The mRNA-like noncoding RNA Gomafu constitutes a novel nuclear domain in a subset of neurons

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Summary
Recent transcriptome analyses have revealed that a large body of noncoding regions of mammalian genomes are actually transcribed into RNAs. Our understanding of the molecular features of these noncoding RNAs is far from complete. We have identified a novel mRNA-like noncoding gene, named Gomafu, which is expressed in a distinct set of neurons in the mouse nervous system. Interestingly, spliced mature Gomafu RNA is localized to the nucleus despite its mRNA-like characteristics, which usually act as potent export signals to the cytoplasm. Within the nucleus, Gomafu RNA is detected as numerous spots that do not colocalize with known nuclear domain markers. Gomafu RNA is extremely insoluble and remains intact after nuclear matrix preparation. Furthermore, heterokaryon assays revealed that Gomafu RNA does not shuttle between the nucleus and cytoplasm, but is retained in the nucleus after its transcription. We propose that Gomafu RNA represents a novel family of mRNA-like noncoding RNA that constitutes a cell-type-specific component of the nuclear matrix.

Key words: mRNA export, Noncoding RNA, Nuclear domains, Nuclear matrix

Introduction
The classical view that RNA molecules are dedicated solely to protein synthesis has been rapidly changing (Mattick, 2003; Andersen and Panning, 2003; Cao et al., 2006). In particular, extensive studies have revealed the importance of the RNA-interference pathway mediated by small RNAs, which controls target gene expression both at the transcriptional and post-transcriptional levels (reviewed by Mello and Conte, 2004; Ambros, 2004). Other examples of functional noncoding RNAs include Xist and roX that control dosage compensation of the sex chromosomes (reviewed by Brockdorff, 2002; Kelley, 2004), the long polyadenylated transcript of Air that controls genomic imprinting of genes located nearby (reviewed by Braidotti et al., 2004), regulators of transcription factors or RNA polymerases such as 7SK, SRA, B2 and Evf1 (reviewed by Goodrich and Kugel, 2006) and 7SL, a key component of signal recognition particles involved in secretion and membrane protein targeting to the endoplasmic reticulum (reviewed by Keenan et al., 2001). In certain noncoding RNAs such as Srg1 or the antisense transcript of Ime4, transcription itself, rather than the transcribed product, is essential for its function to regulate the expression of neighboring genes (Martens et al., 2004; Hongay et al., 2006). Besides these characterized noncoding RNAs, recent transcriptome analyses of higher vertebrates have identified a large number of transcripts with unknown function, most of which possess mRNA-like characteristics [i.e. multiple exons and a poly(A) tail at the 3'-terminus] but do not encode conserved peptide sequences (Ota et al., 2004; Carninci et al., 2005; Cheng et al., 2005). In Drosophila, a number of mRNA-like noncoding RNAs are expressed in a temporary and spatially regulated manner during development. However, the physiological functions of these mRNA-like noncoding RNAs as well as their molecular features are largely unknown.

The nucleus of higher eukaryotes is highly organized and consists of functionally distinct domains that contain machineries for specific processes such as transcription, splicing, ribosome processing and assemblies (reviewed by Spector, 2001; Misteli, 2000; Lamond and Earnshaw, 1998). These domains include the nucleolus, interchromatin granule clusters (IGCs, or nuclear speckles), paraspeckles, PML bodies and Cajal bodies (or coiled bodies). Unlike cytoplasmic organelles, these nuclear domains are not separated by a lipid membrane and are identified by the presence of specific marker proteins. Each domain also contains a particular group of RNA molecules; rRNAs are transcribed and processed in the nucleolus, snRNAs are enriched in the IGCs or Cajal bodies (reviewed by Spector, 2001; Lamond and Earnshaw, 1998) and A-to-I edited double-stranded RNAs are recruited to the paraspeckles (Prasanth et al., 2005), suggesting that each discrete domain plays a unique role in RNA metabolism within the nucleus. Although extensive proteomic approaches have provided a comprehensive view of the protein components of nuclear domains (Saitoh et al., 2004; Andersen et al., 2002), little is known about the RNA constituent, especially regarding the precise component of nuclear poly(A)+ hnRNAs recognized
by an oligo dT probe (reviewed by Hall et al., 2006; Lamond and Spector, 2003).

