Alternative splicing and nonsense-mediated mRNA decay regulate mammalian ribosomal gene expression

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ABSTRACT

Messenger RNAs containing premature stop codons are generally targeted for degradation through nonsense-mediated mRNA decay (NMD). This mechanism degrades aberrant transcripts derived from mutant genes containing nonsense or frameshift mutations. Wild-type genes also give rise to transcripts targeted by NMD. For example, some wild-type genes give rise to alternatively spliced transcripts that are targeted for decay by NMD. In *Caenorhabditis elegans*, the ribosomal protein (rp) L12 gene generates a nonsense codon-bearing alternatively spliced transcript that is induced in an autoregulatory manner by the rpL12 protein. By pharmacologically blocking the NMD pathway, we identified alternatively spliced mRNA transcripts derived from the human *rpL3* and *rpL12* genes that are natural targets of NMD. The deduced protein sequence of these alternatively spliced transcripts suggests that they are unlikely to encode functional ribosomal proteins. Overexpression of rpL3 increased the level of the alternatively spliced rpL3 mRNA and decreased the normally expressed rpL3. This indicates that rpL3 regulates its own production by a negative feedback loop and suggests the possibility that NMD participates in this regulatory loop by degrading the non-functional alternatively spliced transcript.

INTRODUCTION

Nonsense-mediated mRNA decay (NMD) is a post-transcriptional pathway that recognizes and rapidly degrades mRNA that contains a premature termination codon (PTC) in its open reading frame (ORF) (1), thereby preventing the synthesis of truncated proteins that could be deleterious to the cell. NMD is evolutionarily conserved in all organisms tested, from yeast to humans (2). The NMD process is well understood in yeast, where it is believed to take place in the cytoplasm (3). *Cis*-acting elements required for PTC recognition include degenerate AU-rich sequences positioned downstream from the PTC (4). Although conforming to the same general strategy as in yeast, there are some relevant differences in the NMD process in higher eukaryotes. Namely, in mammalian cells NMD can occur either in the cytoplasm or in association with the nucleus (5), and is triggered when a stop codon lies >55 nt upstream from an exon-exon junction (6,7). The positions of exon junctions are marked on the mRNA by an exon junction complex (EJC) which is deposited 20–24 nt upstream from exon-exon junctions and is exported to the cytoplasm together with the spliced mRNA (8). A physiological stop codon has no downstream EJC, whereas a PTC is followed by at least one EJC (9).

The genetics of NMD has been studied in *Saccaromyces cerevisiae* and *Caenorhabditis elegans*. Three yeast (*Upf1–Upf3*) (10,11) and seven *C.elegans* (*smg1–7*) (12,13) genes are known to play an essential role in NMD. The smg2, smg3 and smg4 proteins, which are orthologues of the *S.cerevisiae* Upf1, Upf2 and Upf3 proteins, respectively, have been characterized (14). Homologues of yeast Upf proteins have been identified also in mammalian cells and their ability to direct aberrant mRNA to NMD has been established (15). However, NMD in higher eukaryotes seems to require a larger number of protein factors and a more complex regulation. In fact, the smg1 protein has not been found in yeast, whereas it is involved in NMD in *C.elegans* and human cells (16).

NMD has been largely studied as a control mechanism that prevents the production of potentially deleterious polypeptides resulting from a mutation in the DNA of germ or somatic cells, or as a result of inefficient splicing during post-transcriptional processing (17,18). However, besides counteracting aberrant

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gene expression, NMD has been implicated in the regulation of normal gene expression (19,20).

