厚生労働科学研究費補助金（医療機器開発推進研究事業）
分担　報告書

糖部開環型三環性ヌクレオシドアナログを導入した蛍光性核酸プローブによる一塩基多型の検出

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研究要旨

糖部開環型三環性ヌクレオシドアナログ (**N**) の蛍光特性を利用した一塩基多型検出法を開発した。三環性アナログ **N** を中央に導入した４本のオリゴ核酸を用いることにより、DNA および RNA 鎖中の四種の一塩基多型を検出可能であることが明らかとなった。

A. 研究目的

上野は昨年の本研究において、糖部開環型三環性ヌクレオシドアナログ (**Q**) を含む DNA プローブを用いることにより一塩基多型の検出に成功している。本年度は、目視により一塩基多型を検出可能な、より長波長の蛍光を発するジアミノ基を導入した糖部開環型三環性ヌクレオシドアナログ (**N**) を含む DNA プローブを合成し、その一塩基多型検出能を検証した。

B. 研究方法

アナログ **Q** の合成中間体を原料とし、そのものにグアニジンを反応させることによりアナログ **N** を合成した。**N** の極大蛍光波長は、**Q** の極大蛍光波長よりも約 30 nm 長波長の 420 nm であった。また、**N** の蛍光強度は溶媒の極性に依存し、極性溶媒中では強く非極性溶媒中では低下した。そこで、本アナログをバルジ型に鎖の中央に導入した DNA プローブを設計・合成した。アナログに隣接する塩基が相補塩基と塩基対を形成する場合には、自由度の高い糖部開環型の三環性アナログ **N** は二重鎖外にフリップアウトし、水中に露出するため蛍光を発する。一方、隣接する塩基が、標的塩基とミスマッチの場合には、よりインターカレートし易い三環性アナログ **N** が二重鎖内に入り込み疎水性条件下に置かれるため蛍光が消光する。これにより一塩基多型を検出可能と考えた。薬剤代謝に関与する CYP2C9 の遺伝子を標的とした。

C. 研究結果

標的が DNA 鎖、RNA 鎖いずれにおいても隣接する塩基が標的塩基とマッチ配

列の場合に最も蛍光が強くなることが判明した。このことから本三環性アナログを用いることによりDNAおよびRNA鎖中の四種の一塩基多型を検出可能であることが明らかとなった。

D. 考察

標的がDNA鎖、RNA鎖いずれにおいても隣接する塩基が標的塩基とマッチ配列の場合に最も蛍光が強くなることが判明した。このことから本三環性アナログ **N** を用いることによりDNAおよびRNA鎖中の四種の一塩基多型を目視により検出可能である。

E. 結論

三環性アナログ **N** を中央に導入したオリゴ核酸は、DNAおよびRNA鎖中の四種の一塩基多型を検出する為の優れた蛍光プローブであることが明らかとなった。

F. 健康危険情報

総括研究報告書に記載

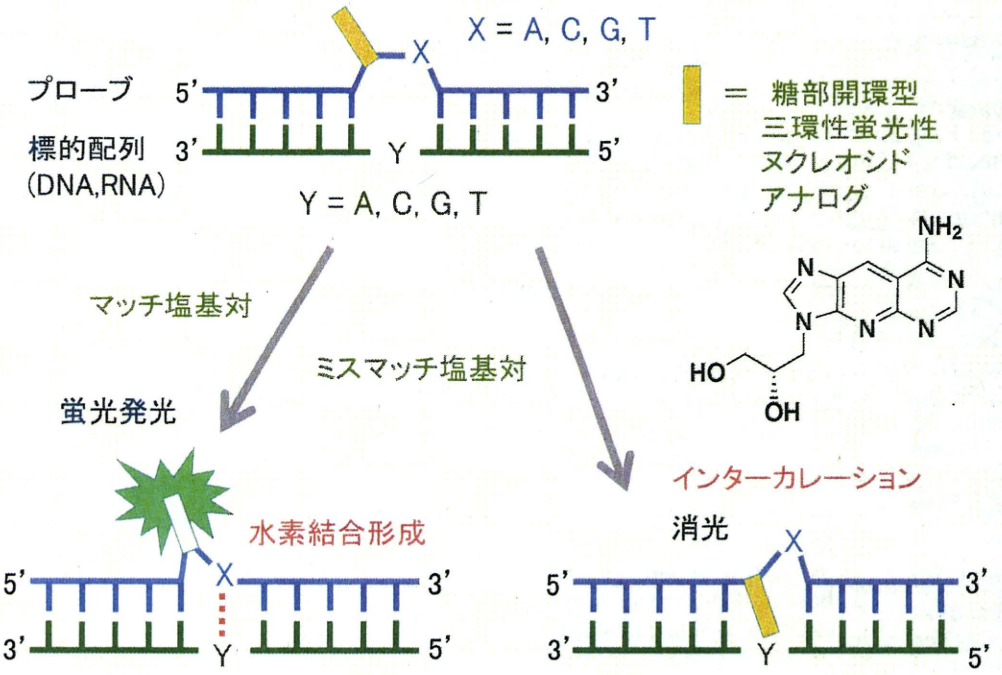
G. 研究発表

学会発表

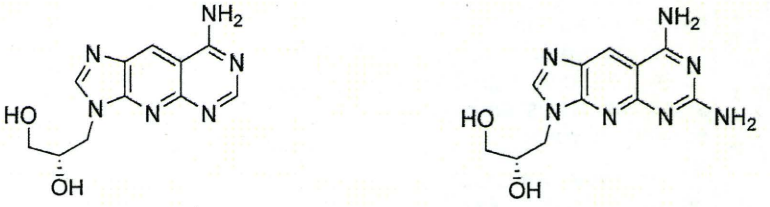
服部 麻由美、上野 義仁、北出 幸夫; 糖部開環型ヌクレオシドアナログを導入した核酸プローブの合成とその一塩基多型検出能; 第 20 回アンチセンスシンポジウム, 講演要旨集 p10, 2010.

H. 知的財産の出願・登録状況

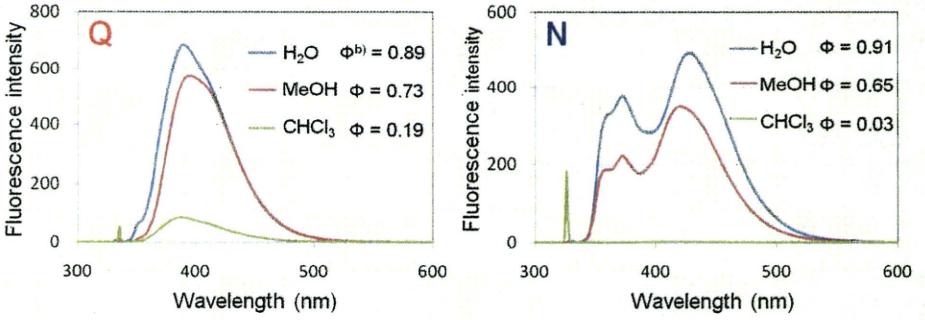
該当なし



三環性アナログQとNの構造とその蛍光特性



Photophysical data of nucleoside analog Q and N[a)]



a) Concentration of analog : 30 μM. b) Φ = fluorescence quantum yield.

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Fujiwara, Y., Kasashima, K., Saito, K., Fukuda, M., Fukao, A., Sasano, Y., Inoue, K., **Fujiwara T.**, Sakamoto, H.	Microtubule association of a neuronal RNA-binding protein HuD through its binding to the light chain of MAP1B	Biochimie	93(5)	817-822	2011
Hokii, Y., Sasano Y., Sato, M., Sakamoto, H., Sakata, K., Shingai, R., Taneda, A., Oka, S., Himeno, H., Muto, A., **Fujiwara, T.**, Ushida C.	A small nucleolar RNA functions in rRNA processing in *C. elegans*	Nucleic Acids Research	38(17)	5909-5918	2010
Ishii J., Fukuda N., Tanaka T., Ogino C., Kondo A.	Protein-Protein interactions and selection: yeast-based approaches that exploit guanine nucleotide-binding protein signaling	FEBS Journal	277	1982-1995	2010
Togawa S, Ishii J, Ishikura A, Tanaka T, Ogino C, Kondo A.	Importance of asparagine residues at positions 13 and 26 on the amino-terminal domain of human somatostatin receptor subtype5 in signalling	Journal of Biochemistry	147(6)	867-873	2010
Iguchi Y, Ishii J, Nakayama H, Ishikura A, Izawa K, Tanaka T, Ogino C, Kondo A.	Control of signalling properties of human somatostatin receptor subtype 5 by additional signal sequences on its amino-terminus in yeast	Journal of Biochemistry	147(6)	875-884	2010
Ishii J., Fukuda N., Tanaka T., Ogino C., Kondo A.	Protein-Protein interactions and selection: yeast-based approaches that exploit guanine nucleotide-binding protein signaling	FEBS Journal	277	1982-1995	2010