During the course of screening for genes expressed in a particular subset of neurons, we have identified a novel noncoding gene, named Gomafu. Despite its mRNA-like characteristics, Gomafu RNA was distributed throughout the nucleoplasm in a spotted pattern, in strong contrast with the cytoplasmic localization of general protein-coding mRNAs. The Gomafu RNA-containing granules did not coincide with known nuclear domains and remained intact after the nuclear matrix preparation. Together with the restricted expression in particular neurons in the nervous system, we propose that Gomafu RNA is a member of a novel family of mRNA-like noncoding RNAs that constitute a cell-type-specific component of the nuclear matrix.

**Results**

Identification of Gomafu RNA – a novel noncoding RNA localized to the nucleus

In the neural retina, six types of neuron and one type of glia are arranged into three nuclear layers, making characteristic laminated structures. To understand the molecular mechanisms underlying specification of each cell type, we initially attempted to identify genes expressed in a distinct set of retinal neurons by screening subtraction libraries prepared from nascent ganglion and cone photoreceptor cells – two early-born cell types in the mouse retina (Fig. 1A). After differential screening and subsequent in situ hybridization, we have identified eight genes in putative ganglion cells and three genes in cone cells with unknown functions, which are expressed in the embryonic day 14 (E14) retina (Fig. 1B,C). We were
especially interested in the subcellular localization of the transcript of clone 1.46, because the hybridization signals were exclusively detected in the nucleus (Fig. 1D) unlike common mRNAs that are usually detected in the cytoplasm. A blast search (http://www.ncbi.nlm.nih.gov/blast/) revealed that the cDNA sequence of clone 1.46 was identical to the 3' region of an EST clone AK028326, which has recently been designated RNCR2 (retinal noncoding RNA 2) (Blackshaw et al., 2004). However, analysis of the mouse genome using NCBI MapViewer (http://www.ncbi.nlm.nih.gov/mapview/) uncovered multiple overlapping EST clones at the 5' flanking region of RNCR2, suggesting that the 3-kb-long EST clone was a partial fragment of the nuclear-localizing RNA. We thus assembled EST clones in silico and an overlapping RT-PCR product in vitro, which led us to predict that cDNA of the nuclear-localizing RNA had a total size of 8.7 kb. Subsequent sequence comparison with the mouse genome revealed that the gene of the nuclear-localizing RNA consisted of seven exons and six introns located on chromosome 5 (Fig. 2A) and did not contain repeat sequences such as LINEs or SINEs (data not shown). Comparison of the EST and genomic sequences also revealed no signs of A-to-I editing, unlike the previously reported nuclear-retained mRNA mCAT2 (Prasanth et al., 2005). To confirm the size of the transcript and its tissue distribution, we performed multiple-tissue northern blot analysis and detected a single band at the expected size (~9 kb) in poly(A)+ RNAs derived from the adult brain but not in the other tissues (Fig. 2B). To further confirm that we obtained a cDNA clone containing the 5' end of the transcript, we carried out northern blot analysis using shorter 5' RNA fragments digested at the defined site using RNaseH (Fig. 2C), which allowed more precise measurement of the length of the transcript. For this experiment, mRNA from E14 mouse brain was hybridized with oligo DNA probes and subsequently treated with RNaseH – an enzyme that specifically digests DNA-RNA hybrids. As a control, RNA was transcribed from the predicted transcription initiation site using chimeric DNA template consisting of the T7 promoter and 5' region of the cDNA we obtained, and the in vitro synthesized RNA was subjected to RNaseH treatment. Both of the short RNA fragments were detected at the same size by northern blot analysis after the RNaseH treatment (Fig. 2C), suggesting that we successfully obtained a full-length cDNA clone. We subsequently carried out RT-PCR analysis and found that at least ten alternatively spliced isoforms existed (Fig. 2D). There are 34 potential open reading frames and the longest one encoded a 179 amino acid peptide sequence (residues 333-872 of AB294525); however, a BLASTp search (http://www.ncbi.nlm.nih.gov/blast/) revealed that none of them showed significant homology with known or predicted proteins in the database (data not shown). Together with its nuclear localization, we concluded that the nuclear-localizing RNA did not encode protein products. Putative polyadenylation signals (ATTAAA) were found at the 3' end of the gene, and the transcripts were enriched in the poly(A)+ fractions as expected (Fig. 2E). We then examined the turnover rate of this transcript to test if it was a component of the metabolically stable, nucleus-restricted poly(A)+ RNA population previously described (reviewed by Lamond and Spector, 2003; Hall et al., 2006). The expression level of the nuclear-localizing RNA was significantly decreased 24 hours after the treatment of α-amanitin (Fig. 2F), and its stability was not significantly different from that of β-actin mRNA (Fig. 2F).