Mitrovich and Anderson (20) showed that unproductive mRNA isoforms for four ribosomal proteins, rpL3, rpL7a, rpL10a and rpL12, accumulate in *C.elegans* mutants that lack a component of the NMD pathway. They also demonstrated that in transgenic worms overexpressing rpL12, a greater proportion of endogenous *rpL12* transcript is spliced unproductively. These results indicated that rpL12 autoregulates its splicing and prompted the concept that one of the functions of NMD surveillance might be to eliminate RNA by-products of gene regulation. Mitrovich and Anderson (21) subsequently extended the surveillance role of NMD in *C.elegans* to transcripts of expressed pseudogenes that encode a small nucleolar RNA (snoRNA). This observation suggested that these transcripts could represent an evolutionary intermediate between snoRNA-encoding host genes that do or do not encode proteins. Alternatively spliced transcripts containing premature termination codons appear to be widespread in mammals, based on analysis of human EST databases (22). Interestingly, 11 ribosomal genes, including human *rpL3*, *rpL10a* and *rpL12*, were predicted to generate aberrant mRNAs.

In this study, we directly examined whether human ribosomal genes give rise to alternatively spliced transcripts subject to NMD. Towards this aim, we analyzed the expression pattern of the rpL3-, rpL7a-, rpL10a- and rpL12-encoding genes. This led to the identification of alternatively spliced nonsense codon-bearing transcripts from the *rpL3* and *rpL12 loci*. We showed that these transcripts are degraded by NMD and we uncovered an autoregulatory mechanism that appears to regulate the level of the alternatively spliced rpL3 transcript.

MATERIALS AND METHODS

Cell culture and transfection conditions

The human lung carcinoma Calu-6 cell line (ATCC, HTB 56; ICLC, HTL97003) was cultured in DMEM containing 10% fetal bovine serum (FBS) with 2 mM L-glutamine and 0.1 mM non-essential amino acids. Cells were grown to 80–90% confluence and then treated with 100 µg/ml cycloheximide for 4 h or with 20 µM wortmannin for 2 h.

The rat PC12 (pheochromocytoma cells) Tet-Off cell line (Clontech) was grown in DMEM supplemented with 5% fetal calf serum (FCS), 10% horse serum, 2 mM L-glutamine and 100 µg/ml G418 (Invitrogen). For stable transfection of these cells, 15 µg of pTRE2-hyg/HA-L3 or pTRE2-hyg/HA-L12 expression plasmids were transfected with lipofectAMINE Reagent, according to the manufacturer's instructions (Invitrogen). After transfection for 24 h, cells were incubated in fresh medium containing 200 µg/ml hygromycin (USB) and 10 ng/ml doxycycline (Sigma). Resistant clones were isolated 15–20 days later and expanded for further analyses. The expression of HA-L3 or HA-L12 was induced upon removal of doxycyclin. For NMD blockage, drug treatment was used as described above.

Origin of probes

The *GAPDH* probe was purchased from Clontech. The *p53* probe was derived from a plasmid generously provided by Dr F. Esposito. The *rpL3* and *rpL12* cDNA probes were generated by RT–PCR of total RNA extracted from HeLa cells, using the SuperScript One-Step RT–PCR kit and gene-specific oligonucleotides. The intronic specific probes, containing the retained region in alternative transcripts, were derived from PCR amplification on the alternative transcript cDNA, using the primer pair 5′-CTCCGCTCGGCTCTG-CCC-3′ (forward) and 5′-CTTCAGGAGCAGAGCAGA-3′ (reverse, Int3R) for *rpL3* intron 3 (see Figure 1B); 5′-GTG-CGTGCTCTGGTTGTG-3′ (forward, Int1F) and 5′-CCTA-GGGCGTGCGGGCTC-3′ (reverse) for *rpL12* intron 1 (see Figure 3B). The HA (5′-AAGAGCGTAATCTGGAACAT-CGTATGGGTACAT-3′) and *rpL3* 3′-untraslated region (3′-UTR) (5′-ATTTTATTGATGAGACCCCACCAGCTGC-ACAAAGTACTCCTGGCA-3′) probes were automatically synthesized.

