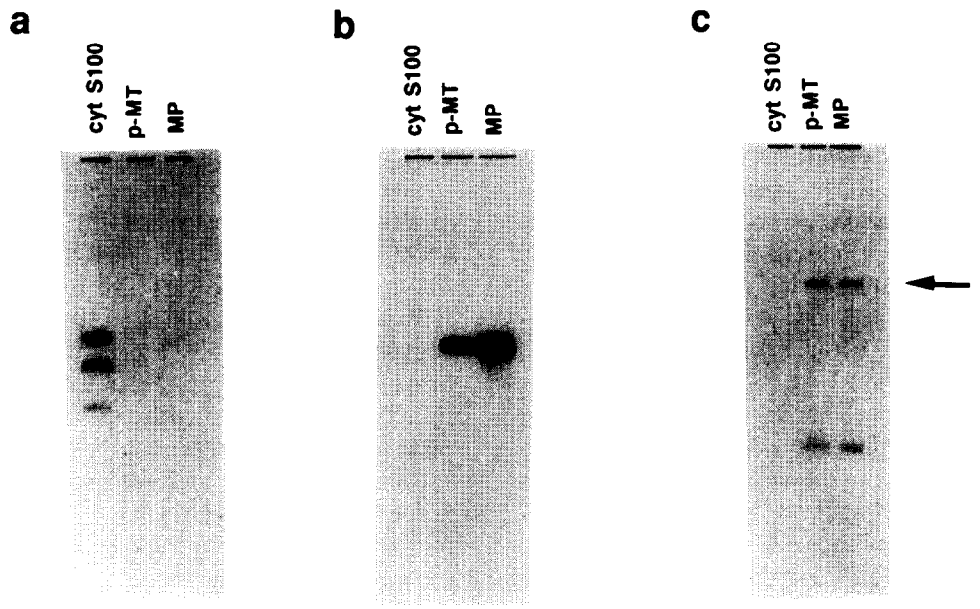


Fig. 4. Analysis of bovine mitochondrial RNA fractions using oligonucleotides complementary to cytoplasmic $tRNA^{Phe}$ (a), mt $tRNA^{Phe}$ (b) and the internal region of 5S RNA (c) as hybridization probes. A mitochondrial fraction purified by sucrose density gradient (p-MT) and a mitoplast fraction (MP) derived from the mitochondrial fraction were treated with RNase A for 10 min as described in the legend to Fig. 3. These two fractions and a cytoplasmic S-100 fraction (cyt S-100) were then treated with phenol and RNAs were recovered. Ten $\mu$g of each RNA fraction was subjected to 10% polyacrylamide gel electrophoresis containing 7 M urea and relevant RNA bands were detected by hybridization using each probe.

that only a part of the band is due to contamination of cytoplasmic tRNAs during the preparation of the organella, because, as shown in Fig. 3, the band strength appears to be larger in mitoplasts than in mitochondria and the band strength seems to decrease as the incubation periods are prolonged. However, the band remaining after 20 min. incubation in mitochondria must be mitochondrial tRNAs, as shown in Fig. 4.

To examine whether exogeneous RNAs are actually digested with RNase A under the above conditions, 6S RNA prepared from *E. coli* was added to the mitochondrial fraction and the mixture was treated with RNase A. After 5 min, the RNA fraction was extracted by phenol treatment and tested for the existence of 6S RNA together with 5S RNA derived from the mitochondria by gel electrophoresis. The result was that whereas the 5S RNA band was retained, the 6S RNA band completely disappeared (data not shown),thus demonstrating that the exogeneous RNAs outside the mitochondiral membrane are not protected by the membrane against RNase attack.

As shown in Fig. 3, high-molecular weight RNAs in both mitochondria and mitoplasts were degraded during incubation with RNase A. Since such a phenomenon is observed even in the absence of RNase (data not shown), it is presumably caused by some endogeneous RNases or processing enzymes contained in the mitochondria. We can exclude the possibility that the apparent increase in the intensities of the 5S RNA bands during incubation with RNase resulted from an increase of RNA fragments with the same chain length as 5S RNA, which are derived from the degradation of the high-molecular weight RNAs, from thefollowing experimental results. First, hybridization using a $^{32}P$-labeled 5S RNA probe (complementary to the internal region of 5S RNA) clearly showed that the intensities of the 5S bands in terms of the autoradiogram correlated well with those monitored by staining (data not shown), suggesting that the 5S RNA bands were not comtaminated by other RNA fragments. Second, the material obtained from the 5S band (in the lane corresponding to 15 min p-MT in Fig. 3a) was not separated further into sub-bands on gel electrophoresis under the different conditions, indicating that the material consists of a single species (data not shown).

