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Evolutionarily conserved autoregulation of alternative pre-mRNA splicing by ribosomal protein L10a

Satomi Takei[1,†], Marina Togo-Ohno[1], Yutaka Suzuki[2] and Hidehito Kuroyanagi[1,*,†]

[1]Laboratory of Gene Expression, Medical Research Institute, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8510, Japan and [2]Department of Medical Genome Science, University of Tokyo, Kashiwa, Chiba 277-8561, Japan

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ABSTRACT

Alternative splicing of pre-mRNAs can regulate expression of protein-coding genes by generating unproductive mRNAs rapidly degraded by nonsense-mediated mRNA decay (NMD). Many of the genes directly regulated by alternative splicing coupled with NMD (AS-NMD) are related to RNA metabolism, but the repertoire of genes regulated by AS-NMD *in vivo* is to be determined. Here, we analyzed transcriptome data of wild-type and NMD-defective mutant strains of the nematode worm *Caenorhabditis elegans* and demonstrate that eight of the 82 cytoplasmic ribosomal protein (*rp*) genes generate unproductively spliced mRNAs. Knockdown of any of the eight *rp* genes exerted a dynamic and compensatory effect on alternative splicing of its own transcript and inverse effects on that of the other *rp* genes. A large subunit protein L10a, termed RPL-1 in nematodes, directly and specifically binds to an evolutionarily conserved 39-nt stretch termed L10ARE between the two alternative 5′ splice sites in its own pre-mRNA to switch the splice site choice. Furthermore, L10ARE-mediated splicing autoregulation of the L10a-coding gene is conserved in vertebrates. These results indicate that L10a is an evolutionarily conserved splicing regulator and that homeostasis of a subset of the *rp* genes are regulated at the level of pre-mRNA splicing *in vivo*.

INTRODUCTION

Alternative splicing of precursor messenger RNAs (pre-mRNAs) expands proteome diversity in multicellular organisms (1). Indeed, recent genome-wide transcriptome analyses revealed that more than 90% of protein-coding genes in humans produce multiple mRNA isoforms (2,3). On the other hand, early bioinformatics analysis of expressed sequence tags (ESTs) and cDNA sequence data predicted that a significant fraction of the alternatively spiced mRNA isoforms have premature termination codons (PTCs) within open reading frames (ORFs) (4–6). Recently, alternative splicing coupled with NMD (AS-NMD), also referred to as regulated unproductive splicing and translation (RUST), has been shown to be one of the mechanisms for regulating gene expression levels by knocking down or knocking out essential factors for NMD (7,8).

Many of well-characterized examples of genes directly regulated by AS-NMD are related to RNA metabolism (9–11). In a number of cases, they are splicing factors and autoregulate their own transcripts to maintain their own abundance in a negative feedback loop. Such examples include general splicing factors such as SR and SR-like proteins (12–16) and heterogeneous nuclear ribonucleoproteins (hnRNPs) (17–20), tissue-specific splicing factors (21–23) as well as core spliceosomal proteins (24,25). Genome-wide search for AS-NMD target mRNAs in *Drosophila* S2 cells by utilizing a custom splicing-sensitive microarray also identified numerous genes related to RNA metabolism such as splicing and translation (26).

In *C. elegans*, critical NMD factors encoded by a series of the *smg* genes are dispensable for viability and fertility (27) unlike in vertebrates (28–30). Therefore, the *smg* mutants have been utilized for characterization of unproductive splice variants or other natural NMD targets *in vivo* (31–36). Splicing-sensitive microarray analysis of 352 known cassette exons in embryonic total RNAs revealed that 30 of them, including 10 in splicing factor genes, produce PTC-containing mRNA isoforms upon alternative splicing, likely representing direct targets for AS-NMD (37). Global analyses of total RNAs from L3 or older worms by genome-scale tiling arrays and massively parallel sequencing revealed that ∼20% of genes likely produce NMD target transcripts and that splicing factor genes are enriched in those that produce PTC-containing NMD targets (38). These large-scale analyses, however, suggested that a large fraction of the natural NMD targets are destabilized independently from PTCs generated upon alternative splicing (37,38) and are related

*To whom correspondence should be addressed. Tel: +81 3 5280 8077; Fax: +81 3 5280 8077; Email: kuroyana.end@tmd.ac.jp
†These authors contributed equally to the work as first authors.

to the presence of upstream ORF (uORFs) or longer 3′ UTRs (38).

Here, we performed mRNAseq analysis of a wild-type strain N2 and the *smg-2 (yb979)* mutant (39) to comprehensively search for NMD target mRNAs generated by alternative splicing in *C. elegans*. In this study, we focus on ribosomal protein (*rp*) genes because we confirmed expression of novel and unproductively spliced mRNAs from four *rp* genes in the *smg-2* mutant in addition to four *rp* genes already known to produce unproductive splice variants in an early study (33). We elucidate homeostatic regulation of all these *rp* genes at the alternative splicing level. We also demonstrate that ribosomal protein L10a, like splicing factors, regulates alternative splicing of its own pre-mRNA in a negative feedback loop by directly binding to an evolutionarily conserved element.

MATERIALS AND METHODS

Worm culture and microscopy

Worms were cultured following standard methods. The strains used are N2 and KH1668: *smg-2 (yb979) I*. Transgenic worms were generated as described previously (40). Images of fluorescence reporter worms were captured using a fluorescence compound microscope (DM6000B, Leica) equipped with a color, cooled CCD camera (DFC310FX, Leica) and processed with Photoshop (Adobe).

RNA preparation, mRNA-seq and RT-PCR

Total RNAs from synchronized worms were extracted as described previously (40,41). mRNA-seq was performed as described previously (41). The sequence data for N2 and the *smg-2* mutant are available in Sequence Read Archive (SRA) under accession numbers DRA000864 (41) and DRA002612 (this study). The numbers of uniquely mapped 35-nt sequence tags for N2 and KH1668: *smg-2 (yb979)* deposited in DRA002612 were 6 583 548 and 6 964 488, respectively. At least one read from either of the strains was mapped to 15 479 (76.3%) of the 20 290 genes in WormBase and 1383 genes (6.8%) were predicted to contain differentially expressed exons by the method described in (41). Total RNAs from cultured mammalian cells were extracted by utilizing Sepasol RNA I Super (nacalai tesque) and RNeasy Mini (QIAGEN). Total RNAs from transfected cells were and treated with RQ1 DNase (Promega) and DNase (QIAGEN). RT-PCR was performed essentially as described previously (40,41). Sequences of the primers used in the RT-PCR experiments are available in Supplementary Table S1. RT-PCR products were analyzed by using BioAnalyzer (Agilent). Sequences of the RT-PCR products were confirmed by direct sequencing or by cloning and sequencing. Sequences of the novel and unproductive mRNAs were deposited in GenBank/EMBL/DDBJ databases under accession numbers LC006013, LC006014, LC006015 and LC006016 for *C. elegans rpl-26*, *rpl-30*, *rps-22* and *ubl-1*, respectively, and LC010245 and LC010246 for human *RPL10A*.