Northern and western blot analyses

Total RNA was extracted using TRIzol reagent (Invitrogen), following the manufacturer's recommendations. Equal amounts (20 µg) of RNA were resolved in a 1.5% (for *rpL3* and *p53* analysis) or 1.7% (for *rpL12* analysis) agarose-formaldehyde gels and transferred to a nylon membrane (Millipore). Hybridizations with radiolabeled probes were performed as described by Church and Gilbert (23). The relative amount of RNA loaded per lane was normalized to that of *GAPDH*. cDNA probes were ^{32}P-labeled by random priming using [α-^{32}P]dCTP. Oligonucleotide probes were 5′ end-labeled with phage T4 polynucleotide kinase (Roche) and [γ-^{32}P]ATP. Signals were detected using a STORM 860 PhosphorImager and quantified using ImageQuant software v5.0 (Molecular Dynamics). Protein samples (100 µg/lane) were subjected to 10% SDS–PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). HA-tagged proteins were revealed with the rabbit HA-probe (Y-11) polyclonal antibody (Santa Cruz Biotechnology) and peroxidase-conjugated horseradish anti-rabbit antibody (Amersham Biosciences). An enhanced chemiluminescence (ECL) system (Amersham Biosciences) was used for detection.

Plasmid construction

The 5′-UTR of the human *rpL3* gene fused to the HA-tag was cloned as double-strand oligonucleotide in the pTRE2-Hyg inducible expression vector (Clontech) to generate the pTRE2-Hyg/HA plasmid. The *rpL3* and *rpL12* ORFs were RT–PCR amplified from HeLa RNA and cloned in pTRE2-Hyg/HA to obtain the pTRE2-Hyg/HA-L3 and pTRE2-Hyg/HA-L12 constructs. All recombinant plasmids were verified by sequencing.