To confirm that the 5S RNA is in fact localized in the inner part of mitochondria, hybridization experiments using oligonucleotide probes complementary to RNAs of either cytoplasmic or mitochondrial origin were carried out. Whereas cytoplasmic low-molecular weight RNA fractions hybridized to the probe for cytoplasmic $tRNA^{Phe}$, RNA fractions from both purified mitochondria and mitoplasts did not (Fig. 4a), suggesting that the mitochondrial RNA fractions were not contaminated by cytoplasmic RNA fractions. On the contrary, the probe complementary to mitochondrial $tRNA^{Phe}$ strongly hybridized to both the mitochondrial RNA fractions but not to the cytoplasmic RNA fraction. These results clearly exclude the possibility of cross-contamination of RNAs between cytoplasm and mitochondria in the preparation of mitohcondria as well as of mitoplasts. Using

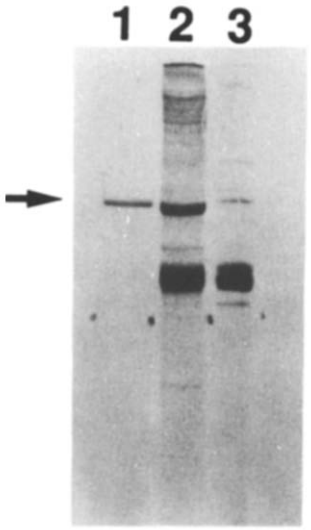


Fig. 5. Detection of 5S RNA-equivalents in chicken and rabbit liver mitoplasts by polyacrylamide gel electrophoresis. Twenty-five $\mu$g each of RNA fractions prepared from chicken (lane 2) and rabbit mitoplasts (lane 3) and 2 $\mu$g of bovine 5S rRNA purified by gel-electrophoresis from a bovine mitochondrial RNA fraction as a size marker (lane 1) were electrophorezed. The arrow indicates the 5S RNA band. The bands were stained with Toluidine blue.

two kinds of probes complementary to 5S RNA, strong bands were detected corresponding to about 120 nucleotides in the RNA fractions of both mitochondria and mitoplasts (Fig. 4c). The shorter band was detected only by using the probe complementary to the internal region of 5S RNA, but not by that complementary to the 3′ region, suggesting that the band contains a 5S RNA fragment which lacks its 3′ region. Thus, it can be concluded that 5S RNA is located within mitochondria.

To determine whether this is also the case for other animal mitochondria, the same experiment was carried out for mitoplasts from rabbit and chicken. The mitoplasts were prepared by the same procedures used for their bovine counterpart, from which RNA fractions were extracted and analyzed. Fig. 5 shows the electrophoretic patterns of mitochondrial RNA fractions from rabbit and chicken. A band corresponding to that of bovine mitochondrial 5S RNA was detected in both cases. The nucleotide sequence of the 5S RNA equivalent from rabbit liver mitochondria was identical to that of the cytoplasmic 5S rRNA (data not shown) These results suggest that 5S RNA generally exists in vertebrate mitochondria.

What is the role of the 5S RNA in mitochondria? It has already been clarified that 5S RNA is not included in ribosomal particles of metazoan mitochondria [23], nor does its gene exist in mitochondrial genomes [1], so it may not be directly related to protein synthesis. When mitoplasts were treated with 0.05% Triton X-100 to solubilize the 5S RNA fractions from membranes or other structural components, 5S RNA was eluted in a region corresponding to a complex with a molecular weight of several tens to a hundred-thousand by Sephacryl S200 gel filtration chromatography. This strongly suggests that 5S RNA is complexed with some proteins.

One possible explanation for the role of 5S RNA in mitochondria is that it is related to signal recognition in the process of protein transport, just like 4.5S RNA in *E. coli* and 7SL RNA in eukaryotic cytoplasm [24]. A second possibility is that 5S RNA is complexed with aminoacyl-tRNA synthetases, or tRNA and ribosomes, and functions to enhance the translation activity of the mitochondrial translation system [25].

To date, nuclear-encoded tRNAs has been reported to be imported into the mitochondria of *S. cerevisiae*, protozoa and plants. It is recognized that the RNA moieties of RNase MRP, which are known to process mitochondrial RNAs, are encoded by nuclear genomes of animals [13]. Recently, the import of tRNAs into mitochondria was demonstrated in vivo in *S. cerevisiae* [6] and in plants [26], as well as in vitro in *S. cerevisiae* [6]. In this case an energy source and cytoplasmic protein factor(s) are required for the tRNA transport, which is similar to the case of protein transport.

*Acknowledgements:* This work was supported by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science and Culture, Japan.

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