RNAi

RNAi experiments by feeding were performed as described previously (42) by utilizing RNAi feeding library (Open Biosystems; Geneservice) for *rpl-1*, *rpl-3*, *rpl-12*, *rpl-26*, *rpl-30*, *rps-22* and *ubl-1*. For knocking down the *rpl-7A* gene, we constructed pL4440-RPL-7A by cloning a genomic DNA fragment spanning from *rpl-7A* exon 4 to exon 5 at the *Eco* RV site of pL4440 vector (from A. Fire) by using In-Fusion HD Cloning Kit (Clontech). *Escherichia coli* strain HT115 (DE3) was transformed with pL4440-RPL-7A and used for RNAi by feeding. Sequences of the primers are available in Supplementary Table S2.

RNAi experiments by soaking were performed essentially as described previously (43,44). The RNAs were synthesized by *in vitro* transcription with T7 (Takara) or SP6 (Promega) RNA polymerase. Sequences of the primers used in amplifying cDNA fragments as templates for the *in vitro* transcription are available in Supplementary Table S3.

Nucleotide sequence alignment

Nucleotide sequences of *C. elegans* introns and orthologous introns were aligned by Clustal V algorithm by utilizing MegAlign module of Lasergene (DNASTAR). Genomic sequences of the genus *Caenorhabditis* were retrieved from WormBase (www.wormbase.org). Databases and accession numbers for the other nucleotide sequences are GenBank/EMBL/DDBJ NC_000006 (human *RPL10A* intron 3), Ensemble ENSGALG00000002644 (chicken *RPL10A* intron 3), Ensemble ENSXETG00000012374 (*X. tropicalis rpl10a* intron 2), Ensemble ENSDARG00000042905 (zebrafish *rpl10a* intron 3) and FlyBase FBgn0036213 (*D. melanogaster RpL10Ab* intron 2). The unproductive splice site for *Drosophila RpL10Ab* is based on a RefSeq sequence NM_168480.

Construction of expression vectors for *C. elegans* genes

Fluorescence *rpl-1* splicing reporter minigenes were constructed essentially as described previously (40,45). Briefly, the *rpl-1* genomic DNA fragment was cloned into Gateway pENTR-L1/R5 vector (Invitrogen) by utilizing BP Clonase II Plus (Invitrogen). Mutagenesis and deletion of L10ARE were performed by utilizing Quickchange II (Stratagene). Expression vectors were constructed by homologous recombination between the genomic DNA cassette, a fluorescent protein cassette in pENTR-L5/L2 vector and a destination vector pDEST-eft-3p or pDEST-myo-3p (46) by utilizing LR Clonase II Plus (Invitrogen). Sequences of the primers used in the plasmid construction are available in Supplementary Table S2.

Expression vectors of RPL-1 and RPL-30 were also constructed by utilizing Gateway system (Invitrogen). RPL-1 and RPL-30 cDNAs were amplified and cloned in pENTR/D-TOPO vector (Invitrogen). The expression vectors were constructed by homologous recombination between the Entry vectors and pDEST-eft-3p or pDEST-Cold-ZZ (sequence information is available upon request to H.K.) with LR Clonase II (Invitrogen). All constructs were confirmed by sequence analysis. Sequences of the primers

used in the construction are available in Supplementary Table S2.

UV crosslinking and electrophoretic mobility shift assay (EMSA)

Recombinant full-length RPL-1 and RPL-30 proteins were expressed as His-ZZ-fused proteins by cold-induction (47) in *E. coli* strain BL21(DE3)pLysS, purified by using Ni-NTA agarose (QIAGEN), quantified by using Micro BCA Protein Assay Kit (PIERCE) and analyzed by CBB staining of neutral PAGE gel (Invitrogen). ^{32}P-labeled RNA probes were prepared as described previously (48). The templates for the *in vitro* transcription were amplified from genomic DNA or the reporter minigenes by PCR. The sequences of the primers used are available in Supplementary Table S3. *In vitro* binding reactions were performed in the presence of 100 ng/μl *E. coli* tRNAs and 50 ng/μl bovine serum albumin in 25 μl of RNA binding buffer (150 mM KCl in 20 mM HEPES-KOH (pH 7.9) with 5% glycerol, 1% Triton X-100, 1 mM DTT and 0.1 mM PMSF) for 30 min at 20°C. UV crosslinking (46) and EMSA (48) were performed essentially as described previously except that the denatured proteins were separated by using NuPAGE gel (Invitrogen).

Construction of expression vectors for mammalian genes

The human *RPL10A* WT reporter cassette was constructed by cloning *RPL10A* genomic DNA fragments spanning from exon 3 to exon 5 into pENTR/D-TOPO vector (Invitrogen). L10ARE was deleted by utilizing Quickchange II (Stratagene) for the *RPL10A* DEL reporter cassette. The reporter minigenes were constructed by homologous recombination between the Entry vectors and pDEST-cDNA3 (45) by utilizing LR Clonase II (Invitrogen). Sequences of the primers used in the construction are available in Supplementary Table S2.

Entry vectors for human L10a and L26 were constructed by cloning cDNA fragments into pENTR/D-TOPO vector (Invitrogen). The Expression vectors were constructed by homologous recombination between the Entry vectors and pDEST-ME18S (H.K.) by utilizing LR Clonase II (Invitrogen). All constructs were confirmed by sequence analysis. Sequences of the primers used in the construction are available in Supplementary Table S2.

Cell culture

HeLa, HEK293T and NIH3T3 cells were cultured in DMEM (nacalai tesque) containing 10% fetal bovine serum (FBS) and penicillin-streptomycin mixed solution (nacalai tesque). HeLa cells were grown to 90% confluence and treated with 100 μg/ml emetin (SIGMA) for 6 h. The human *RPL10A* reporter minigenes and the expression vectors were co-transfected at the ratio of 1:3 into NIH3T3 cells by utilizing FuGENE HD (Roche).

RESULTS

Eight *rp* genes produce unproductively spliced mRNAs in *C. elegans*

To identify unproductive splice variants stabilized in the NMD-defective mutant, we compared mRNA-seq data to search for genes whose mRNAs appeared to be differentially spliced between the wild-type strain N2 and the *smg-2 (yb979)* mutant. For this purpose, we sequenced poly(A)$^+$ RNAs from synchronized L1 larvae because we previously noticed enrichment of neuron-specific minor variants at this stage (45) and successfully identified 24 alternative splicing events regulated by a neuron-specific splicing factor UNC-75 in the same way (41). We estimated significance of difference in inclusion levels between N2 and *smg-2* for each exon in all RefSeq gene models (41), and noticed enrichment of the *rp* gene exons in the candidates (11 out of top 50). We therefore inspected distribution of the sequence reads for each of all the 82 cytoplasmic ribosomal protein genes in *C. elegans* (Ribosomal Protein Gene Database (RPG), http://ribosome.med.miyazaki-u.ac.jp/), and analyzed splicing patterns of candidate transcripts in N2 and the *smg-2* mutant by reverse transcription-polymerase chain reaction (RT-PCR). Sequencing of the RT-PCR products revealed that *rpl-26* and *rpl-30*, encoding 60S subunit proteins L26 and L30, respectively, and *rps-22* and *ubl-1*, encoding 40S subunit proteins S22 and S27a, respectively, have *smg-2*-specific mRNA isoforms (Figure 1) in addition to the four *rp* genes known to express unproductive mRNAs (33). The novel *smg-2*-specific mRNA isoforms contain PTCs (Figure 1), consistent with the idea that they are unproductive mRNAs and are rapidly degraded in the wild-type by NMD.