The genomic organization (multiple exons with introns) and
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the presence of the poly(A) tail of this nuclear-localizing RNA fulfilled the feature of mRNAs transcribed by RNA polymerase II (pol II), although mRNAs are normally exported to the cytoplasm after removal of their intron sequences (reviewed by Stutz and Izaurralde, 2003; Reed and Hurt, 2002; Cullen, 2003). We named this atypical mRNA-like noncoding gene Gomafu after the spotted distribution of its transcripts within the nucleus as described below (‘Gomafu’ means ‘spotted pattern’ in Japanese). Blast searches on the human genome revealed homologous sequences in a syntenic region in chromosome 22q12. A number of EST clones were mapped to the locus, suggesting that human Gomafu was actually transcribed. No homologous sequences were found in the genome of non-mammalian organisms.

Gomafu expression starts in differentiating neural progenitor cells and persists in a subset of postmitotic neurons

We next examined the expression pattern of the Gomafu gene during retinal development. Expression was initiated in a subpopulation of cells at the central region of the E11.5 retina (Fig. 3A), when postmitotic retinal ganglion cells are first formed. The number of Gomafu-expressing cells increased gradually as development proceeded (Fig. 3B) and the expression was confined to the retinal ganglion and amacrine cells in the mature retina at postnatal day 7 (P7) (Fig. 3C). The expression of Gomafu was not restricted to the retinal neurons, but was widely observed in the neural tissues (Fig. 3D-I). Whole-mount in situ hybridization in E8.5 and E9.5 embryos showed intense expression in the entire nervous system (Fig. 3D,E). In the brain, the expression persisted throughout the embryonic stages to adulthood (Fig. 3F-I). At the stage when most of the progenitor cells become postmitotic (P7), Gomafu was expressed in a particular subset of neurons including mitral cells in the olfactory bulb (Fig. 3J,J′), pyramidal cells in the layer 5 of the cerebral cortex (Fig. 3K,K′), CA1 pyramidal neurons in the hippocampus (Fig. 3L,L′), and large neurons in the pontine nucleus (Fig. 3M,M′). In all cases, the Gomafu transcript was exclusively concentrated in the nucleus, but not in the cytoplasm (Fig. 3J-M).

To distinguish whether Gomafu was expressed in proliferating neural progenitor cells or postmitotic neurons during earlier stages, we double-stained the E14 retina for

Fig. 3. Expression pattern of Gomafu during development analyzed by in situ hybridization. (A-C) Gomafu expression in the retina at E11.5 (A), P0 (B), and P7 (C). Note that the expression was restricted to the amacrine (am) and retinal ganglion cells (gc) at P7 (C). The inset in (A,B) shows higher magnification view. Arrowheads show the nuclear localization of the transcript. (D,E) Whole-mount in situ hybridization stained for Gomafu transcript at E8.5 (D) and E9.5 (E). Signals were observed in the entire neural tissues. Inset in E shows the signals obtained with the sense probe. (F-I) Gomafu expression in the brain at E14 (F), P0 (G), P7 (H), and adult (I). The horizontal line with intense staining in I is derived from artifactual folding of the section. (J-M,J′-M′) Higher magnification images of the section shown in H. The signals were observed in the nucleus of mitral cells in the olfactory bulb (J,J′), pyramidal neurons of layer V in the cerebral cortex (K,K′), CA1 pyramidal neurons in the hippocampus (L,L′), and large neurons in the pontine nucleus in the hindbrain (M,M′). Nuclear counterstaining signals for DAPI are shown in J′-M′ in light blue. (N,O) The marginal region of E14 retina was double labeled for Gomafu RNA (green) and a proliferating cell marker PCNA (magenta in N-N′) or BrdU (magenta in O-O′). Note that most of the Gomafu-expressing cells are post mitotic and labeled green in the merged images (N,O). Arrowheads indicate cells positive for both Gomafu RNA and the proliferation markers. Bars, 20 µm (A-C,J-O); 500 µm (D-I).
Gomafu and the proliferating-cell marker PCNA (Fig. 3N). A majority of Gomafu-expressing cells in the retina did not express PCNA, suggesting that they were nascent postmitotic neurons (Fig. 3N). A small population of PCNA-positive cells (10±1.5%; n=10) co-expressed Gomafu at lower levels (arrowheads in Fig. 3N-N’), suggesting that a small number of progenitor cells, presumably those undergoing the final S phase before dividing to produce postmitotic neurons, started to express Gomafu. A similar result was obtained when proliferating cells were labeled with BrdU (Fig. 3O-O’). These results suggested that Gomafu expression begins in differentiating progenitor cells and continues in particular types of postmitotic neurons.