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Research paper

Microtubule association of a neuronal RNA-binding protein HuD through its binding to the light chain of MAP1B

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ABSTRACT

RNA-binding proteins (RBPs) play a vital role in the post-transcriptional regulation of gene expression during neuronal differentiation and synaptic plasticity. One such RBP family, the neuronal Hu protein family, serves as an early marker of neuronal differentiation and targets several mRNAs containing adenine/uridine-rich elements. Recently, we reported that one of the neuronal Hu proteins, HuD stimulates cap-dependent translation through interactions with eIF4A and poly (A) tail. Nevertheless, little is known with respect to how neuronal Hu proteins contribute to the local translation of target mRNAs in neuronal differentiation. Here, we found that neuronal Hu proteins, but not the ubiquitously expressed HuR protein, directly interact with the light chain of microtubule-associated proteins MAP1B (LC1). We also show that HuD simultaneously binds both RNA and LC1 *in vitro* and that it tightly associates with microtubules in cells in an LC1-dependent manner, raising the possibility that HuD recruits target mRNAs to microtubules. These results uncover the neuronal binding partners for neuron-specific Hu proteins and suggest the involvement of Hu proteins in microtubule-mediated regulation of mRNA expression within neuronal processes.

1. Introduction

Accumulating evidence suggests that RNA-binding proteins (RBPs) play crucial roles in regulating the expression of various neuron-specific genes at the post-transcriptional level [1,2]. The presence of mRNAs and ribosomes in neuronal dendrites implies that the translation of these mRNAs is essential for neuronal differentiation and some forms of synaptic plasticity [3,4]. A recent study indicated that several RBPs, including neuronal Hu proteins, are specifically expressed in neurons in the mouse brain [5]. Furthermore, it now appears that neuronal Hu proteins are essential for proper neuronal development and plasticity [4,6–8].

Hu proteins were initially identified as autoimmune antigens in human paraneoplastic neurologic disorders, and are highly conserved RNA-binding proteins among vertebrates [9–12]. Hu proteins show extensive similarity with the *Drosophila* ELAV (embryonic lethal abnormal visual system) protein [9,13], which is required for the development and maintenance of the *Drosophila* nervous system, and is involved in post-transcriptional regulation of a neuronal genes [12,14–16]. There are four members of the Hu family in vertebrates, each being expressed from distinct genes [12]. HuB, HuC, and HuD are specifically expressed in neurons [9,11,17–22]. In contrast, HuR is ubiquitously expressed [11,23]. All four Hu proteins contain three RNA-binding domains (RBDs) of the RNP-consensus sequence and a linker region separating the two N-terminal RBDs (RBDs 1 and 2) from the C-terminal RBD (RBD 3) [11–13,24]. Although the RBDs are conserved among all of the Hu proteins, the linker regions are somewhat diverse. Previous studies

Abbreviations: MAP1A, microtubule-associated protein 1A; MAP1B, microtubule-associated protein 1B; LC1, microtubule-associated protein 1B Light Chain; LC2, microtubule-associated protein 1B light chain; mRNP, messenger ribonucleoproteins.

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revealed that mammalian Hu proteins bind specifically to AU-rich elements (AREs) in the 3′ untranslated regions of various mRNAs [25]. RBD1 and RBD2 are responsible for the binding to AREs [22,26], whereas RBD3 contributes to poly(A)-binding of Hu proteins [22,27]. Currently Hu proteins are thought to recognize specific ARE-containing target mRNAs by binding to both AREs and to the poly(A) tail. Hu protein binding is thought to regulate the translation and/or stability of the bound mRNAs. Indeed, several putative target mRNAs of Hu proteins were reported to play crucial roles in cell differentiation and proliferation [26,28–37]. We have previously shown that HuD enhances cap-dependent translation in a eIF4A- and poly(A)-dependent manner [38]. We have also shown that the HuD needs to interact with both eIF4A and the poly(A) tail to induce neurite outgrowth in PC12 cells [38]. However, the mechanism of how the neuron-specific Hu proteins function in neuronal process extension pathway remains unclear.

In this study, we set out to find the neuronal binding partners for HuD by yeast two-hybrid screening. We show here that the light chain of microtubule-associated protein MAP1B (LC1) interacts with neuronal Hu proteins. Additional *in vitro* and *in vivo* analyses show that neuron-specific Hu proteins bind the light chains of both MAP1A and MAP1B, and form a ternary complex of LC1-HuD-RNA. Moreover, HuD associates with the microtubules network in a LC1-dependent manner. These findings provide novel insight into how neuron-specific Hu proteins associate with microtubules and their roles in neurons.

2. Materials and methods

2.1. Yeast two-hybrid screening

Yeast two-hybrid screening was carried out using the MATCHMAKER GAL4 Two-Hybrid System 2 (Clontech). A cDNA fragment encoding a mutant mouse HuD (HuDmt), which was described previously [39], was PCR-amplified using appropriate synthetic primers, introduced into the two-hybrid vector, pGBKT7, and transformed into yeast strain AH109. A mouse brain cDNA library (Clontech) was used for screening of proteins interacting with the mutant HuD.

2.2. Cell culture and transfection

PC12 and HeLa cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (For HeLa cells) and 5% horse serum (for PC12 cells).

PC12 cells were transiently transfected with 8 μg of plasmid DNA and 10 μl of Lipofectamine 2000 (Invitrogen) per 6 cm dish. HeLa cell were transiently transfected with 1.5 μg of plasmid DNA and 6 μl of PolyFect Transfection Reagent (QIAGEN) per 3.5 cm dish. The cells were harvested for biochemical analyses 24 h after transfection.

2.3. Plasmid construction and preparation of recombinant proteins

The plasmids encoding T7-tagged Hu proteins were described previously [40]. The plasmids encoding GST-Hu proteins were made by introducing the same cDNA fragments as in T7-tagged Hu plasmids followed by the segment encoding six histidine residues into the expression vector pGEX3X (GE). The plasmids encoding FLAG-tagged mouse LC1 and LC2 were made by introducing cDNA fragments corresponding to the carboxy-terminal 250 amino acids of MAP1B and the carboxy-terminal 222 amino acids of MAP1A into pFLAG-CMV (SIGMA), respectively. For the plasmids encoding myc-tagged LC1 and LC2 and the plasmids encoding MBP-LC1 and MBP-LC2, the same cDNA fragments as in FLAG-tagged LC plasmids were introduced into pCDNA3.1 (Invitrogen) and pMAL-c2 (New England BioLabs), respectively. Plasmids encoding GST- and MBP-fusion proteins were transformed into *Escherichia coli* XL2. GST- and MBP-fusion proteins were induced with 1 mM IPTG for 3 h and affinity-purified with glutathione-Sepharose 4B (GE) and nickel-agarose (QIAGEN), respectively.

2.4. In vitro binding experiment

GST pull-down assay was performed as described previously [39] and the bound proteins were separated by SDS-PAGE and immunoblotted with anti-myc monoclonal antibody (Roche) or anti-MBP antibody (GE). Poly(U) pull-down assay was performed as described previously [39] with a modification that the binding buffer contained 2 mg/ml heparin.

2.5. Immunological analyses

For immunoprecipitation analysis, HeLa cells transfected with FLAG-LC1 together with either T7-GFP, T7-HuD or T7-HuDmt were lysed in TNE buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and immunoprecipitated with anti-T7 monoclonal antibody (Novagen). The precipitated proteins were separated by electrophoresis and immunoblotted with anti-FLAG monoclonal antibody (SIGMA). For indirect immunofluorescence analysis to detect endogenous Hu proteins and LC1, NGF-differentiated PC12 cells were fixed with 4% formaldehyde and then incubated with HuD specific 16C12 antibody and anti-LC1 monoclonal antibody (Sigma), respectively. Subsequently, the cells were incubated with FITC-labelled anti-mouse IgG secondary antibody (DAKO) and analyzed using a fluorescence microscope (Olympus BX51). Detection of detergent-insoluble T7-HuD and FLAG-LC1 was performed according to the method [38] with some modifications: transfected cells were treated with 0.2% Triton X-100 for 1 min at room temperature, washed two times with PBS containing 10 μg/ml RNase A, 1 unit/ml RNase I for 5 min at room temperature, and were incubated with anti-T7 antibody and anti-FLAG rabbit polyclonal antibody (Sigma). Subsequently, the cells were incubated with Alexa 488-labelled anti-mouse IgG and Alexa 546-labelled anti-rabbit IgG secondary antibodies (Molecular Probe) and analyzed using a confocal laser-scanning microscope (Zeiss LSM5 PASCAL).