Homeostatic regulation of the *rp* genes by alternative pre-mRNA splicing

As ribosomal proteins are principally RNA-binding proteins present in the nucleus and many splicing regulators autoregulate production of their own unproductive mRNA isoforms, we tested possibilities that the eight ribosomal proteins are involved in the splicing regulation of their own pre-mRNAs. To systematically knockdown each of the *rp* genes, we fed synchronized L4 larvae of the *smg-2* mutant with bacteria expressing double-stranded RNA targeting the *rp* gene and cultured the worms for another 6, 12, 24 or 36 h. In the control experiments with bacteria carrying an empty vector, total amounts of the two isoforms and as well as ratios of the productive isoforms to the total remained rather constant (Figure 2A, left panels, Supplementary Figure S1 and Figure 2B, blue lines) regardless of substantial growth of the worms during this period. When *rpl-1* is knocked down, the total amount of the *rpl-1* mRNAs gradually decreased along with time (Figure 2A, top right panel), confirming that *rpl-1* was effectively knocked down. Noticeably, the ratio of the productive *rpl-1* mRNA to the total gradually increased (Figure 2A, top right panel and Figure 2B, top left panel), likely compensating for the reduction in the total amount. In contrast, the ratios of the productive mRNA isoforms gradually decreased for *rpl-7A* (Figure 2A, bottom right panel) and the other six *rp* genes (Supplementary Figure S1 and Figure 2B, top pan-

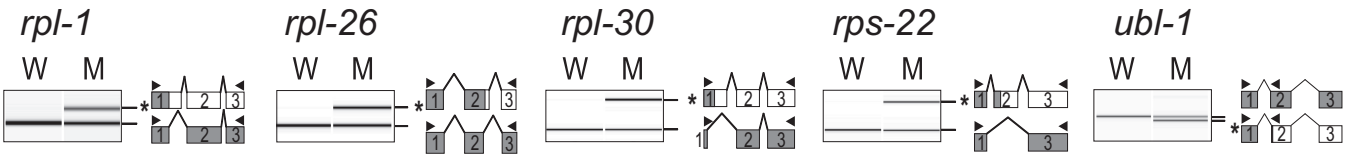

Figure 1. RT-PCR analysis confirms expression of newly identified unproductive mRNA variants from four ribosomal protein genes *rpl-26*, *rpl-30*, *rps-22* and *ubl-1* in the *smg-2* mutant of *C. elegans*. Gel-like presentation of the RT-PCR analysis of the *rp* gene transcripts in total RNAs from synchronized L1 larvae of the wild-type strain N2 (W) and the *smg-2 (yb979)* mutant (M). Schematic structures of the mRNAs are indicated. Numbered boxes indicate exons. Coding regions are colored in gray. Arrowheads indicate positions and directions of the PCR primers. Asterisks indicate the unproductive mRNA variants detected only in the *smg-2* mutant.

els). Analogous results were obtained when any one of the other seven *rp* genes were knocked down: increase in the ratio of the productive mRNA isoforms for the targeted gene and decrease for the rest (Supplementary Figure S1 and Figure 2B). These results indicated that expression of these *rp* genes are regulated by their own products either directly or indirectly at the level of alternative splicing, which contributes to the homeostasis of the *rp* gene products.

A 40-nt stretch is highly conserved in orthologous introns of genes encoding ribosomal protein L10a in metazoans

It has been reported that nucleotide sequences of the alternatively spliced introns of the *rpl-1*, *rpl-3*, *rpl-7a* and *rpl-12* genes are exceptionally conserved (66–93% identity) among related nematode species *C. briggsae* and *C. remanei* compared to those of constitutive introns (33). We compared genomic sequences of the *rpl-26*, *rpl-30*, *rps-22* and *ubl-1* genes between *C. elegans* and related nematodes and found that the alternative introns of these genes are also highly conserved (68–84% identity) except for *C. japonica rps-22* (53%) (Supplementary Figure S2), suggesting that alternative splicing regulation is also conserved for these genes.

During the course of nucleotide sequence comparison, we found that a 39-nt stretch residing between the two alternative 5′ splice sites of *rpl-1* intron 1 in *C. elegans* is conserved in orthologous introns of orthologous genes encoding ribosomal protein L10a in vertebrates and a fly (Figure 3). Here, the orthologous introns are defined as those that reside at exactly the same position in the coding sequences for the evolutionarily conserved ribosomal proteins. In contrast, such an evolutionarily conserved stretch was not found for *rpl-3* intron 1, although analogous alternative splicing regulation of the orthologous introns has been reported for the human *RPL3* gene (49) and predicted for the *Drosophila RpL3* gene (FlyBase, http://flybase.org). We therefore focused on the *rpl-1* gene in elucidating mechanisms for autoregulation of alternative pre-mRNA splicing in the following sections.

Fluorescence *rpl-1* splicing reporters for visualizing alternative splicing regulation *in vivo*

In order to visualize the splicing patterns of the *rpl-1* mRNAs *in vivo*, we constructed a pair of wild-type (WT) *rpl-1* fluorescence reporter minigenes that contain a genomic fragment spanning from exon 1 through exon 2 (Figure 4A). The minigenes were designed so that expression of red (RFP) and green (GFP) fluorescent proteins indicated the use of the upstream (or productive) and the downstream (or unproductive) splice sites, respectively (Figure 4A). When the *rpl-1* WT reporter minigenes were expressed under the control of a ubiquitous promoter, RFP was predominantly expressed and GFP was modestly expressed without apparent tissue-specificity (Figure 4B). Since the reporter worms were fairly sick, we utilized a body wall muscle-specific promoter to drive expression of the reporter (Supplementary Figures S3 and S4A) in the following experiments.

To test splicing regulation of the *rpl-1* WT reporter, we knocked down *rpl-1*, *rpl-26* or *rpl-30* in the reporter worms. As expected, the productive splice site was predominantly selected upon knockdown of the endogenous *rpl-1* gene, whereas the unproductive splice site was preferred upon knockdown of *rpl-26* or *rpl-30* (Figure 4C). Expression of the fluorescent proteins was consistent with the splicing change of the reporter mRNAs (Supplementary Figure S4). Next, we tested the effects of RPL-1 overexpression on the *rpl-1* WT reporter. In contrast to the knockdown experiment, transgenic expression of RPL-1 dramatically increased expression of GFP (Figure 4D), while expression of RPL-30 had little effect (Figure 4E). These results indicated that the fluorescence *rpl-1* WT reporter specifically reflected the negative feedback regulation of the endogenous *rpl-1* gene.