Gomafu RNA is localized to a novel nuclear domain
To gain further insight into the sub-nuclear localization of Gomafu RNA, we detected the hybridized probes without enzyme-mediated signal amplification. The high-resolution fluorescent in situ signals were observed under a confocal microscope. In the E14 retinal cells, Gomafu RNA was detected as numerous spots scattered throughout the nucleus (Fig. 4A). These spots were also observed in mitotic cells, in which the signals did not coincide with condensed chromosomes detected by a DNA-binding dye Sytox (Fig. 4A, inset). Higher magnification images revealed that Gomafu RNA was preferentially found in a region where weaker DNA signals were observed (arrows in Fig. 4B-B”). Since this expression pattern was reminiscent of IGCs containing a number of proteins essential for mRNA splicing (reviewed by Hall et al., 2006), we next tried to simultaneously detect Gomafu RNA and an IGC marker SC35 on a single optical section of the confocal images. However, we observed no overlap in the localization of Gomafu RNA and immunolabeling with the anti-SC35 antibody (Fig. 4C). In addition, actinomycin D treatment, which induces formation of large IGCs (Huang et al., 1994), did not affect the spotted distribution of Gomafu RNA (Fig. 4D), further suggesting that Gomafu RNA was not localized to the IGCs. We also failed to detect co-localization of Gomafu RNA with the nucleolus or PML bodies, visualized by antibodies against fibrillarin or PML, respectively (Fig. 4E,F).

To determine whether Gomafu RNA was localized to a novel nuclear domain, we performed double-labeling experiments using HeLa cells expressing Gomafu cDNA under the control of the CAG promoter (Niwa et al., 1991). The use of the human cell line allowed us to examine additional antibodies for nuclear domains, which are available for human antigen but do not cross-react with the mouse antigen. As in intact retinal cells, the exogenous Gomafu-RNA in HeLa cells was exclusively observed within the nucleus in a spotted pattern (Fig. 4G-K), indicating that the nuclear localization of Gomafu RNA is not dependent on the endogenous Gomafu promoter or its intron sequences. The fluorescent signals of Gomafu RNA did not overlap with immunolabeling for IGCs, paraspeckles, nucleolus, PML bodies or Cajal bodies (Fig. 4G-K).

To date, at least three mRNA-like noncoding RNAs are known to be expressed in the mouse nervous system: (1) Xist RNA that paints the X-chromosome in the female nucleus and controls dosage compensation (Clemson et al., 1996); (2) Evf1/2 RNA that regulates expression of a neighboring gene Dlx5 or Dlx6 (Feng et al., 2006); (3) Air RNA that is necessary for genomic imprinting of neighboring genes (Braidotti et al., 2004). However, none of these noncoding RNAs overlapped with the spotted signals of Gomafu RNA (Fig. 4L-N), suggesting that Gomafu RNA comprises a unique population of mRNA-like noncoding RNAs within the nucleus. Taken
Spliced, mature Gomafu RNA accumulates in the nuclear matrix

Since inefficient splicing leads to an accumulation of mRNA precursors in the nucleus (Custodio et al., 1999), we examined whether nuclear Gomafu RNA was properly processed by performing in situ hybridization using probes against intron sequences of the Gomafu gene. The intron probes detected one or two foci in the nucleus, which presumably corresponded to the site of its transcription (Fig. 5A,A′, arrows). The spotted pattern was observed only when we used exon-specific probes (Fig. 5A,A′), indicating that the correctly spliced form of Gomafu RNA, but not its precursor product, spreads from its transcription site and accumulates in the nucleus. These observations were consistent with the northern blot analysis, where we detected essentially a single band of the mature product without major precursor or processed products (Fig. 2B).

The nuclear matrix is a biochemical fraction that remains after extractions with nonionic detergents, DNasel and high-salt buffers, and the fraction contains a number of regulatory proteins involved in transcription, splicing, replication and DNA repair (Stein et al., 2003; Pederson, 2000; Berezney, 1991). We therefore examined whether Gomafu RNA was associated with this fraction in primary cultures of E14 retinal cells. The successful preparation of the nuclear matrix was confirmed by the disappearance of DNA detected with Sytox staining (Fig. 5C). The spotted pattern of Gomafu RNA was essentially unchanged in these extracted cells, suggesting that Gomafu RNA is a component of the nuclear matrix (Fig. 5B,C). To further confirm its localization to the nuclear matrix, we prepared a mouse neuroblastoma cell line Neuro2A expressing Gomafu. The exogenous Gomafu RNA also remained intact in the cell line after the nuclear matrix preparation (Fig. 5D,E), suggesting that Gomafu RNA itself, but not its genomic locus or promoter sequences, possesses nuclear-matrix-binding properties. Northern blot analysis using RNA from each biochemical fraction revealed that Gomafu RNA was highly insoluble and almost exclusively enriched in the nuclear matrix fraction (Fig. 5F).