3. Result and discussion

3.1. Identification of LC1 as a HuD-interacting protein

The yeast two-hybrid screening for the proteins that interact with RNA-binding proteins often result in many false positives, possibly because of indirect and non-specific RNA-bridged interactions by the RNA-binding activity of Hu proteins in cells. To circumvent such a problem, we used a mutant HuD protein that cannot bind RNA (HuDmt, [39]) as a bait for two-hybrid screening for the binding partners for HuD in neurons. After screening of 8×10^5 transformants from a mouse brain cDNA library, we obtained two positive clones that encode the carboxy-terminal 206 and 172 amino acids of MAP1B (Fig. 1A). MAP1B is a polyprotein that is expressed predominantly in the neuronal cells and is involved in microtubule stability [41]. The light chain of MAP1B (LC1), consisting of the carboxy-terminal part of 250 amino acids, is produced together with the heavy chain after proteolytic cleavage. Since the clones that we isolated corresponded to the majority of LC1, it was suggested that full-length LC1 also interacts with HuD.

To confirm whether this interaction occurs in mammalian cells, we first performed cotransfection with T7-tagged HuD and FLAG-tagged LC1 into HeLa cells. Immunoprecipitation using anti-T7

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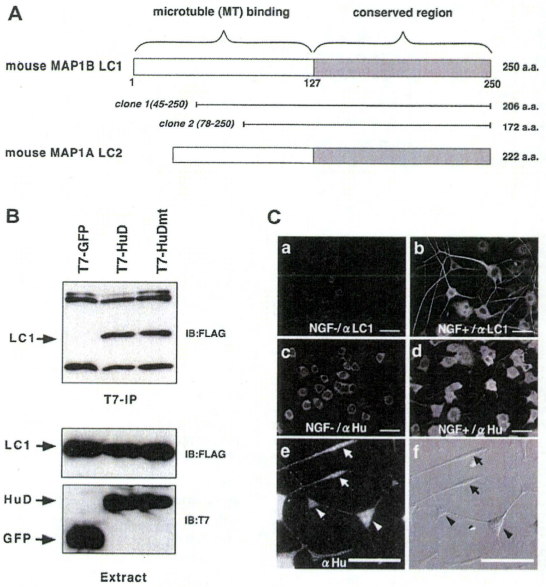

Fig. 1. Interaction between HuD and LC1 and their expression patterns in PC12 cells. A. Schematic representation of the light chains of MAP1B and MAP1A, LC1 and LC2. The LC1 regions encoded by the two-hybrid clones (clone 1 and clone 2) isolated are indicated. B. Specific coimmunoprecipitation of LC1 with HuD. FLAG-tagged LC1 was cotransfected with either T7-tagged GFP, HuD or HuDmt into HeLa cells. Extracts from the transfected cells were immunoprecipitated with anti-T7 antibody and the precipitates were immunoblotted (IB) with anti-FLAG antibody (T7-IP). For quantitative controls, each extract was immunoblotted with either anti-FLAG antibody or anti-T7 antibody (Extract). C. Colocalization of endogenous LC1 with neuron-specific Hu proteins. Undifferentiated (NGF–, a, c) and differentiated (NGF+, b, d, e: 5 days after addition of NGF) PC12 cells were immunostained with anti-LC1 antibody (a, b) or 16C12 antibody (c, d, e). Nomarski view of panel (e) is shown (f). Arrows and arrowheads indicate apical and branching regions of neuronal processes, respectively. Scale bars, 10 μm.

antibody followed by western blotting with anti-FLAG antibody showed that both wild-type and mutant HuD interact with LC1 (Fig. 1B). We then performed immunoprecipitation with monoclonal antibodies for endogenous neuron-specific Hu proteins and LC1 to evaluate the relevance of the interaction in PC12 cells, but failed to detect any interaction. This is possibly due to the monoclonal antibody binding perturbing the interaction between HuD and LC1. Thus, we examined endogenous expression of LC1 and HuD in PC12 cells before and after differentiation by nerve growth factor (NGF) (Fig. 1C). Immunostaining with LC1-specific monoclonal antibody showed that endogenous LC1 increased greatly after NGF stimulation and localized predominantly within neurites, indicating its association with microtubules. Endogenous HuD proteins also increased after NGF stimulation and localized within both the cytoplasm of cell bodies and neurites. Within neurites, they accumulated at the branching and apical regions. The localization patterns of the HuD and LC1 were somewhat different, but both were present in neurites, suggesting that HuD interacts with LC1 in PC12 cells.

3.2. Direct binding of the light chains to neuron-specific Hu proteins

LC2, the light chain of another microtubule-associated protein MAP1A, shows 49% amino acid sequence identity to LC1, and in particular, the carboxy-half region is highly conserved between the two light chains (76% identity) [43]. Thus, to test whether the LC1 binding to HuD is reproducible *in vitro* and whether LC2 also binds HuD, we performed GST pull-down assays using purified recombinant GST-HuD and purified light chains fused with maltose-binding protein (MBP). GST pull-down assays followed by western blotting with anti-MBP antibody showed that the binding between HuD and LC1 or LC2 is specific and direct (Fig. 2A). Next we wished to identify the region in LC1 that mediates the HuD interaction. Since there is less homology between the N-terminal region of LC1 and LC2, we reasoned that the C-terminal highly conserved region of LCs is involved in the binding to HuD. To examine this possibility, we performed GST-pull down assays using N-terminal and C-terminal deletion LC1 proteins and found that C-terminal but not with N-terminal region of LC1 copurifies with GST-HuD. In sum, we conclude that the highly conserved C-terminal region of LC1 is responsible for HuD binding.

Next, we tested whether only neural Hu proteins are bound to LC1. To address this question, we performed GST pull-down assays using MBP-LC1 and GST fusions of HuB, HuC and HuR. The results showed that LC1 also binds two other neural Hu proteins (HuB and HuC), but not HuR (Fig. 3). LC2 also bound the neural Hu proteins specifically (not shown).

3.3. Formation of the ternary complex between HuD, LC and RNA

Since HuD binds RNAs with AU-rich element (ARE), we examined whether the light chain binding and the RNA-binding are

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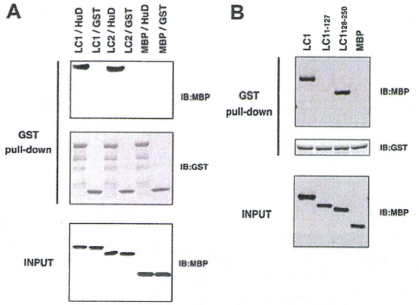

Fig. 2. Direct protein–protein interactions by HuD with LC1 and LC2. A. RNA-independent interactions between HuD and LCs. Purified MBP-LC1, MBP-LC2 and MBP were incubated with GST-HuD or GST proteins. GST pull-downs were examined for copurification of LCs by immunoblotting anti-MBP antibody (upper panel) and for pull-down efficiency by immunoblotting with anti-GST antibody (middle panel). Input MBP-LC1 and MBP proteins were shown in lower panel. B. C-terminal conserved region is required for LC1 to associate with HuD. Recombinant GST-HuD was incubated with the indicated MBP-LC1 proteins. GST-pull-downs were examined for copurification of LC1 proteins by immunoblotting with anti-MBP antibody (upper panel) and for pull-down efficiency by immunoblotting with anti-GST antibody (lower panel). Input LC1 proteins were shown below.