L10ARE is essential for the splicing regulation of the *rpl-1* splicing reporter *in vivo*

To test the involvement of the evolutionarily conserved 39-nt stretch between the two alternative splice sites in the regulation of the *rpl-1* splicing reporter, we constructed a pair of deletion mutant (DEL) minigenes in which the entire stretch was deleted (Figure 4A). The *rpl-1* DEL reporter worms also expressed both of RFP and GFP (Supplementary Figures S3 and S5A) but were no longer affected by knockdown of *rpl-1*, *rpl-26* or *rpl-30* (Figure 4F and Supplementary Figure S5) or by overexpression of RPL-1 (Figure 4G). These results indicated that the conserved stretch is an essential *cis*-element for the splicing regulation of the *rpl-1* reporter. We therefore designated the stretch as ribosomal protein L10a regulatory element (L10ARE).

RPL-1 protein directly and specifically recognizes L10ARE *in vitro*

To test whether RPL-1 protein can directly and specifically recognize *rpl-1* pre-mRNA, we performed *in vitro* binding

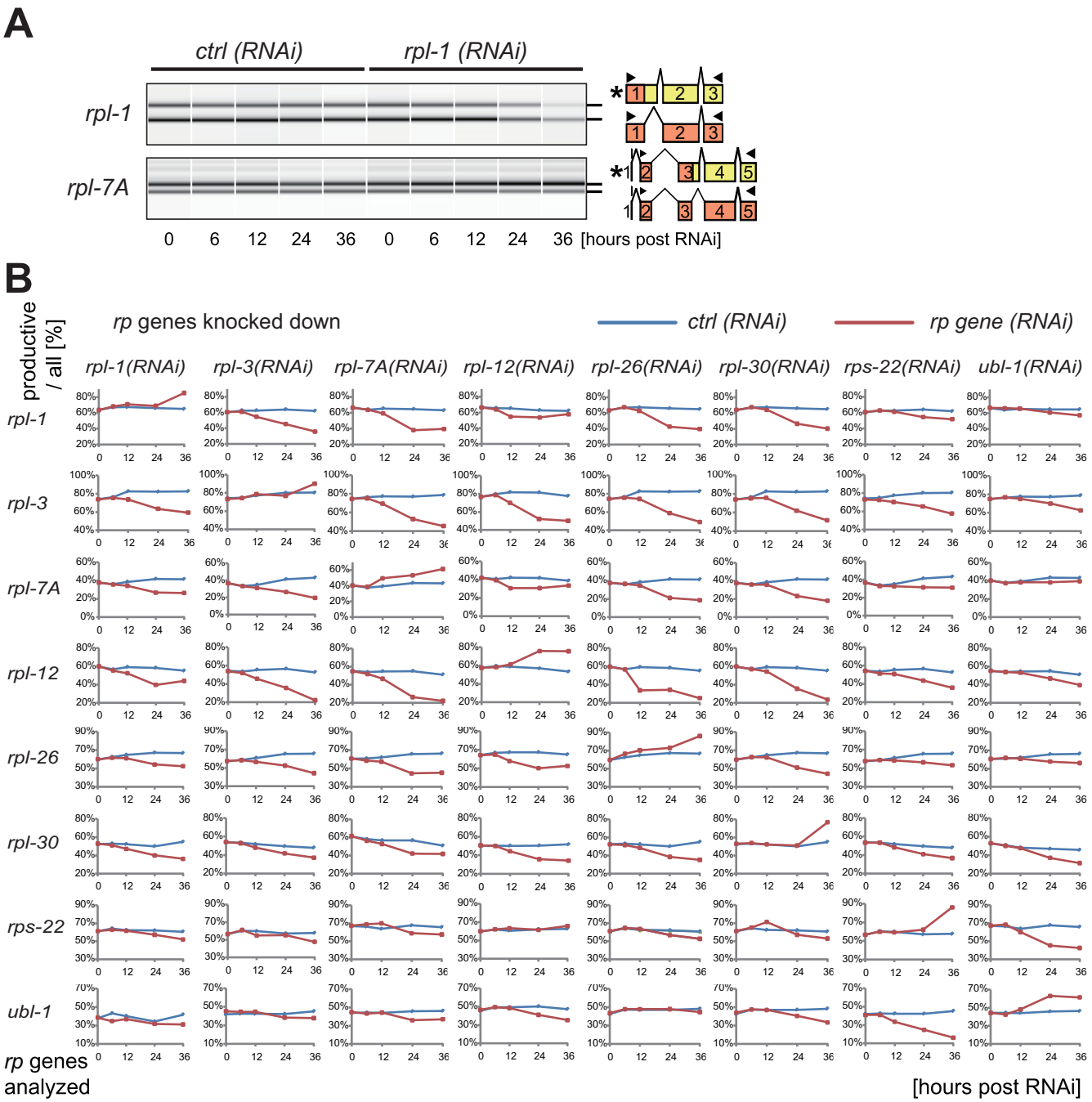

Figure 2. Knockdown of each of the *rp* genes expressing the unproductive mRNAs oppositely affects productive splicing of its own transcript and of the other *rp* gene transcripts. (**A**) Time course analysis of the *rpl-1* (top) and the *rpl-7A* (bottom) transcripts in total RNAs from synchronized *smg-2* worms fed with bacteria for control (left) or *rpl-1 (RNAi)* (right) by RT-PCR. Schematic structures of the mRNAs are indicated on the right. Coding regions are colored in orange. Arrowheads indicate PCR primers. Note that the *rpl-7A* forward primer overlaps an exon–exon junction. (**B**) Summary of the RT-PCR analysis presented in Figure 2A and Supplementary Figure S1. A red line in each graph indicates the change in the molar ratio of the productive mRNA isoform to the sum of the two isoforms of the *rp* gene indicated on the left after feeding with the bacteria for RNAi of the *rp* gene indicated at the top. A blue line indicates the change in worms fed with the control bacteria in a parallel experiment. X-axis indicates the time of feeding for knockdown in hours.