RT–PCR analysis and sequencing

To identify the human alternative transcripts, RT–PCRs were performed using the primers shown in Figures 1B and 3B. Total RNA extracted from Calu-6 cells was treated with RQ1-Rnase-free DNaseI (Promega) to eliminate any genomic DNA and RT was carried out on 2−5 µg of RNA using the antisense oligonucleotides Int3R and Ex2R (5′-CAGGGCA-GAAGTGGCACC-3′) to synthesize the *rpL3* and *rpL12* cDNA fragments, respectively, and Superscript II RT (Invitrogen) according to the manufacturer's instructions. The PCR
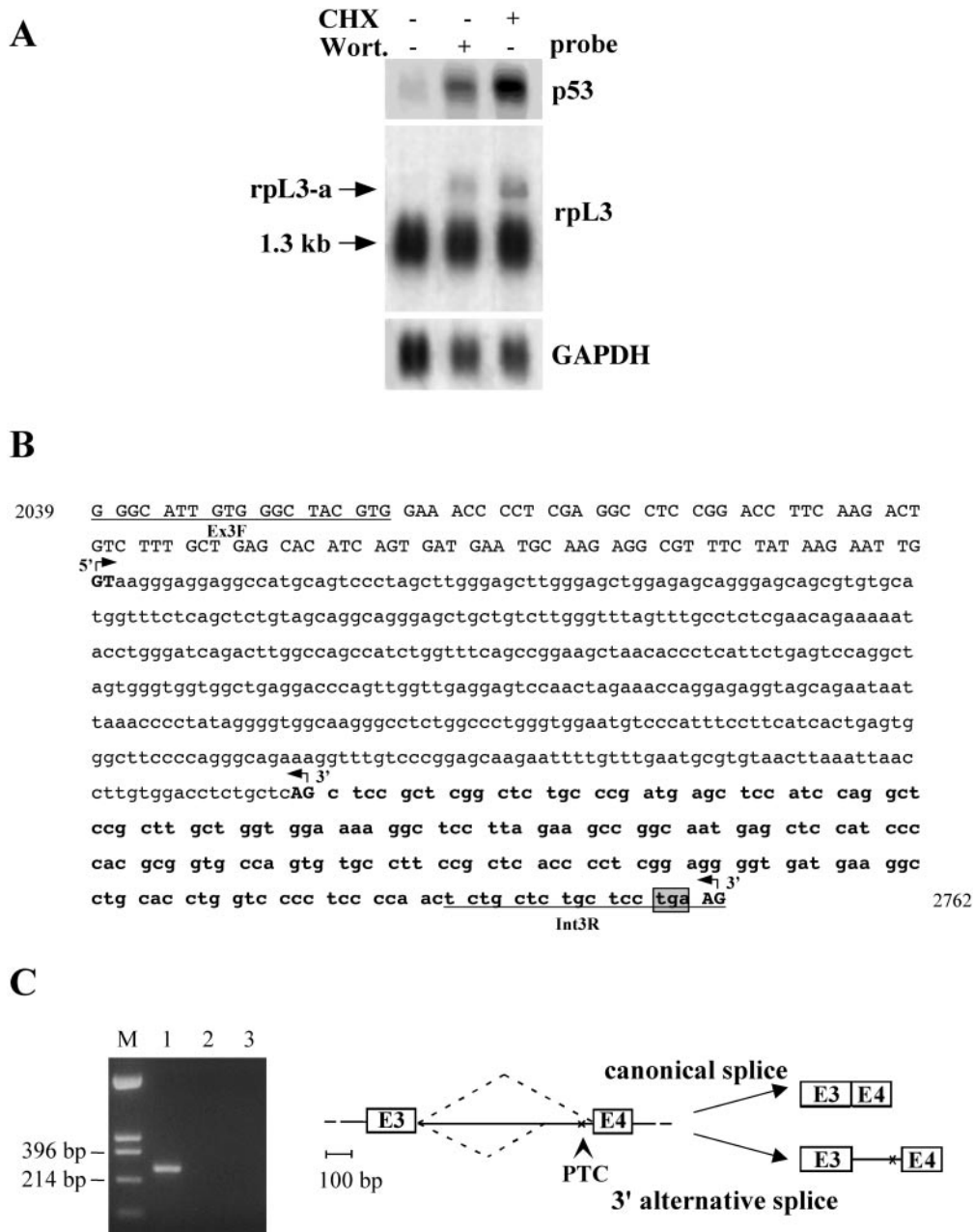
Figure 1. Identification of NMD sensitive isoforms of the human *rpL3*. (**A**) Analysis of *p53* and *rpL3* expression in Calu-6 cells after incubation with CHX (4 h) or wortmannin (Wort.) (2 h). Total RNA purified from treated or untreated cells was hybridized with the cDNA specific probes as indicated. The size of canonical mRNA is indicated; rpL3-a indicates the newly identified isoform. The quantity of RNA was normalized using a GAPDH-specific probe. A representative result of three independent experiments is shown. (**B**) Nucleotide sequence of *rpL3* intron 3 with 5′ flanking exonic sequences. Lower case letters refer to intronic nucleotides; upper case letters refer to exonic nucleotides. The intronic sequence retained by alternative splicing is indicated in bold. The primers used for RT–PCR are underlined. Canonical and alternative splice sites (GT/AG), as determined by sequence analysis of RT–PCR products, are shown in bold upper case letters and indicated by 5′ or 3′ arrows. The ORF of exonic and retained intronic sequences is shown. Nucleotides encoding the PTC are boxed and shaded in gray. The nucleotide positions according to the corresponding NCBI/GenBank sequences (accession no.: AL022326) is indicated. (**C**) Left panel RT–PCR assays of RNA from Calu-6 cells. Amplifications were performed with the following primers: lane 1, Ex3F/Int3R (rpL3-specific); lane 2, reaction was carried out as in lane 1 but omitting the RT step. As negative control, amplification was performed without cDNA (lane 3). The base pairs of HinfII-digested pUC18 (lane M) are indicated on the left. Right panel, schematic drawing of alternative splicing of human *rpL3*; intron 3 and flanking exons 3 and 4 (E3, E4) are shown.

amplifications were performed according to standard procedures. In brief, alternative cDNA products were amplified for 35 cycles, with annealing at 54°C (for *rpL3* amplification) or 58°C (for *rpL12* amplification), using the primer couples Ex3F (5′-GGGCATTGTGGGCTACGT-3′)/Int3R for *rpL3* and Ex2R/Int1F for *rpL12*.