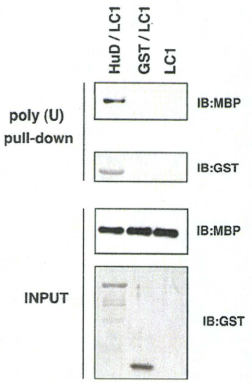

Fig. 4. Simultaneous binding of HuD to RNA and LC proteins *in vitro*. MBP-LC1 was incubated with GST-HuD or GST and pulled down with poly(U)-Sepharose beads, followed by immunoblotting with anti-MBP antibody. Lower panel showed that GST-HuD, but not GST, was pulled down with poly(U)-Sepharose beads. Input proteins were shown below.

compatible for HuD. To do so, we utilized the affinity of HuD for poly(U) RNA, which is carried by the first and second RNA-binding domains, representing its ARE-binding activity [44]. After mixing GST-HuD with either MBP-LC1, the reaction mixtures were pulled down with poly(U)-Sepharose beads followed by western blotting with anti-MBP antibody (Fig. 4). The results showed that LC1 were precipitated by poly(U)-Sepharose beads only in the presence of GST-HuD. This clearly showed that HuD can bind *in vitro* the light chains and RNA simultaneously, suggesting a similar ternary complex is formed in cells which is composed of HuD, LC and ARE-containing mRNA.

3.4. *LC1-dependent association of HuD with microtubules*

It was reported that LC1 associates with microtubules in the absence of its heavy chain when it was transiently expressed in non-neuronal cells [45,46]. In addition, it was suggested that HuB associates with microtubules in neuronal cells [47]. We then examined whether HuD also associates with microtubules and, if so, whether LC1 is involved in the association. To do so, we utilized a detergent-resistant property of microtubules with microtubule-associated proteins [45]. In this experiment, cells transfected with T7-tagged HuD with or without FLAG-tagged LC1 were mildly treated with a detergent before fixation and immunostaining with anti-T7 antibody (Fig. 5A). After the treatment, HuD remained in the cytoplasmic region only when it was coexpressed with LC1, whereas the control GFP disappeared even in the presence of LC1. LC1 remained filamentously in the cytoplasmic region, indicating its tight association with microtubules as described previously [45]. Moreover, the HuD co-localized precisely at the LC1 filaments after detergent treatment (Fig. 5B). On the basis of these results, we suggest that HuD associates with microtubules via its LC binding.

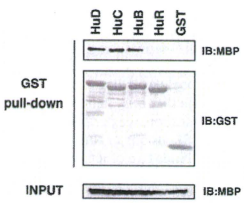

Fig. 3. LC1 specifically binds to neural Hu proteins. Purified MBP-LC1 was incubated with GST-HuB, GST-HuC, GST-HuD, or GST-HuR. GST pull-downs were examined for copurification of LC1 by immunoblotting with anti-MBP antibody (upper panel) and for pull-down efficiency by immunoblotting with anti-GST antibody (lower panel). Input LC1 proteins were shown below.

3.5. *Possible roles of neuron-specific Hu proteins*

Microtubules, whose dynamics are modulated by microtubule-associated proteins, play an important role in elaboration and maintenance of neuronal processes. Based on the timing of their expression, MAP1A and MAP1B are thought to regulate neuronal process extension during neuritogenesis and in mature neurons, respectively [48]. We have shown here that LC1 and LC2 are neuronal binding partners for the neuron-specific Hu proteins (Figs. 1 and 2). The specific binding of the light chains to neuron-specific Hu proteins parallels our previous finding that only the neuron-specific Hu proteins have an activity to induce neurite outgrowth when they are overexpressed in PC12 cells [40,49]. This suggests an important role of the light chains in the mechanism of the neurite induction by neuron-specific Hu proteins. In this connection, since functional analyses of LC1 revealed that it stabilizes microtubules and that the LC1 activity is inhibited in the presence of the MAP1B heavy chain, it was proposed that the heavy chain might act as the regulatory subunit of the MAP1B complex to control LC1 activity [50]. If this is the case, one possible role of neuron-specific Hu proteins may be to compete with the heavy chain inhibitory effect on LC1 and to promote microtubule stability in neuronal processes.

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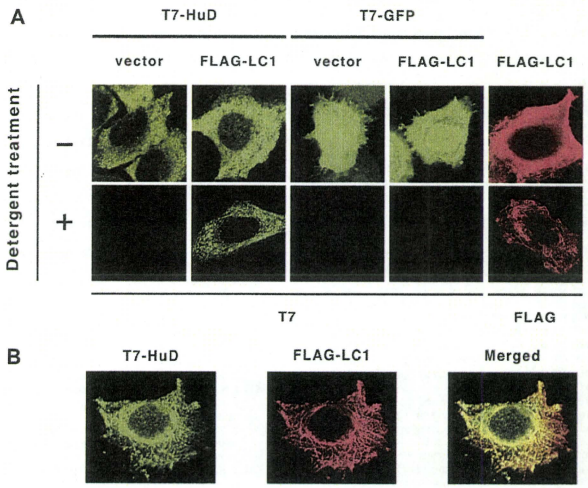

Fig. 5. LC1-dependent microtubule association of HuD. A. HeLa cells were transfected with T7- and FLAG-tagged proteins indicated above. Twenty-four hours after transfection, cells were treated with (+) or without (−) detergent solution, fixed, immunostained with anti-T7 antibody or anti-FLAG-antibody and analyzed by a confocal laser-scanning microscope. B. A HeLa cell expressing both T7-HuD and FLAG-LC1 was treated with detergent solution, fixed, immunostained with anti-T7 antibody (green) or anti-FLAG-antibody (red) and analyzed by a confocal laser-scanning microscope. Merged view is shown on the right (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Another possible and attractive role of neuron-specific Hu proteins is that they may be involved in microtubule-mediated regulation of transport and/or translation of some ARE-containing mRNAs, since HuD forms a ternary complex including one of the light chains and RNA *in vitro* (Fig. 4). This idea is consistent with LC1-dependent granular distribution of HuD on the LC1-associated microtubules in cells (Fig. 5). There is growing evidence that localization of mRNA and its regulated translation are important elements in determining cell polarity and identity in many organisms [51]. In particular, the microtubule network is known to serve as a major railway system for transport and localization of mRNA in neurons [52]. Thus, HuD may act as an adaptor for long-distance transport of some ARE-containing mRNAs along the neuronal processes. In fact, we previously demonstrated that HuD specifically interacts with TAP/NXF1, the primary mRNA nuclear export receptor and suggested that HuD is an adapter molecule for TAP/NXF1 involved in specific nuclear export and translational regulation in nerve cells [42]. On the other hand, Tretyakova et al. reported and proposed the model that LC1 tethers the mRNAs that form the mRNP (messenger ribonucleoproteins) complexes with TAP/NXF1 and several kinds of RNA binding proteins to microtubules [53]. Moreover, HuD and Tau mRNA were identified within same RNPs [54] and HuD was co-localized with GAP-43 mRNA [55]. Recently, we reported that HuD stimulates cap-dependent translation in a eIF4A- and poly(A)-dependent manner and this ability is prerequisite for neurite-inducing activity of HuD [38]. Taken together, we suggest that HuD may make a contribution to localized translation in neuron.

Finally, identification of mRNAs that associate with HuD-LC1 complex will provide important insights into the roles of Hu proteins in neuronal cells.

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A small nucleolar RNA functions in rRNA processing in *Caenorhabditis elegans*

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ABSTRACT

CeR-2 RNA is one of the newly identified *Caenorhabditis elegans* noncoding RNAs (ncRNAs). The characterization of CeR-2 by RNomic studies has failed to classify it into any known ncRNA family. In this study, we examined the spatiotemporal expression patterns of CeR-2 to gain insight into its function. CeR-2 is expressed in most cells from the early embryo to adult stages. The subcellular localization of this RNA is analogous to that of fibrillarin, a major protein of the nucleolus. It was observed that knockdown of C/D small nucleolar ribonucleoproteins (snoRNPs), but not of H/ACA snoRNPs, resulted in the aberrant nucleolar localization of CeR-2 RNA. A mutant worm with a reduced amount of cellular CeR-2 RNA showed changes in its pre-rRNA processing pattern compared with that of the wild-type strain N2. These results suggest that CeR-2 RNA is a C/D snoRNA involved in the processing of rRNAs.

INTRODUCTION

The ribosome is an essential component of the cell and is deeply involved in the regulation of cell growth, proliferation and differentiation (1–7). Despite its significance, little is known about the biogenesis of the ribosome. In eukaryotes, hundreds of nonribosomal proteins and small nucleolar RNAs (snoRNAs) are involved in ribosome assembly, and it is thought that they are elaborately coordinated to form a functional ribosome in response to cell circumstances (8,9).