Gomafu RNA escapes nuclear export

We next asked whether Gomafu RNA shuttled between the nucleus and cytoplasm or whether it remained within the nucleus after its transcription. To distinguish between these possibilities, we carried out a heterokaryon assay using Neuro2A cells expressing Gomafu and the other Neuro2A cells expressing a nuclear-localizing EGFP (NLS-EGFP). These transfectants were fused with polyethylene glycol and the resultant heterokaryons were subjected to fluorescent in situ hybridization. As expected, NLS-EGFP was detected in all nuclei in the heterokaryon (Fig. 6A′,B′,C′). However, Gomafu RNA was observed only in a subset of nuclei (arrowheads in Fig. 6A′,B′,C′). These results suggest that Gomafu RNA escapes nuclear export and remains in the nucleus after its transcription.

Because the Gomafu transcript possesses multiple stop codons, it is a potential target of nonsense-mediated decay (NMD), which causes rapid degradation of cytoplasmic mRNAs that contain premature stop codons (Maquat, 2004). Thus, it could be argued that the nuclear accumulation of Gomafu RNA was due to its instability once it is exported to the cytoplasm, rather than its active retention within the nucleus. To test this possibility, we prepared primary cultured cells derived from E14 brain and treated them with cycloheximide, which blocks protein translation and thus accordingly inhibits NMD, a process dependent on translation (Carter et al., 1995). Poly(A)+ RNAs were then purified from cytoplasmic or nuclear fractions, and the expression of Gomafu RNA was examined by northern blot. As a control, we examined the expression of a spliced product of a small nucleolar RNA (snoRNA) host gene UHG (Frischmeyer and Dietz, 1999), which is rapidly degraded by the NMD mechanism. In a control culture, Gomafu RNA was highly enriched in the nuclear fraction as expected (Fig. 6D). Whereas the UHG transcript was almost undetectable in the control cytoplasmic fraction, the cycloheximide treatment induced a rapid increase in the amount of the spliced UHG transcript (Fig. 6D). By contrast, no increase in the amount of

![Fig. 5.](https://example.com/fig5.png) Intron-removed mature Gomafu RNA is localized to the nuclear matrix. (A–A′) Localization of Gomafu RNA in the nuclei of E14 retinal cell detected with probes against exon (green, A′) and intron (magenta, A″) sequences. Note that intron probes detected two foci (arrows), whereas exon probes detected a number of dots diffusely distributed throughout the nucleus. (B–E) Distribution of Gomafu RNA (green) and Sytox-stained DNA (magenta) in the control (B,C) and nuclear-matrix-extracted (D,E) cells. E14 retinal cells (B,C) or Neuro2A cells transfected with a Gomafu cDNA expression vector (D,E) were subjected to fluorescent in situ hybridization. Note that Gomafu RNA remained intact after nuclear matrix preparation. (F) Northern blot analysis of Gomafu RNA in the biochemical fractions. Gomafu RNA was highly insoluble and was mostly fractionated into the nuclear matrix. Bars, 2 μm.
of the nuclear matrix is sensitive to RNase treatment (Nickerson, 2001). We thus assume that Gomafu RNA represents a novel family of mRNA-like noncoding RNAs that act as cell-type-specific components of the nuclear matrix, which controls gene expression or DNA metabolism characteristic of the cell-type. Further studies to identify proteins interacting with Gomafu RNA will be crucial to test this intriguing possibility.