To identify the mouse *rpL12* alternative transcript, a RT–PCR was performed as described above, using RNA extracted from heart tissue and the following primer couple: mInt1F (5′-GTGCGTGCCCTCGCCGCG-3′)/mEx2R (5′-CAGACCCAGAGGACCGAT-3′) (see Figure 5B). The annealing step in the amplification reaction was performed

at 50°C. PCR products were resolved on ethidium bromide-stained 2% agarose gels, and analyzed by dideoxy sequencing.

Sequence analysis and comparison

Sequences of rp genes were aligned using the CLUSTAL W algorithm (24), then adjusted manually. Sequence identity for the individual *rpL3* introns and exons was established by using the web-based EMBOSS infoalign program.

RESULTS

Blockade of NMD upregulates alternatively spliced rp transcripts

To determine whether the mammalian *rpL3*, *rpL7a*, *rpL10* and *rpL12* genes give rise to alternatively spliced transcripts containing premature termination codons, we exploited the observation that NMD-regulated mRNAs accumulate in cells exposed to NMD-blocking drugs. In this study, we used wortmannin, an inhibitor of the human smg1 kinase (16), cycloheximide (CHX), an inhibitor of protein synthesis that represses NMD (25), and the human cell line Calu-6 (26) in which a nonsense mutation in the *p53* gene results in the production of a PTC-containing mRNA that is an NMD substrate (16). Total RNA was prepared from drug-treated and non-treated cells and analyzed in northern blotting experiments. As shown in Figure 1A, the aberrant p53 mRNA was barely detectable in non-treated cells, and very pronounced in treated cells, which is consistent with the observations made by Yamashita *et al.* (16). Having shown that NMD was blocked, we examined whether rp transcripts were induced. While we did not detect induction of alternative human *rpL10a* nor *rpL7a* transcripts, we did observe a novel 1.5 kb *rpL3* transcript induced by cycloheximide and wortmannin. In fact, like the aberrant p53 mRNA, this minor species was barely detectable in untreated cells and very pronounced in treated cells (Figure 1A). In contrast, the level of the canonical 1.3 kb rpL3 mRNA was not affected by these treatments, as its level was virtually unchanged, as occurs for the control GAPDH transcript (Figure 1A). We named the alternative rpL3 transcript 'rpL3-a'.

NMD-substrate transcripts of rp genes are generated by alternative splicing events that lead to retention of intronic sequences

As indicated above, some human rp genes have been predicted to produce NMD-candidate mRNA isoforms (22). More specifically, three alternative mRNA isoforms of the *rpL3* gene have been predicted to contain a PTC, one of which includes a putative transcript that has a molecular size larger than the canonical (i.e. productive) mRNA. In such a case, the use of the proposed splice sites would result in an alternative mRNA isoform that retains some intronic sequences from intron 3. In an attempt to obtain experimental proof of this predicted alternative mRNA isoform, we evaluated whether rpL3-a mRNA accumulates in treated cells. For this purpose, we examined the *rpL3* gene using RT–PCR analysis with Calu-6 RNA as template, and primers flanking the proposed alternatively spliced intron (Figure 1B). After amplifications,

a cDNA species was detected (Figure 1C, left panel), thereby providing experimental evidence that alternative splicing had occurred within the candidate intron of *rpL3*.

Sequencing analysis of the RT–PCR product revealed the site of the splicing event that caused the retention of a portion of the intron. In detail, 3′ alternative splicing in intron 3 of *rpL3* generated a larger transcript that retained the 3′-most 180 nt of the intron (Figure 1B, also see schematic representation in Figure 1C, right panel). The size of the alternative mRNA species is consistent with the apparent molecular size of the NMD-sensitive mRNA species identified for rpL3 (Figure 1A). Inclusion of the alternatively spliced region led to the generation of an in-frame translation termination codon in the *rpL3* transcript localized >50–55 nt upstream from the 3′-most exon-exon junction. The latter feature characterizes PTCs responsible for conveying the aberrant mRNA to the NMD pathway.