Four RNA species are involved in eukaryotic ribosomes: the 18S ribosomal RNA (rRNA), which is a component of the small subunit (40S), and the 5.8S rRNA, 28S rRNA (25S or 28S, depending on the organism) and 5S rRNA, which are components of the large subunit (60S). The 18S, 5.8S and 28S rDNAs are aligned in tandem and cotranscribed by RNA polymerase I in the nucleolus (10). The primary transcripts contain extra sequences designated the 5′ external transcribed spacer (ETS), 3′-ETS, internal transcribed spacer 1 (ITS1) and ITS2, which are removed to produce the mature 18S, 5.8S and 28S rRNAs (11). Studies of rRNA maturation in yeast, frogs and mammals have shown that there are many similarities and differences in the pathways of pre-rRNA processing among species (6,12–15).

Nine snoRNAs (U3, U14, U17/E1/snR30, snR10, U8, U22, MRP, E2 and E3) are related to pre-rRNA processing. U3, U14 and U17/E1/snR30 are evolutionarily conserved snoRNAs that are required for the maturation of 18S rRNA (16–18). MRP snoRNAs are found in a

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

variety of eukaryotic organisms. According to yeast analyses, this RNA functions in the cleavage of ITS1. However, it is not yet clear whether MRP snoRNA is involved in rRNA maturation in mammals and other organisms too. Similarly, snR10 has been identified in several organisms, but it has not been confirmed that this RNA functions in the cleavage of pre-rRNAs other than in yeast. The remaining snoRNAs, U8, U22, E2 and E3, have only been found in vertebrates to date. U3, U14, U8 and U22 snoRNAs share some features with C/D snoRNAs, and U17/E1/snR30, snR10, E2 and E3 RNAs share features with H/ACA snoRNAs (6,17–23).

Caenorhabditis elegans is a good model to study how various physiological phenomena occur based on the molecular systems in cells. However, little is known about *C. elegans* ribosome biogenesis. Until recently, the cleavage sites of the pre-rRNAs remained unclear (24). U3 is the only snoRNA that probably functions in pre-rRNA cleavage in *C. elegans* (25). Although several RNomic studies have suggested candidates for snR10, U14, U17 and MRP RNA homologs in *C. elegans* (23,26,27), there is no biochemical or genetic evidence that they are involved in pre-rRNA processing. Moreover, it is unclear whether RNAs homologous to U8, U22, E2 or E3 are expressed in *C. elegans*.

In our previous study, we identified 19 novel ncRNA candidates in *C. elegans* (28,29). Seven showed the characteristic secondary structure of the modification-guiding C/D or H/ACA snoRNAs (28). None of the remaining 12 candidates showed marked similarity to any known ncRNA sequence in the database. Here, we show that one of these RNAs, designated CeR-2 RNA and also known as CeN21 or Ce9 (26,30), has several characteristics of a C/D snoRNA and is likely to function in rRNA processing.

MATERIALS AND METHODS

Caenorhabditis elegans strains and culture

Worms were grown and maintained by standard procedures (31). Strain MT16939 containing a *cer-2a* mutant allele (*n5007*), which lacks a region encompassing nts 8 428 510–8 429 125 of chromosome IV, was generated by ethane methyl sulfonate mutagenesis. The *cer-2a* (*n5007*) worms were outcrossed to N2 animals six times before analysis. The *cer-2a* (*n5007*) worms were genotyped by polymerase chain reaction (PCR) using the primers cer-2a-1129 (5′-CCACAAGCTTTCATTTAGAGG-3′) and cer-2a+300 (5′-TTTACAATTGTTGATTACGTTTTTA CCTC-3′). The positions and directions of these primers are shown in Figure 4A.

Plasmids

The plasmid pT7CER2aSP6 was designed to express CeR-2 RNA from a T7 promoter and to express an antisense CeR-2 RNA from an SP6 promoter. The DNA fragments were amplified by nested PCR. The first PCR was performed with a *C. elegans* genomic DNA template and primers CeR2aT7F1 (5′-CGACTCACTATAGTCTTCA GTATGGGTCA-3′) and CeR2aSP6R (5′-AGGTGACA CTATAGTTCAGAATCGGGCTGG-3′), which contain T7 promoter and SP6 promoter sequences (underlined), respectively. The PCR mixture was then used as the template for the second PCR, which was performed with the primers EcoRIT7 (5′-AAAGAATTCTAATACGACT CACTATA-3′) and PstISP6 (5′-AAACTGCAGATTTAG GTGACACTATA-3′). The resulting DNA fragment was digested with *Eco*RI and *Pst*I and ligated into the same sites of pUC19. Clones pT7U18SP6 and pT7U17SP6 were prepared by the same procedures. The primers used for the first PCR were U18(T7)F 5′-CGACTCACTATAGTGGC AGTGATGATCACAAATC-3′, U18(SP6)R 5′-AGGTG ACACTATAGTGGCTCAGCCGGTTTTC-3′, U17(T7)F 5′-CGACTCACTATAGCTCGACATGTGA CTAGCG-3′ and U17(SP6)R 5′-AGGTGACACTATAG ATTTGTAATTTGCATGGTTTG-3′. EcoRIT7 and PstISP6 were used as the second PCR primers. The clone containing part of the rRNA precursor sequence has been described previously (24).

Northern hybridization

Total RNAs from N2 and MT16939 were extracted with TRIzol Reagent or the PureLink RNA Mini Kit (Invitrogen). The RNAs were resolved on formaldehyde-containing 1.0% agarose gel or by 7 M urea/6% polyacrylamide gel electrophoresis and blotted onto Biodyne Plus membrane (Pall Corporation). The blot was hybridized with RNA probes prepared with the DIG RNA Labeling Kit (Roche). The templates for RNA synthesis were amplified by PCR from the clones as described earlier. The probes used for detecting the pre-rRNA intermediates have been described previously (24). Immunoprecipitation with anti-2,2,7-trimethylguanosine (TMG) antibody K121 was based on a previous work (25).

RNA fluorescence *in situ* hybridization of small RNAs and immunofluorescence analysis

Specimens for RNA fluorescence *in situ* hybridization and immunofluorescence analysis were prepared as described previously (32,33). The RNA probes were prepared using the DIG RNA Labeling Kit (SP6/T7) or with MEGAscript/SP6 (Ambion, Inc.) and fluorescein-12-UTP (Enzo Industries, Inc.). The DIG haptens were detected by Cy3-conjugated IgG fraction monoclonal mouse anti-DIG antibody (1:400 dilution; Jackson ImmunoResearch Laboratories, Inc.; lot 59998) or fluorescein-conjugated anti-DIG Fab fragment (1:25 dilution; Roche). Cy3-conjugated Affinipure goat anti-mouse IgG (H+L) antibody (1:400 dilution; Jackson ImmunoResearch Laboratories, Inc.) or Alexa-488-conjugated anti-fluorescein/Oregon Green rabbit polyclonal IgG (1:100 dilution; Molecular Probes, Inc.) was used as the secondary antibody. The signals of the fluorescein-labeled RNA probes were enhanced with Alexa-488-conjugated anti-fluorescein/Oregon Green rabbit antibody and anti-rabbit chicken IgG antibody (1:100; Molecular Probes, Inc.). Fibrillarin (FIB-1) was visualized with the anti-FIB-1 antibody 38F3 (1:400 dilution; EnCor Biotechnology Inc.) and Cy3-conjugated

anti-mouse antibody. The nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). The signals were observed under a light fluorescence microscope (Olympus, BX60) or a confocal laser microscope (Leica, DMI6000, TCS SP5 and Olympus, FV1000D, FV10-ASW).

RNA interference

The template DNAs for *in vitro* transcription were generated by PCR using yk cDNA clones (*nop10* [C25A1.6], yk1472f12; *nop56* [K07C5.4], yk1604e03), which were kind gifts from Dr Yuji Kohara. Oligonucleotides containing the T7 promoter sequence were used as primers (T7ME774FW, 5′-TTTTAATAAT ACGACTCACTATAGCTTCTGCTCTAAAAGCTGC G-3′ and T7ME1250RV, 5′-TAAAGATAATACGACTC ACTATAGTGTGGGAGGTTTTTTCTCTTAG-3′). Sense and antisense RNAs were synthesized using MEGAscript/T7 (Ambion). The resulting RNAs were annealed to generate double-stranded RNAs. The double-stranded RNAs (1 μg/μl) were injected into L4 worms.