In eukaryotic cells, nucleocytoplasmic transport of transcribed RNAs is specifically and differentially regulated through interactions with a distinct set of protein complexes depending on each RNA species. Although the precise mechanisms by which mRNAs are distinguished from other RNA molecules in terms of the nuclear export pathway remain unclear, the splicing event plays an essential role in the recruitment of an export factor Tap/Nxf1 to the exon-exon junction complex of mature mRNA, promoting its rapid export to the cytoplasm (reviewed by Stutz and Izaurralde, 2003; Reed and Hurt, 2002; Cullen, 2003). The length of the RNA is also proposed to be a identifying feature for mRNA (Masuyama et al., 2004). Given that the Gomafu gene consists of multiple exons and is a long transcript, it should be recognized as mRNA and exported to the cytoplasm without a special mechanism for its retention in the nucleus. How Gomafu RNA escapes the export pathway despite its mRNA-like characteristics remains to be investigated. Since exogenous Gomafu RNA is also localized to the nucleus of transfected cell lines, it is unlikely that its promoter contents or intron sequences are responsible for the nuclear localization. The nuclear retention signal of Gomafu RNA has so far been unable to be narrowed down into a particular region, but it seems to be distributed redundantly throughout the transcript (M.S. and S.N., unpublished observation). We failed to detect intermolecular homologous sequence within Gomafu RNA and thus the nuclear retention machinery assumingly recognizes higher-order structures rather than predictable secondary structures or particular sequence motifs. This may account for the lack of a predictable gene family for Gomafu based upon the Blast sequence search.

Xist RNA is a well-characterized mRNA-like noncoding RNA that is retained in the nucleus and controls dosage compensation and genomic imprinting of mammalian X chromosomes (reviewed by Brockdorff, 2002). roX is a functional counterpart of Xist in Drosophila (reviewed by Kelley, 2004), and both transcripts ‘paint’ the X chromosome and recruit a transcriptional regulatory complex to the chromosome in cis (i.e. to the chromosome where it is transcribed). Air RNA is another mRNA-like noncoding RNA in the nucleus, and it controls genomic imprinting of genes located nearby (reviewed by Braidotti et al., 2004). Since all three nuclear mRNA-like noncoding RNAs are involved in epigenetic processes, it could be possible that Gomafu RNA plays a similar role. Genomic imprinting is usually regulated by elements on the same chromosome: however, there is no known imprinted gene on chromosome 5 where Gomafu is located, according to a current database (http://www.mgu.har.mrc.ac.uk/research/imprinting/). In addition, transcripts of the abovementioned epigenetic noncoding genes accumulate near the site of their transcription or chromosome of its own, whereas Gomafu RNA is distributed throughout the nucleoplasm. We therefore speculate that Gomafu and the

cytoplasmic Gomafu RNA was observed in the cells treated with cycloheximide (Fig. 6D). Thus, the nuclear localization of Gomafu RNA is not a consequence of the degradation of its cytoplasmic transcripts by the NMD pathway, rather it is actively retained in the nucleus.

Discussion
We have identified a unique mRNA-like noncoding gene named Gomafu, which is expressed in a distinct set of postmitotic neurons. Unlike general protein-coding mRNAs, Gomafu RNA escapes nuclear export and forms a number of spots throughout the nucleoplasm, which do not coincide with known nuclear domain markers. Gomafu RNA is highly insoluble and remains intact after nuclear matrix preparation. Although the ultrastructural entity of the nuclear matrix and its physiological relevance has long been controversial (reviewed by Pederson, 2000; Nickerson, 2001), this biochemical fraction contains a wide spectrum of factors essential for control of transcription, splicing, replication and DNA repair (reviewed by Stein et al., 2003). It is noteworthy that the nuclear matrix contains a number of cell-type-specific components as well as abundant non-chromatin proteins that are ubiquitously expressed (Fey and Penman, 1988). In addition, the integrity of the nuclear matrix is sensitive to RNase treatment (Nickerson, 2001). We thus assume that Gomafu RNA represents a novel family of mRNA-like noncoding RNAs that act as cell-type-specific components of the nuclear matrix, which controls gene expression or DNA metabolism characteristic of the cell-type. Further studies to identify proteins interacting with Gomafu RNA will be crucial to test this intriguing possibility.

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epigenetic noncoding RNAs play different roles in different domains in the nucleus.

Among the increasing number of mRNA-like noncoding RNAs with unknown function, some are concentrated to the nucleus. These include hsrO (Prasanth et al., 2000), Rian (Hatada et al., 2001), Ks-1 (Sawata et al., 2002), AncR1 (Sawata et al., 2004), Evf-1 and Evf-2 (Feng et al., 2006) and Pinc (Ginger et al., 2006). However, transcripts of Evf-1 and Pinc are not restricted to the nucleus, but are also detected in the cytoplasm depending on the cell type. As for hsrO, Rian, Ks-1, and AncR1, the probes detect the undisplaced form of the transcripts, leaving the possibility that the nuclear signals are derived from intron-containing, unprocessed transcripts retained in the nucleus. To our knowledge, Gomafu RNA is the first example of genuine mRNA (i.e. intron-removed and polyadenylated) that accumulates within the nucleus to form a compartment that does not coincide with any known nuclear domains or particular chromosomes. Earlier studies showed that a large fraction of poly(A)+ RNAs form insoluble structures in the IGCs (Carter et al., 1991; Huang et al., 1994), the molecular composition of which in terms of RNA species has not been characterized (reviewed by Lamond and Spector, 2003; Hall et al., 2006). Together with our finding that Gomafu RNA constitutes a novel nuclear domain in the nuclear matrix in particular cell types, further studies on these matrix-associated noncoding RNAs will uncover a new biological process controlled by RNA, which so far has been underestimated owing to the lack of specific markers.