A DNA probe spanning sequences from the *rpL3* intron 3 retained by alternative splicing, and thus specific for the alternative isoform, was hybridized to RNA extracted from Calu-6 cells treated or not with NMD-blocking drugs and fractionated on agarose gel. As shown in Figure 2A (left panel), in the presence of drugs, the specific probe revealed an mRNA species, whose size and accumulation mode were identical to those of the NMD-sensitive isoform shown in Figure 1A. The levels of this mRNA are shown in the panel on the right.

Upon withdrawal of CHX and wortmannin, the rpL3-a mRNA decreased progressively and reached a (low) steady-state level in 6 h, whereas the canonically spliced mRNA was unaffected by drug removal (Figure 2B). These findings confirm that the alternative transcript is NMD substrate. In fact, it accumulates consequent to NMD block, and is rapidly degraded upon NMD restoration.

An NMD candidate mRNA larger than the canonical mRNA has also been predicted for the *rpL12* gene (22). In this case, alternative splicing of the primary transcript leads to retention of some intron 1 sequences. We thus looked for NMD-targeted rpL12 mRNA isoforms using the same experimental strategy described for *rpL3*. In a northern analysis, an *rpL12* cDNA probe revealed a major mRNA species of 0.63 kb in untreated as well as in cells treated with NMD-blocking drugs, and a minor species of an apparent size of 0.8 kb, which accumulated after NMD inhibition (rpL12-a in Figure 3A). An intron 1 probe specifically detected the NMD-sensitive mRNA isoform (Figure 3A). An RT–PCR experiment (data not shown) with Calu-6 cell RNA as template, and primers flanking intron 1 followed by sequencing analysis of the amplification product identified the start site of the splicing event: a 5′ alternative splicing in intron 1 of *rpL12* generated an mRNA isoform that contained 210 bp of the 5′ region of the intron (Figure 3B and C).

In summary, the human *rpL12* and *rpL3* genes generated two alternative mRNAs: a canonical transcript that is not affected by NMD, and an isoform produced by alternative splicing that results in incomplete removal of introns 3 and 1, respectively (see schematic representation in Figures 1C and 3C). The alternative splicing introduces PTCs, and the stability of these transcripts increases when NMD-mediated degradation is inhibited.
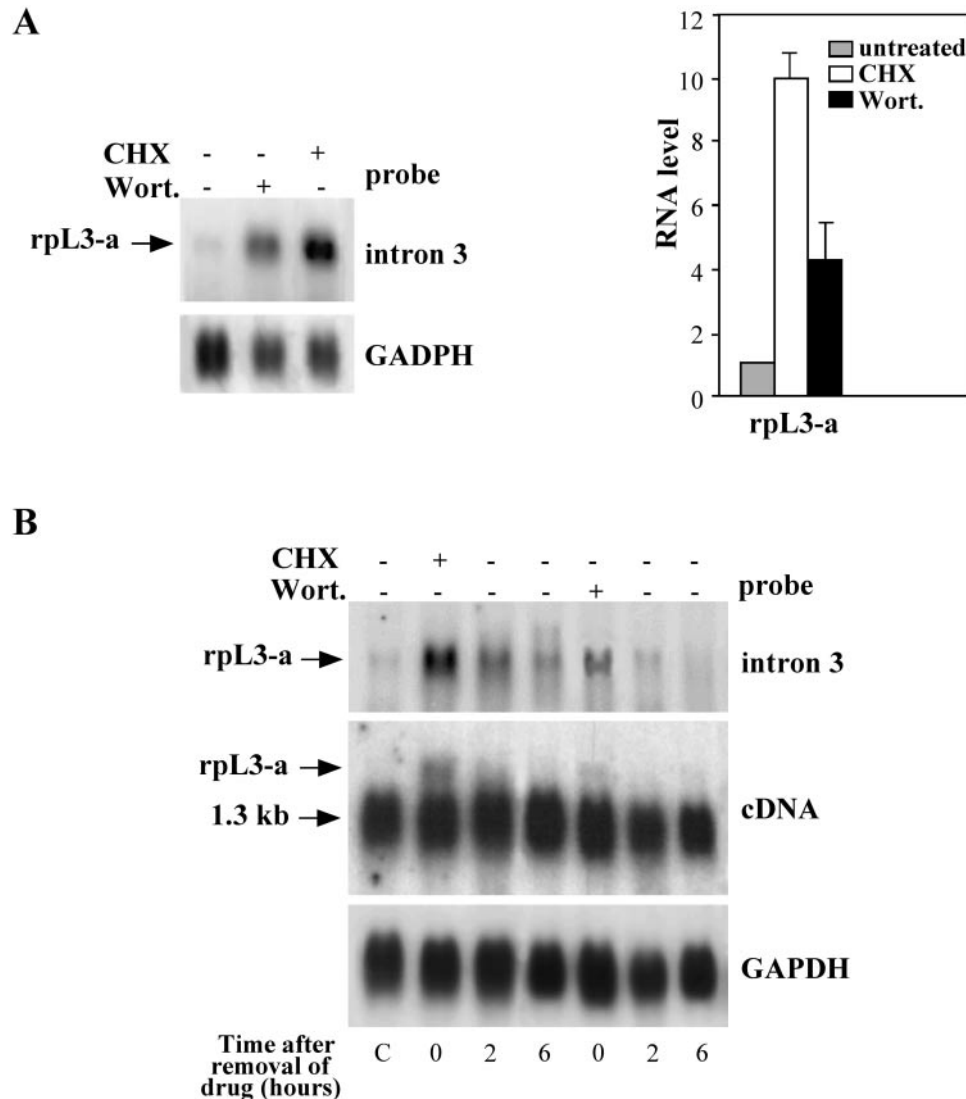
A