RESULTS AND DISCUSSION

Isolation of CeR-2 RNA

CeR-2 RNA is one of the 19 small novel candidate ncRNAs isolated from *C. elegans* (28,29). This RNA is encoded in the intergenic region of chromosome IV. A homologous gene is found on the same chromosome: the former is designated *cer-2a* and the latter is designated *cer-2b* (chromosome IV, nts 4 880 726–4 880 851, complement). A BLAST search showed that the genomes of nematodes closely related to *C. elegans*, *C. briggsae* (CB3; 2006), *C. brenneri* (6.0.1 contigs), *C. remanei* (15.0.1 supercontigs) and *C. japonica* (3.0.2 supercontigs), have two, six, three and two homologs of CeR-2, respectively, but no similar sequence was found in any other organism. Figure 1A shows the sequence alignment of the CeR-2 RNA gene with its homologs, constructed using Align X of the software package Vector NTI ver. 9 (Invitrogen). There are two highly conserved sequences: one corresponds to the sequence of CeR-2 RNA itself and the other is located ~30–40-bp upstream from the CeR-2-RNA-coding region. The upstream sequence (−32 to −69 in Figure 1A) is similar to the proximal sequence element, which functions as a promoter in U snRNA genes (34–37). This suggests that CeR-2 RNA is transcribed by RNA polymerase II and has a TMG cap at its 5′ end. To examine this possibility, we performed immunoprecipitation with the anti-TMG antibody K121 against the total RNAs extracted from mixed-stage worms. The anti-TMG-precipitated RNAs were then subjected to Northern blot analysis (Figure 1B). As a control for immunoprecipitation specificity, U6 snRNA, which does not have a TMG cap (25), was also monitored by Northern blot analysis. Most of the CeR-2 RNA was detected in the K121 precipitate, whereas the majority of the U6 snRNA was detected in the supernatant. This indicates that most CeR-2 RNAs in the cell have a TMG cap at their 5′ ends, like other *C. elegans* small RNAs, such as the U snRNAs, SL RNAs (38) and U3 snoRNA (25).

Spatiotemporal expression patterns and subcellular localization of CeR-2 RNA

We examined the spatiotemporal expression patterns and subcellular localization of CeR-2 RNA by *in situ* hybridization. As shown in Figure 2A, CeR-2 is expressed in most cells in L1 larvae and it continues to be expressed until adulthood in both the somatic and germline cells. Consistently, Northern hybridization of total RNA revealed that CeR-2 is expressed constitutively during the four larval stages and the adult stage (Figure 2B).

To determine the subcellular localization of CeR-2, we inspected the large intestinal nucleus (Figure 2C). Foci of CeR-2 RNA were detected inside the nucleus and completely overlapped with the signals for FIB-1 (Figure 2C). This indicates that CeR-2 RNA localizes in the nucleolus. Thus, CeR-2 RNA shares the most characteristic feature of the snoRNAs that function in pre-rRNA processing or rRNA modification.

Changes in the nucleolar localization of CeR-2 RNA by knockdown of the C/D snoRNP gene

There are two major snoRNA families, the C/D snoRNA and H/ACA snoRNA families. Four core proteins interact specifically with the RNAs of each family: fibrillarin/NOP1, NOP56, NOP58 and a 15.5-kDa protein with C/D snoRNAs, and dyskerin/NAP57, NHP2, NOP10 and GAR1 with H/ACA snoRNAs (39). We expected that some of these proteins would interact with CeR-2 RNA and contribute to its function and nucleolar localization. Therefore, we knocked down the expression of the *C. elegans* C/D snoRNP gene *nop56* (K07C5.4) and the H/ACA snoRNP gene *nop10* (C25A1.6). The effects of RNA interference (RNAi) were assayed by *in situ* hybridization and immunofluorescence.

As expected, knockdown of *nop56*, which encodes a C/D snoRNP-specific protein, markedly reduced the signals of CeR-2 RNA and FIB-1 (Figure 3A). In several cells of the *nop56* (*RNAi*) worm, both signals were observed in a limited region at the periphery of the nucleolus (Figure 3A and B, arrows). U18 snoRNA, a typical C/D snoRNA, was used as an internal control to monitor the knockdown effect of *nop56*. The level of nucleolar localization was reduced by the reduction of Nop56 but not by the reduction of Nop10. The effect of *nop10* knockdown was confirmed by the reduction of the U17 H/ACA-type snoRNA (Figure 3C). It is likely that CeR-2 RNA is a member of the C/D snoRNA family and functions in the nucleolus together with C/D snoRNPs. A recent study based on a microarray indicated that the knockdown of *nop58* or *snu13* leads to a severe reduction in CeR-2 RNA (CeN21 RNA in refs. 26 and 40). This also supports our prediction that CeR-2 RNA is a C/D snoRNA.

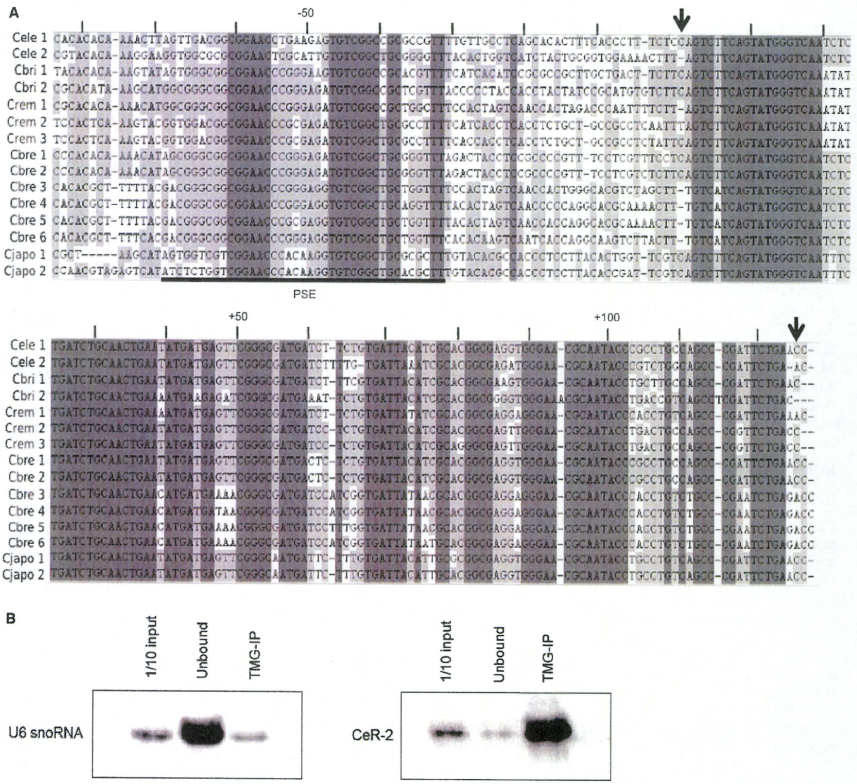

Figure 1. Sequence alignment of CeR-2 RNA genes. (**A**) Sequence alignment of *cer-2a* and its homologs. The sequences conserved among all 15 homologs are shaded in dark gray. Partially conserved sequences are shaded in light gray. Cele1, *cer-2a*; Cele2, *cer-2b*; Cbri1, *C. briggsae* homolog of *cer-2a*; Cbri2, *C. briggsae* homolog of *cer-2b*; Crem1-3, three homologs found in *C. remanei*; Cbre1-6, six homologs found in *C. brenneri*; Cjapo1 and Cjapo2, two homologs found in *C. japonica*. The putative 5′- and 3′-terminal nucleotides of the RNA-coding region in *cer-2a* are indicated by arrows. (**B**) *Caenorhabditis elegans* TMG-capped RNA was precipitated with the anti-TMG antibody K121. Northern hybridization was performed with antisense probes of CeR-2 RNA and U6 snRNA against the precipitate (TMG-IP) and the supernatant (Unbound). A 1/10 amount of total RNA input was also separated on the same denaturing gel and blotted onto a nylon membrane (1/10 input).

A mutant lacking *cer-2a* shows an altered accumulation pattern of pre-rRNAs

To determine the function of CeR-2 RNA, we produced a mutant strain lacking *cer-2a* and designated it MT16939. This deletion mutant of *cer-2a* (*n5007*) lacks a 618-bp sequence on chromosome IV (Figure 4A and B). Northern blot analysis indicated that CeR-2 RNA was reduced by about half to one-third in *cer-2a* mutants compared with that in wild-type N2 worms (Figure 4C). The remaining CeR-2 RNA signal on Northern blots originated from *cer-2b*, a homolog of *cer-2a*, with a 98% identical sequence. The homozygous mutant of *cer-2a* showed a slow growth phenotype and abnormal fertilization, especially in old adults. We tried to generate a mutant of *cer-2b*, but were unsuccessful.