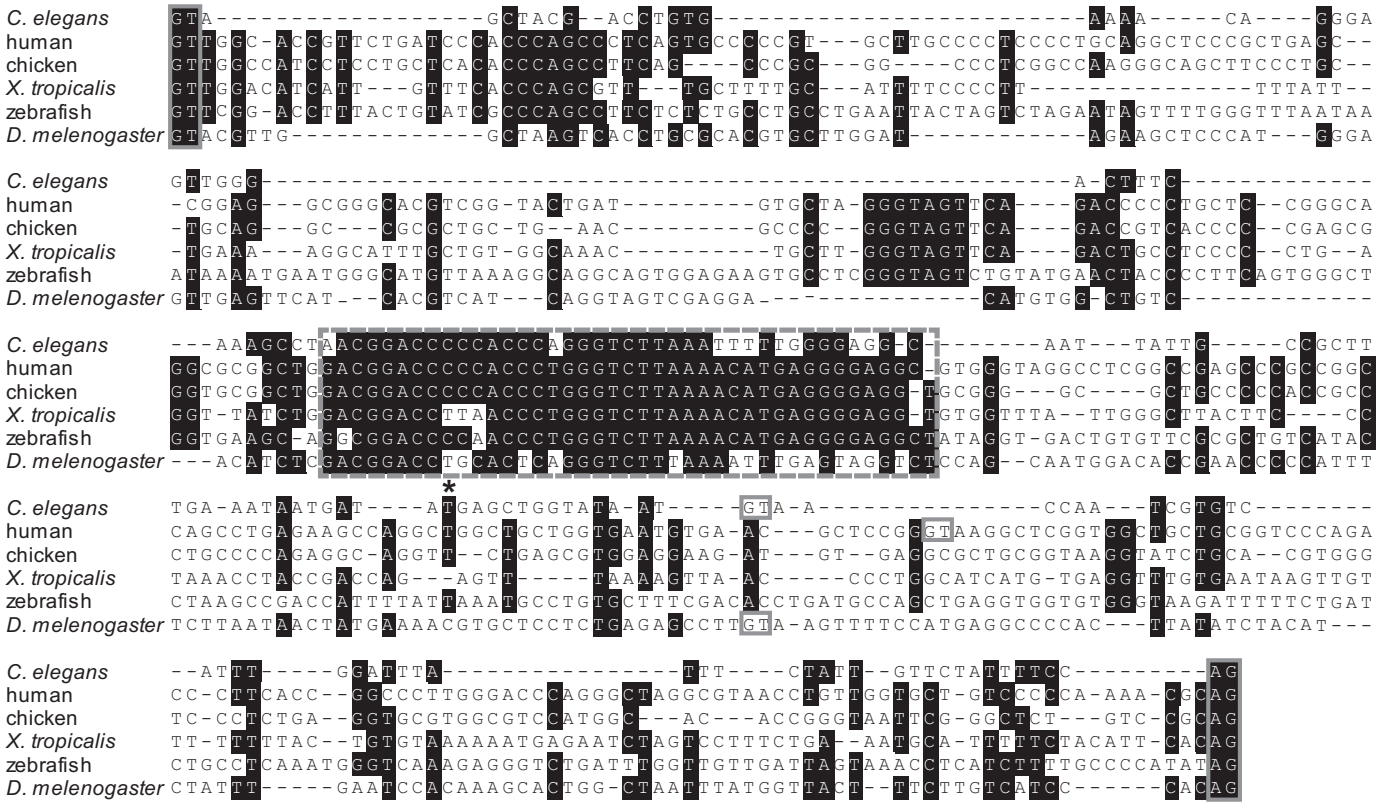

Figure 3. Nucleotide sequence alignment of *C. elegans rpl-1* intron 1 and orthologous introns in human, chicken, the Western clawed frog *Xenopus tropicalis*, zebrafish and the fruit fly *Drosophila melanogaster*. These introns were considered to be orthologous because they reside at exactly the same position, i.e. between the second and the third nucleotides of a specific codon, encoding Arg53 in *C. elegans*. Nucleotides conserved in four or more species are shaded in black. Confirmed splice sites are boxed in gray. A highly conserved 40-nt stretch (L10ARE) is boxed with a dashed gray line. An asterisk indicates the T residue converted to C in the *rpl-1* splicing reporter minigenes shown in Figure 4.

assays. We prepared four radiolabeled RNA probes covering the entire *rpl-1* fragment cloned in the *rpl-1* WT reporter minigenes (Figure 5A and Supplementary Figure S6A) and recombinant His-ZZ-fused full-length RPL-1 and RPL-30 proteins (Supplementary Figure S6B). RPL-1 strongly bound to probe 3, moderately to probe 1 and weakly to probes 2 and 4 in UV crosslinking experiments, whereas RPL-30 was only faintly crosslinked with these probes (Figure 5B, top panel). Consistent results were obtained in EMSAs (Supplementary Figure S6C). These results indicated that RPL-1 can directly bind to *rpl-1* pre-mRNA in a sequence-dependent manner.

As probe 3 was the only fragment that contains the L10ARE stretch, we next asked whether L10ARE is required for RPL-1 to specifically recognize the *rpl-1* pre-mRNA by using probes 5 and 6 that have sequences derived from the WT and the DEL reporter minigenes, respectively (Figure 5A and Supplementary Figure S6A). RPL-1 was strongly crosslinked with probe 5 and weakly to probe 6, whereas RPL-30 was only weakly crosslinked with probe 5 (Figure 5B, bottom panel). Consistent results were obtained in the EMSAs (Supplementary Figure S6C). These results indicated that the RPL-1 protein directly and specifically binds to the *rpl-1* pre-mRNA via L10ARE *in vitro*.

Autoregulation of alternative pre-mRNA splicing of the *RPL10A* gene is conserved in mammals

To ask whether L10ARE-mediated splicing autoregulation by L10a is evolutionarily conserved in metazoans, we first tried to detect a novel and unproductive splice variant mRNA from the human *RPL10A* gene. The putative L10ARE stretch resides in intron 3 and the unproductive variant was expected to have a PTC and be degraded by NMD. We therefore treated HeLa cells with emetine, an inhibitor of protein biosynthesis, to stabilize NMD target mRNAs (50), which was confirmed by stabilization of an unproductively spliced *RPL3* mRNA (49) (Figure 6A, bottom panel). In addition to the productive *RPL10A* mRNA, we discovered two novel and emetine-stabilized *RPL10A* mRNA isoforms: one utilizing a novel 5′ splice site downstream of the putative L10ARE stretch and one with intron 3 retention (Figure 6A, top panel and Supplementary Figure S7). These results indicated that the alternative splice donor site does exist in intron 3 of the human *RPL10A* gene (Figure 3) for the unproductive mRNA rapidly degraded by NMD.

To analyze splicing regulation of the human *RPL10A* gene, we constructed a wild-type (WT) *RPL10A* reporter minigene that contains a genomic fragment spanning from exon 3 through exon 5 (Figure 6B). When the minigene was expressed in NIH3T3 cells, either of the two alterna-