Materials and Methods

Differential screening of cell-type-specific genes

To obtain cDNAs from single cells, representative RT-PCR was carried out as previously described (Brady and Iscove, 1993) with slight modifications. Briefly, E13.5 mouse retinas were dissociated into single cells using the Papain Dissociation System (Worthington Biochemical) and stained with Hoechst 33342 and propidium iodide (PI). Hoechst-positive, PI-negative living cells were collected using FACs (FACS Vantage SE, Beckton Dickinson) into a well of 96-well PCR tubes containing 5 μl ice-cold cell lysis buffer (1.6%RoverTara Ace buffer, 0.8% Nonidet P-40, 1 ng of oligo dT12 primer, 20 μM of dNTPs, 10 μg of RNase inhibitor (Toyobo, Tokyo). Lysis was carried out at 65°C for 1 minute, and 1st strand synthesis was started by adding 3 μl of an enzyme mixture containing 1 U AMV reverse transcriptase (PerkinElmer) and 100 U RoverTaraAce (Toyobo). After a 15-minute incubation at 42°C, the enzyme was heat-inactivated at 65°C for 10 minutes. 8 μl of a tailing buffer (2X TdT buffer (Toyobo), 0.5 mM dATP, and 15 U TdT (Toyobo)) was subsequently added to the cdNA solutions and they were further incubated for 15 minutes at 42°C to add poly(A) sequences. PCR was carried out according to the conditions described previously (Brady and Iscove, 1993) using the modified AL1 primer (5'-ATTGGATCCAGGGCCTGGTCAGCAGAAGATTCGCGGCAAATG-3') containing a Mmel restriction site to remove the primer sequences afterward (see below). To identify each cell type, the expression of Math5 and Cnx was examined by Southern blot using an aliquot of the amplified cdNA products. After the retroviral identification of retinal ganglion cells (Math5+ and cone photoreceptor cells (Cnx+), the cdNA from each cell was digested with Mmel and purified with the QiAqua gel extraction Kit (Qiagen) to remove flanking primer sequences. Suppression-PCR based subtractive PCR was carried out using the cdNAs obtained from single cells and PCR-Select cdNA Subtraction Kit (BD Biosciences) according to a previous report (Fujimura et al., 2006). The amplified PCR products were then subcloned into pCR2 (Invitrogen) to make a mini plasmid library, which were differentially screened with probes prepared from each cdNA. Clones that were positive for either of the probes were selected for further analysis, and their endogenous expression was confirmed on histological sections by in situ hybridization.

cDNA cloning and vector construction

EST clones AK035540 and AK028326, corresponding to the 5' and 3' regions of Gomafu RNA, respectively, were obtained from RIKEN Genome Sciences Center, Japan. A cdNA fragment overlapping with the 2 EST clones was amplified by RT-PCR using the primer set: 5'-GAATTCGCGCCGGCTCTGCAGGAGGACGTG-GCTAGGCCCTCTG-3' and 5'-GATACTACTGTTGGAAGAGGCCTTGCTAGTT-GACCGCCAAT-3'. The fragments were subcloned into pCR2 (Invitrogen), and the sequence was confirmed using the mouse genome database. The PCR fragment and two EST clones were then assembled using苇elt and Hazel II and yielded pBSKS-Gomafu that contains full-length Gomafu cdNA (AB300594). To generate pCaGo:Gomafu-pa, the pBSKS-Gomafu was digested with Sfil, blunt-ended and the cdNA fragment was subcloned into pCa-pa (Niwa et al., 1991). To identify splicing isoforms of Gomafu, RT-PCR was carried out using the following primer sets: 5'-CACAGGGCCGGCCAGAATTCGCAACTC-3' and 5'-GTTAGCGCAAGCAGGAACATTTG-3' for the third intron; 5'-TTGGCCGGGAAGAATAAGAC-3' and 5'-GTGTACCAGGACACCTG-3' for the fourth intron. The amplified fragments were subcloned into pCR2 and used as templates. Other EST clones used in this study are AK035537 for Evf-1, AK039861 for Xist and AK032756 for Air.