Because CeR-2 RNA exhibits the characteristics of a box C/D-type snoRNA, it was expected that CeR-2 RNA would function in guiding the 2′-*O*-methylation of

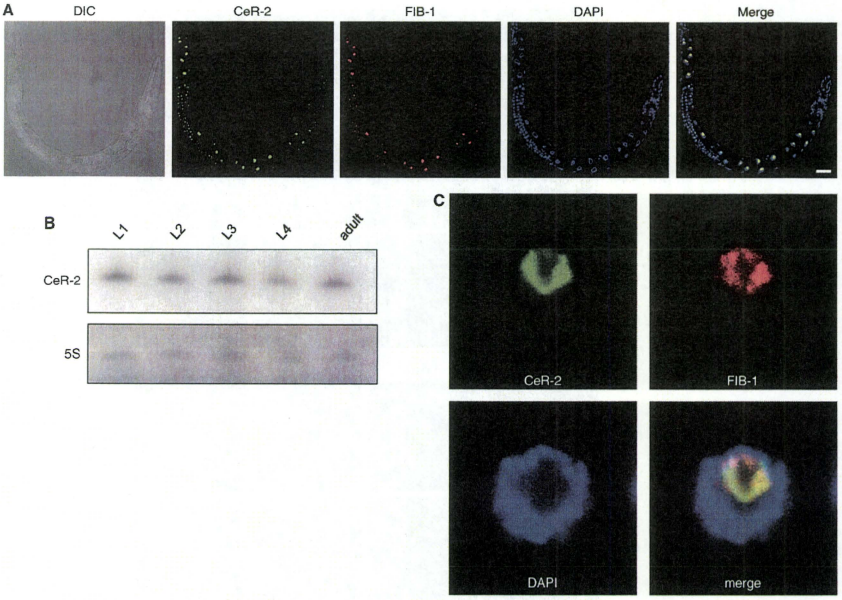

Figure 2. Spatiotemporal expression patterns of CeR-2 RNA. (**A**) CeR-2 RNA was detected by *in situ* hybridization (CeR-2, green). FIB-1 was costained with an anti-FIB antibody (FIB-1, red). DIC, Nomarski differential interference contrast microscopic image; DAPI, DNA visualized with DAPI staining; Merge, merged images of CeR-2, FIB-1, and DAPI. Scale bar, 5 μm. (**B**) Northern hybridization of CeR-2 RNA against total RNAs prepared from the larvae of each stage (L1 to L4) and adults. The 5S rRNA band on the blot was detected with methylene blue staining. (**C**) CeR-2 RNA foci in the nucleus overlapped with FIB-1 foci. The stained images of an intestinal nucleus are magnified. CeR-2 RNA (green) and FIB-1 (red) were stained with DAPI.

rRNAs and/or in processing pre-rRNAs (1–6,41,42). One important structural feature of modification guiding C/D snoRNAs is that the region upstream from the D or D′ box encompasses 10–21 bp complementary to the target rRNA around the modification site. When the duplex is <9 bp or contains substantial AU or GU pairs, methylation becomes less efficient (42). Therefore, searching for a complementary sequence to an rRNA sequence longer than 10 bp is one way to assess the function of a C/D snoRNA in guiding the 2′-*O*-methylation of rRNA. We searched for a complementary sequence between CeR-2 RNA and *C. elegans* rRNAs that was longer than 10 bp. However, no such continuous sequences were found, which reduced our expectation that CeR-2 RNA functions in guiding the modification of rRNAs.

An outline of *C. elegans* rRNA processing was established in a previous study (Figure 5A) (24). In N2 worms, five pre-rRNA processing intermediates were detected and designated a, b, c, c′ and d. We designed four probes (probes 3, 4, 5 and 6), with reference to the study of Saijou *et al.* (24), to detect each intermediate. Figure 5B shows the results of Northern hybridization of RNA extracts from N2 (wild-type) worms and MT16939 (*cer-2a* [*n5007*]) worms with these probes. Intermediate c′ accumulated more in MT16939 than in N2, as shown in the results for probes 4, 5 and 6 (Figure 5B, lanes 4, 6 and 8, respectively). The accumulation of c′ indicates that the efficiency of processing the large subunit rRNA precursor into 5.8S and 26S rRNAs was reduced in the mutant after the cleavage of the pre-rRNA in ITS1. The results for probe 3 showed reductions in intermediates b and/or d in the mutant (Figure 5B, lanes 1 and 2, respectively). Intermediate d, detected with probe 4, did not differ significantly between N2 and MT16939 (Figure 5B, lanes 3 and 4, respectively), which indicates that intermediate b, which is a precursor of 18S rRNA, was reduced in the mutant. Therefore, MT16939 exhibited changes in the accumulation patterns of the rRNA precursors. This suggests that CeR-2 RNA is involved in the processing of pre-rRNAs, although it is

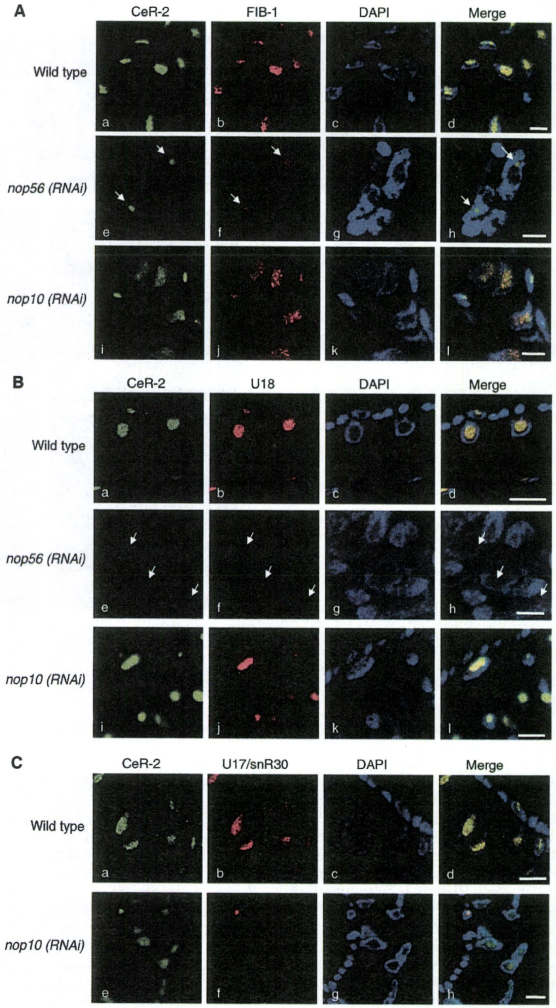

Figure 3. Knockdown of *nop56* reduced the nucleolar localization of CeR-2 RNA. Each nucleolar factor was observed by *in situ* hybridization or immunofluorescence analysis. The white arrows indicate the accumulation of CeR-2 RNA, FIB-1 or U18 snoRNA in the foci, which newly appeared in the nucleoplasm after the knockdown of *nop56*. Scale bars, 5 μm. (**A**) Costaining of CeR-2 RNA (green, panels a, e and i) and FIB-1 (red, panels b, f and j) in *nop56* (RNAi) and *nop10* (RNAi) worms. (**B**) Costaining of CeR-2 RNA (green, panels a, e and i) and U18 C/D snoRNA (red, panels b, f and j) in *nop56* (RNAi) worms and *nop10* (RNAi) worms. (**C**) Costaining of CeR-2 RNA (green, panels a, e and i) and U17/snR30 H/ACA snoRNA (red, panels b, f and j) in *nop10* (RNAi) worms. Panels c, g and k, DAPI staining; panels d, h and l, merged images.