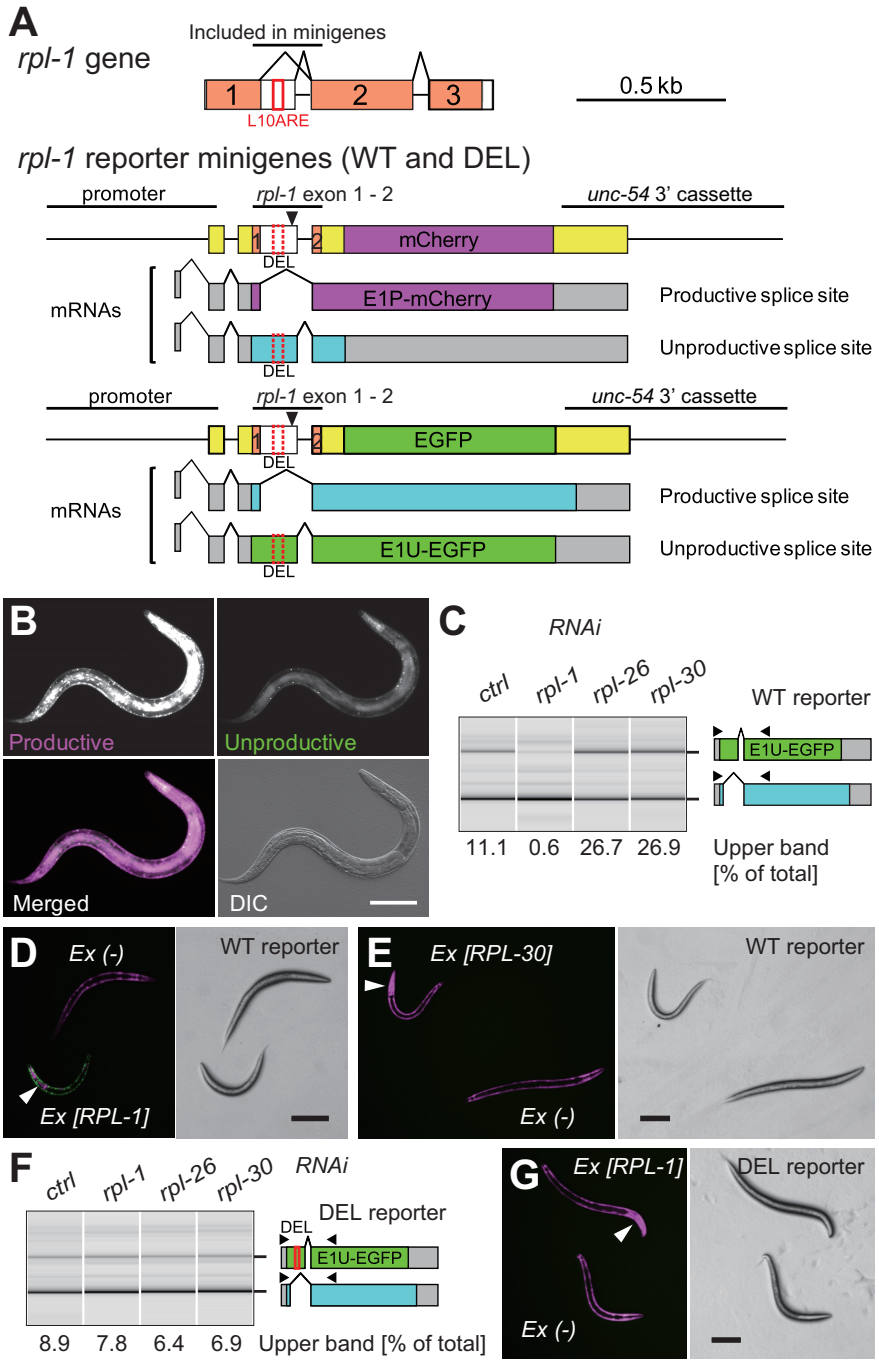

Figure 4. Fluorescence *rpl-1* splicing reporters represent splicing regulation of the endogenous *rpl-1* gene. (**A**) Schematic structures of the *rpl-1* gene (top), WT and DEL pairs of the *rpl-1* splicing reporter minigenes and mRNAs derived from them (bottom). Boxes indicate exons. Coding regions of the *rpl-1* gene are colored in orange. L10ARE is boxed in red and is deleted in the *rpl-1* DEL reporter minigene pair. Note that mCherry and EGFP cDNAs are in frame when the upstream (productive) and downstream (unproductive) splice sites, respectively, are selected. Coding regions for mCherry—(E1P-mCherry) and EGFP—(E1U-EGFP) fusion proteins and non-fluorescent proteins are colored in magenta, green and light blue, respectively. Arrowheads in the minigenes indicate positions of the T residue artificially converted to C for disrupting a termination codon. (**B**) Fluorescence microphotographs of a reporter worm expressing the *rpl-1* WT reporter minigenes under the control of the *eef-1A.1* promoter. Black-white presentation of a red channel image with a red filter (Productive) and a green channel image with a green filter (Unproductive), a merged image with pseudo colors (mCherry in magenta and EGFP in green) and a differential interference contrast image (DIC). (**C**) RT-PCR analysis of mRNAs derived from the *rpl-1* WT reporter minigene carrying EGFP in worms treated with buffer (*ctrl*) or dsRNA for *rpl-1*, *rpl-26* or *rpl-30*. Schematic structure of the mRNAs and molar ratios of the upper band are indicated. (**D** and **E**) Fluorescence (left) and bright field (right) microphotographs of *rpl-1* WT reporter worms ectopically expressing either (D) RPL-1 or (E) RPL-30 in the body wall muscles. Arrowheads indicate expression of a co-injected pharynx::RFP marker in the transgenic worms. Non-transgenic worms (*Ex (-)*) are included in the images for comparison. The fluorescence images are pseudo-colored as in (B). (**F**) RT-PCR analysis of mRNAs derived from the *rpl-1* DEL reporter minigene carrying EGFP in worms treated with buffer (*ctrl*) or dsRNA for *rpl-1*, *rpl-26* or *rpl-30*. The data are shown as in (C). (**G**) Microphotographs of an *rpl-1* DEL reporter worm ectopically expressing RPL-1 in the body wall muscles with the pharynx::RFP marker (arrowhead). The images are processed as in (D and E). Scale bars in (B, D, E and G), 100 μm.

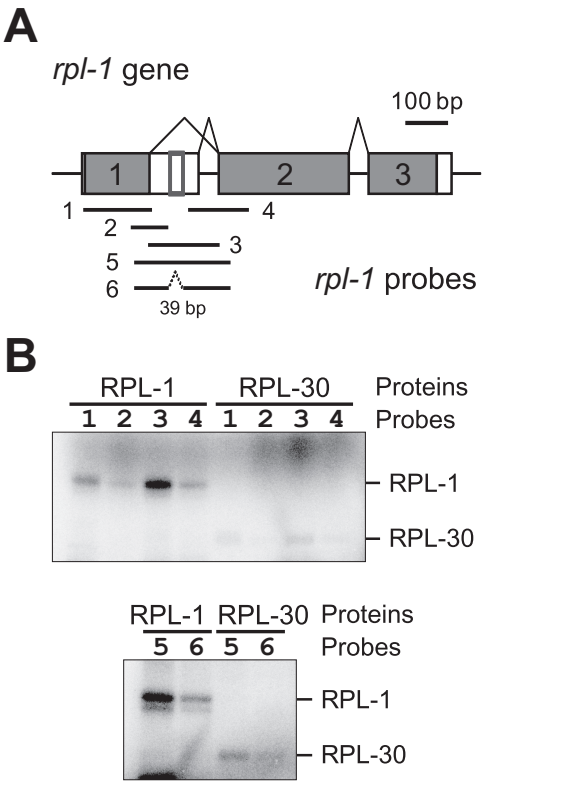

Figure 5. Recombinant RPL-1 protein directly and specifically recognizes L10ARE *in vitro*. (**A**) Schematic presentation of ^{32}P-labeled *rpl-1* RNA probes 1 to 6. L10ARE is boxed in grey. (**B**) UV-crosslink experiments utilizing probes 1 to 4 (top) and probes 5 and 6 (bottom) incubated with 2.5 μM of recombinant RPL-1 or RPL-30.

tive splice donor sites were utilized (Figure 6C, lanes 1–3). When co-expressed with L10a, the upstream (or productive) splice site was less efficiently selected (lane 2) compared to co-expression with RFP (lane 1) or L26 (lane 3). These results indicated that L10a specifically switches the donor site for *RPL10A* intron 3 from the upstream to the downstream sites in a similar way to *C. elegans rpl-1* autoregulation.