In situ hybridization and immunohistochemistry

Digoxygenin- or FITC-labeled RNA probes were prepared using RNA labeling mixture (Roche) and T3, T7 or SP6 RNA polymerase (Roche) according to the manufacturer’s instructions. For detecting transiently expressed RNAs, additional RNaseH treatment and northern hybridization was carried out in a buffer containing 20 mM HEPES, pH 7.5, 50 mM KCl, 10 mM MgCl2, 1 mM DTT and 100 U/ml RNaseH (Toyobo, Tokyo) at 37°C for 30 minutes. For detecting mouse SC53 antigen, it was essential to treat the samples through the whole-mount in situ hybridization procedure described previously (Suzuki et al., 1997). After hybridization and washing steps, the samples were fixed, sectioned, and further processed for the signal detection. The hybridized probes were detected with standard immunohistochemical procedures and the following antibodies were used dependent on each application: alkaline phosphatase conjugated sheep anti-mouse antibodies, rabbit anti-DIG (Chemicon) for double staining with rabbit primary antibodies, rabbit polyclonal antibodies against DIG antibody (Roche) and Cy3-conjugated anti-mouse antibody (Chemicon) for co-staining with mouse antibodies. The other antibodies were used dependent on each application; alkaline phosphatase conjugated sheep anti-DIG antibody (Roche) for NBT/BCIP or FastRed/HNPP chromogenic reactions, a mouse monoclonal anti DIG antibody (Roche) and Cy3-conjugated anti-mouse antibody (Chemicon) for double staining with rabbit primary antibodies, rabbit polyclonal antibodies against FITC (Invitrogen) and Alexa Fluor 488-conjugated anti-rabbit antibody for double staining with mouse antibodies. To detect intranuclear signals, the signals were amplified using TSA Kit2 (Invitrogen) according to the manufacturer’s instructions. The other antibodies were used as follows: PC10 (mouse anti-PCNA, Transduction Lab.), BU33 (mouse anti-BrDU, Sigma), SC35 (mouse anti-SC35, Sigma), 38F3 (mouse anti-fibrillarin, Abcam), p6 (mouse anti-collin, Sigma), 36.1-104 (mouse anti-PML, MBL), PG-M3 (mouse anti-PML, Santa Cruz), rabbit anti-PS1 (a kind gift from Angus Lamond, University of Dundee, UK), rabbit anti-GFP (MBL). For detection of DNA, samples were incubated with SYTOX Green (Cambrex). Fluorescent images were obtained using a confocal microscope (LSM Pascal, Zeiss) or an epifluorescent microscopy (BX51, Olympus) equipped with a CCD camera (DP70).

Cell cultures, fractionations and heterokaryon assays

All cells were grown in a 1:1 mixture of DMEM and Ham F12 (Nissui, Japan) supplemented with 10% fetal bovine serum (DHI0). To prepare primary cultures of mouse cells, E12.5 mouse brains or E14 retinas were dissociated into single cells and plated into a culture dish (Nunc) or chamber slides (Culture Slide, BD Falcon). To remove the nuclear membranes, cells were incubated in a protocol described previously (Huang et al., 1994). To block mRNA transcription, α-amanitin (Sigma) was added at a concentration of 50 μg/ml. To block protein synthesis, cycloheximide (Sigma) was added at a concentration of 20 μg/ml and cells were incubated for the indicated time. To obtain nuclear or cytoplasmic RNAs, cultured cells were washed with HBSS and lysed in CSK buffer. The cell lysates were centrifuged at 7000 g for 2 minutes to prepare cytoplasmic and the nuclear fractions. Total RNA from each fraction was isolated using TRizol reagent (Invitrogen) and was subjected to northern analyses.

For heterokaryon assays, Neuro2A cells stably expressing Gomafu or NLS EGFP
were prepared by a standard method. 5×10^5 NLS EGFP-expressing neuro2A cells were transferred onto cover slips (22 mm²) Matsunami, Japan) and cultured overnight at 37°C. They were overlaid with 7×10^5 Gomafu-expressing neuro2A cells and then the cells were fused with 50% PEG 4000PBS for 2 minutes at room temperature. The heterokaryons were further cultured overnight in DM 10 and subsequently processed for in situ hybridization.

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