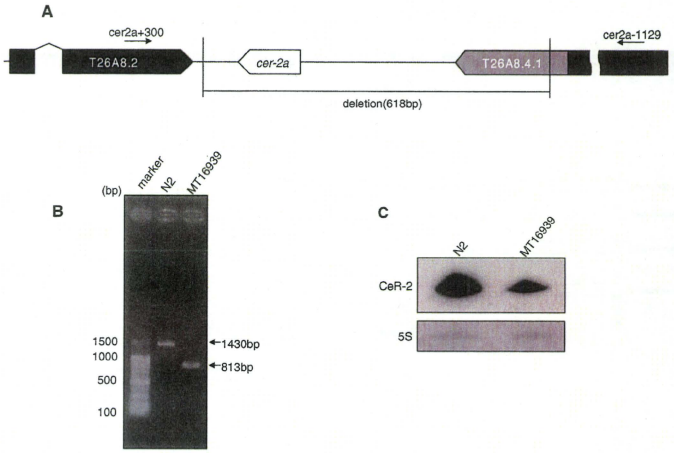

Figure 4. CeR-2 RNA expression in MT16939. (**A**) Genomic map around *cer-2a* of MT16939 on chromosome IV. The primers cer-2a-1129 and cer-2a+300 refer to the PCR primers used for genotyping N2 and MT16939. There are two putative protein genes, T26A8.2 and T26A8.4.1, adjacent to *cer-2a*. (**B**) Genotyping of N2 and MT16939 worms by single-worm PCR. Marker, 100-bp DNA ladder; N2, PCR product amplified from the genomes of N2 worms; MT16939, PCR product amplified from the genomes of MT16939 worms. The PCR products are indicated by arrows with their putative lengths (bp). (**C**) Northern hybridization of total RNAs from N2 and MT16939 with the CeR-2 RNA antisense probe. The 5S rRNA band on the blot, stained with methylene blue, is shown subsequently.

still possible that CeR-2 RNA guides the modification of rRNAs.

Because MT16939 lacks a part of the 3′ untranslated region (UTR) of the gene upstream from *cer-2a* (T26A8.4.1), it is possible that T26A8.4.1 affects the processing of rRNAs. The homozygous mutation of T26A8.4.1 is lethal and it is therefore difficult to analyze the pre-rRNA patterns. We tried to detect pre-rRNAs in worms in which T26A8.4.1 was knocked down. Although no obvious changes in the pre-rRNA pattern were observed, we cannot completely rule out the possibility that T26A8.4.1 is relevant to rRNA processing.

U8 and U22 snoRNAs are C/D-type snoRNAs related to the cleavage of rRNA processing. They have been identified only in vertebrates and their homologs have not been found in invertebrates to date. Some features of CeR-2 RNA shown here revealed similarity to those of U8 snoRNAs: both RNAs are TMG-capped, have features of C/D snoRNAs, and are involved in the cleavage of ITS2. In addition, the 5′-terminal sequence of CeR-2 RNA has the potential to base pair with that of *C. elegans* 26S rRNA, as U8 snoRNA base pairs with the 5′ terminus of 28S rRNA (Figure 6). There is a sequence similar to the conserved LSm binding motif of U8 snoRNA in the second stem-loop (Figure 6). Thus, CeR-2 RNA is an excellent candidate to be a U8 ortholog.

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Conflict of interest statement. None declared.

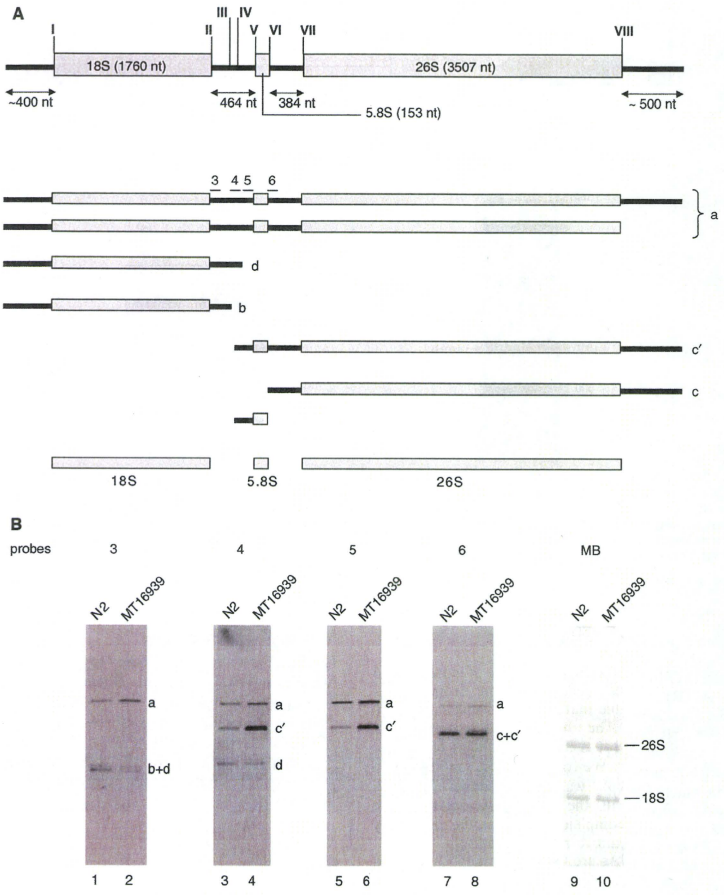

Figure 5. pre-rRNAs in MT16939. (**A**) Schematic representation of pre-rRNA processing pattern. Probes 3, 4, 5, and 6 used for the hybridization are indicated by bars above intermediate **a**. The cleavage sites (I–VIII) are indicated along the precursor **a** with reference to the study of Saijou *et al.* (24). The length of the rDNA is based on nucleotide data for GenBank accession number X03680. (**B**) Comparison of the pre-rRNA patterns of N2 and MT16939. Northern hybridization of the RNAs from N2 (lanes 1, 3, 5 and 7) and MT16939 (lanes 2, 4, 6 and 8) with each probe. Intermediates a, b, c, c′ and d indicate the pre-rRNAs shown in Figure 5A. The membrane was stained with methylene blue (lanes 9 and 10, MB), and the 26S and 18S rRNA bands are shown.

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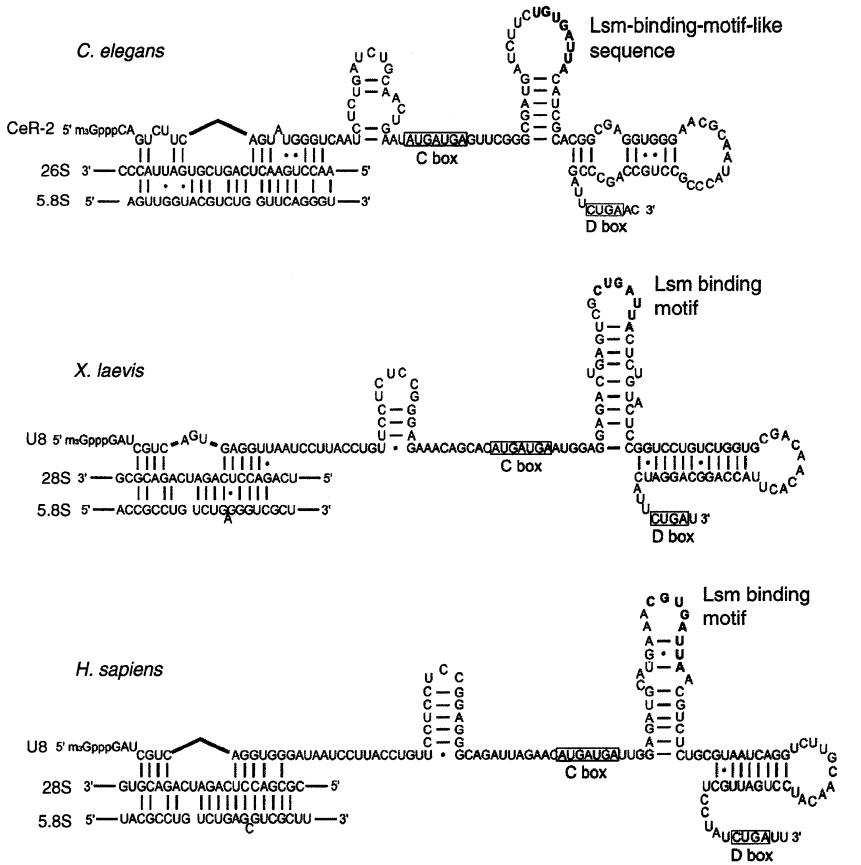

Figure 6. Comparison of the predicted secondary structures of CeR-2 RNA and vertebrate U8 snoRNAs. Potential base pairing between the 5′ region of CeR-2 RNA and the 5′ region of 26S rRNA is shown and compared with that between U8 snoRNA and 28S rRNA of *Xenopus laevis* and *Homo sapiens* (20,43). There are three stem-loops in the remaining 3′ part of CeR-2 RNA, which appear in the secondary structure of U8 snoRNAs in similar regions: one upstream from the C-box and the other two between the C-box and the D-box (open rectangles). The LSm binding motif is a conserved octameric sequence located in the loop of the second stem-loop in U8 snoRNAs (44). Similar sequences (six of the conserved eight nucleotides, LSm-binding-motif-like sequence) are also found in the loop of the second stem-loop of CeR-2 RNA (bold letters).

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