To test whether the autoregulation of the *RPL10A* gene is dependent on the putative L10ARE stretch, we constructed a deletion mutant (DEL) reporter minigene in which the stretch was entirely deleted (Figure 6B). The splicing pattern of the DEL reporter transcript was less significantly affected by L10a overexpression (Figure 6C, lanes 4–6), confirming that L10a autoregulates alternative splicing of the *RPL10A* pre-mRNA primarily via L10ARE in mammals like in *C. elegans*.

DISCUSSION

In the present study, we analyzed the mRNA-seq data from the WT and the NMD-deficient *smg-2* mutant strains of *C. elegans* to search all the 82 cytoplasmic *rp* genes for AS-NMD target mRNAs and identified eight *rp* genes, including four known genes (33), as regulated by AS-NMD. In the early study, Mitrovich and Anderson demonstrated that transgenic expression of RPL-12 protein affected alternative splicing of the endogenous *rpl-12* pre-mRNA in *C. ele-*

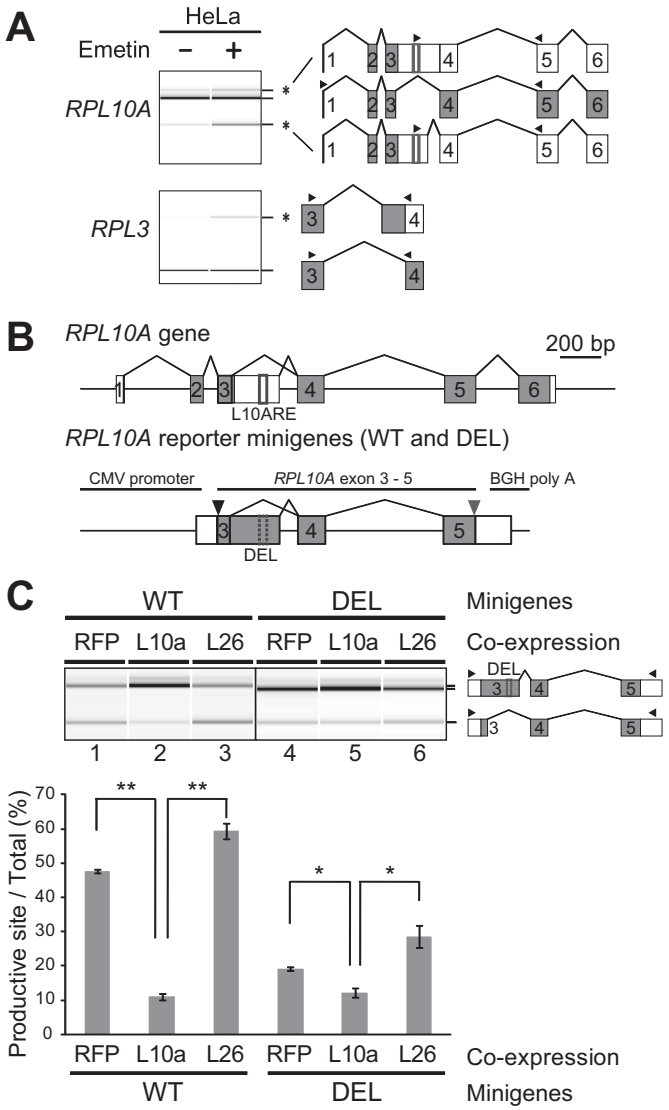

Figure 6. L10ARE-mediated splicing autoregulation by L10a is evolutionarily conserved in mammals. (**A**) RT-PCR analysis of *RPL10A* (top) and the *RPL3* (bottom) mRNAs in total RNAs from HeLa cells treated with vehicle (–) or 100 μg/ml emetin (+) for 6 h. Schematic structures of the mRNAs are indicated on the right as in Figure 1. Asterisks indicate the unproductive mRNAs stabilized by the emetin treatment. Gray boxes indicate L10ARE. Arrowheads indicate positions and directions of the PCR primers. *RPL10A* exon 1 forward primer was used at 5-fold lower concentration. (**B**) Schematic structures of the human *RPL10A* gene (top) and *RPL10A* WT and DEL splicing reporter minigenes (bottom). Boxes indicate exons and the coding region of the *RPL10A* gene is colored in gray. The 40-nt long L10ARE stretch is boxed in gray and is deleted in the DEL minigene. Exonic regions in the minigenes derived from the endogenous *RPL10A* gene are in gray. A potential initiation codon in exon 3 (black arrowhead) was disrupted in the minigenes to preclude unexpected translation and NMD of mRNAs derived from the minigenes. An artificial termination (gray arrowhead) was introduced in exon 5 to preclude potential non-stop decay (NSD) of the mRNAs. (**C**) Gel-like presentation (top) and a summary (bottom) of RT-PCR analysis of the mRNAs derived from the *RPL10A* splicing reporter minigenes co-expressed with mRFP1 (RFP), human ribosomal protein L10a or L26 in NIH3T3 cells. Averages of molar ratios of the splicing isoforms utilizing the upstream splice site to the sum of the two isoforms are indicated. Error bars indicate s.e.m. (n = 3). *0.001 < P < 0.05; **P < 0.001 (Student's t-test).

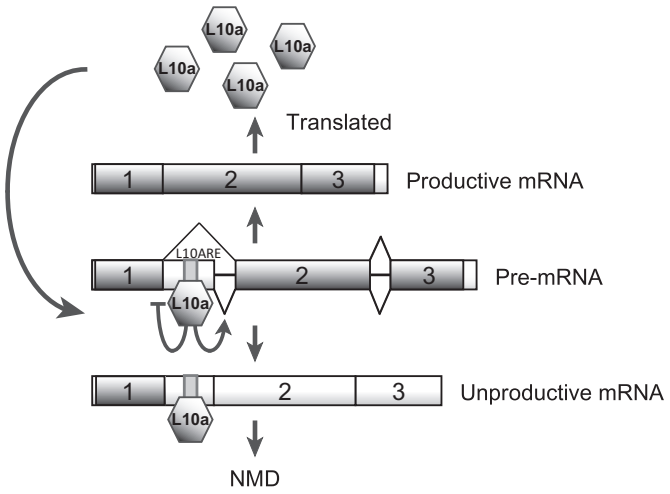

Figure 7. A model for autoregulation of *rpl-1* pre-mRNA splicing for quantity control of ribosomal protein L10a. See Discussion for details.

gans (33), suggesting autoregulation of splice site selection by a ribosomal protein in metazoans. We demonstrated here by knocking down the endogenous *rp* genes that alternative pre-mRNA splicing of not only *rpl-12* but all the eight *rp* genes with the AS-NMD isoforms are actively regulated for homeostatic control of their expression levels. We also demonstrated that RPL-1 can directly bind to its own pre-mRNA via the evolutionarily conserved element L10ARE to switch the 5′ splice sites of intron 1 for negative feedback regulation of its own expression like splicing regulators (Figure 7).

AS-NMD has been reported for some of the *rp* genes in other organisms. Global search for AS-NMD target mRNAs in *Drosophila melanogaster* S2 cells by utilizing a custom splicing-sensitive microarray identified five *rp* genes as regulated by AS-NMD (26). Among them, only *RpL10Ab*, encoding L10a, is overlapping with those in *C. elegans* as reported here. In human, AS-NMD mRNAs have been reported for *RPL3* and *RPL12* (49) besides *RPL10A* demonstrated here. These three genes were characterized after *C. elegans* orthologues and therefore global search for AS-NMD target *rp* genes in mammals are yet to be done. In *Saccharomyces cerevisiae*, most of protein-coding genes lack introns yet the *rp* genes are unusual in that they are over-represented among intron-containing genes (51,52). The introns in the *RPS21B*, *RPS14B*, *RPS9A* and *RPS9B* genes function to repress rather than to increase steady-state mRNA levels (51), suggesting regulatory roles for these introns. Thus, a limited subset of the *rp* genes are regulated at the splicing level in each organism. The genes encoding L10a are unique among the *rp* genes in that they commonly express AS-NMD target mRNAs in metazoans. Orthologues of L10a in Bacteria and Archaea, termed L1, have also been reported to autoregulate their own expression by directly binding to their own polycistronic mRNAs to repress translation (53,54), suggesting the physiological significance of fine-tuning gene expression of this evolutionarily conserved protein. L10a is modeled as a part of highly flexible L1 stalk comprised of helices 76, 77 and 78 of 28S rRNA in eukaryotic ribosomes (55) after crystal structure of prokaryotic ribosomes (56). The nucleotide sequence or predicted secondary structure of L10ARE, however, is not related to those of the L1 stalk. As the mode of target RNA recognition by L1 is considered to be highly conserved during evolution (54), other element(s) outside of L10ARE may also be involved in the specific recognition of its own pre-mRNA by L10a. Consistent with this idea, the entire nucleotide sequences between the two 5′-splices sites are highly conserved within the genus *Caenorhabditis* (33).

We demonstrated an extraribosomal function of L10a as a sequence-specific splicing regulator. Similar extraribosomal and autoregulatory functions have been described in the literature for some of the ribosomal proteins in eukaryotes, most of which rely on their properties as sequence- and structure-specific RNA-binding proteins. *S. cerevisiae* L30 (57) and human S13 (58) have been demonstrated to bind to their own pre-mRNAs to repress excision of their introns. Yeast L30 also represses translation of its own mature mRNA (59). *Xenopus laevis* L4 somehow represses splicing its own pre-mRNA to promote endonucleolytic cleavage (60). What is unique to L10a, however, is that it can alter splice site choice, although molecular mechanisms for splicing autoregulation by human *RPL3* have not yet been fully elucidated (49,61,62). Considering their ubiquitous expression as house-keeping genes and high conservation of their amino acid sequences, it is reasonable to suggest that ribosomal proteins can potentially regulate homeostasis of gene expression as sequence- and structure-specific RNA-binding proteins. As L10ARE is 45 nucleotides apart from the upstream 5′-splice site in *C. elegans* and further apart in other species shown in Figure 3, repression of the upstream sites may not be merely due to steric hindrance of spliceosome assembly. L10ARE is required for the specific binding of L10a *in vitro* (Figure 5 and Supplementary Figure S6) and splicing regulation by L10a *in vivo* (Figures 4 and 6), but not sufficient to switch splice site choice in a heterologous context (data not shown), suggesting that other elements as well as *trans*-acting factors may be involved in the splicing autoregulation by L10a.

We revealed unexpected crossregulation of the AS-NMD events between the eight *rp* genes in *C. elegans*. Crossregulation between paralogous genes via direct and specific binding has been reported for ribosomal proteins yeast S9 (51), S14 (63) and mouse L22/L22-like1 (64) as well as for a family of splicing regulators (65), which may be explained by duplication of genes that had already acquired autoregulation mechanisms. The crossregulation of the AS-NMD events between the *rp* genes is reminiscent of yet in good contrast to a recent report that RBFOX2 crossregulates AS-NMD of other splicing regulator genes in mouse embryonic stem cells (66). In the case of RBFOX2, it controls autoregulation of other splicing regulators by directly binding to canonical UGCAUG motifs in target pre-mRNAs (66). In the case of the *C. elegans rp* genes, on the other hand, it appears that not a single gene is dominant to the others but all are equivalent and mutually dependent. A likely mechanism, assuming that each of the ribosomal proteins can somehow autoregulate AS-NMD of its own transcript, is that the apparent crossregulations are all indirect secondary

effects caused by excess amounts of free ribosomal proteins upon knockdown of one of the *rp* genes. This idea is supported by the results demonstrating that the ratios of the productive mRNAs more prominently decreased upon lack of proteins in the same subunit than lack of those in the other subunit (Figure 2B).

As the ribosomes are abundant in cells and are assembled through a complex series of highly regulated steps (67,68), it is generally considered that the cells need to produce equimolar amounts of ribosomal proteins. Indeed, recent bioinformatics analysis of mRNA-seq data from various cell lines and tissues indicated that the molar ratio of mRNAs varies less than 3-fold for most of the human *rp* genes, with little tissue specificity (69). Heterozygous mutations in many of the *rp* genes have been reported to cause remarkable common phenotypes in metazoans presumably due to haploinsufficiency. In human, haploinsufficiency in any of 10 *rp* genes are considered to be the cause of Diamond–Blackfan anemia (DBA) (70). In zebrafish, 17 out of 28 lines with heterozygous mutations in the *rp* genes are prone to develop malignant peripheral nerve sheath tumors (71,72). In *Drosophila*, haploinsufficiency in 64 of 88 cytoplasmic *rp* genes are proven to cause or likely cause Minute phenotype (73). It is still unclear why a small or a large subset of the *rp* genes are prone to the common haploinsufficiency phenotypes while others are not. Remarkably, the genes encoding L10a were excluded from such genes prone to the haploinsufficiency phenotypes, consistent with the idea that autoregulation of gene expression by AS-NMD can compensate for lack of one allele to some extent. A particular subset of the *rp* genes especially sensitive to changes in gene dosage may have acquired the autoregulation mechanisms in each organism.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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