B

Figure 2. Expression of alternatively spliced mRNA of the *rpL3* gene (rpL3-a mRNA). (**A**) Left panel: northern blots of total RNA from untreated Calu-6 cells and from the same cells after incubation with either CHX or Wort. The probe used for hybridization is specific for the portion of intron retained by alternative splicing. Right panel: rpL3-a mRNA was quantified by PhosphorImager, normalized to GAPDH levels and expressed in a graph as a ratio to endogenous levels in untreated cells. Numerical values are the average of three independent experiments, reported with SDs. (**B**) Decay of the drug-stabilized rpL3-a mRNA. Total RNA was isolated from Calu-6 cells before treatment (lane C), after treatment with CHX or with Wort., and 2 and 6 h after drug removal. Northern blot was performed with the indicated probes.

rpL3 and *rpL12* alternative splicing is conserved among mammalian species

Alternative splicing transcripts of these genes could result from a systematic error of the splicing machinery. Alternatively, non-canonical splicing could be functionally relevant for gene expression, and thus regulated to modify the level of the functional ribosomal protein. To test these hypotheses we investigated whether alternative splicing of the type observed in human cells occurs in other organisms. To this aim, we compared the sequences of the human affected introns with the introns of orthologue genes from other eukaryotes.

Using the CLUSTAL W algorithm, we aligned the sequence of human *rpL3* intron 3 with each intron in the *C.elegans* and *Drosophila melanogaster rpL3* genes, and with the corresponding intron in the murine and bovine *rpL3* genes. While

we found a low degree of sequence identity between *C.elegans* and *D.melanogaster rpL3* introns, there was a high degree of sequence identity in *rpL3* intron 3 in mammals (Figure 4A). In particular, the early portion of the intron (2144–2557 nt) shows a sequence identity of about 50%, whereas the remaining 3′ region (2558–2762 nt) is nearly identical (nucleotide identity of about 90%) in the three species. This region contains the alternatively spliced sequence corresponding to 2583–2762 nt. Thus, the portion of the intron retained by alternative splicing in the human *rpL3* mRNA isoform is more conserved than the portion excised.

To determine whether this degree of conservation applied to other introns of the gene, we compared the whole intron–exon sequence of the *rpL3* gene in human, bovine and rodent species. As expected, exon sequences are highly conserved, the nucleotide identity ranging from 80 to 90%, whereas intron
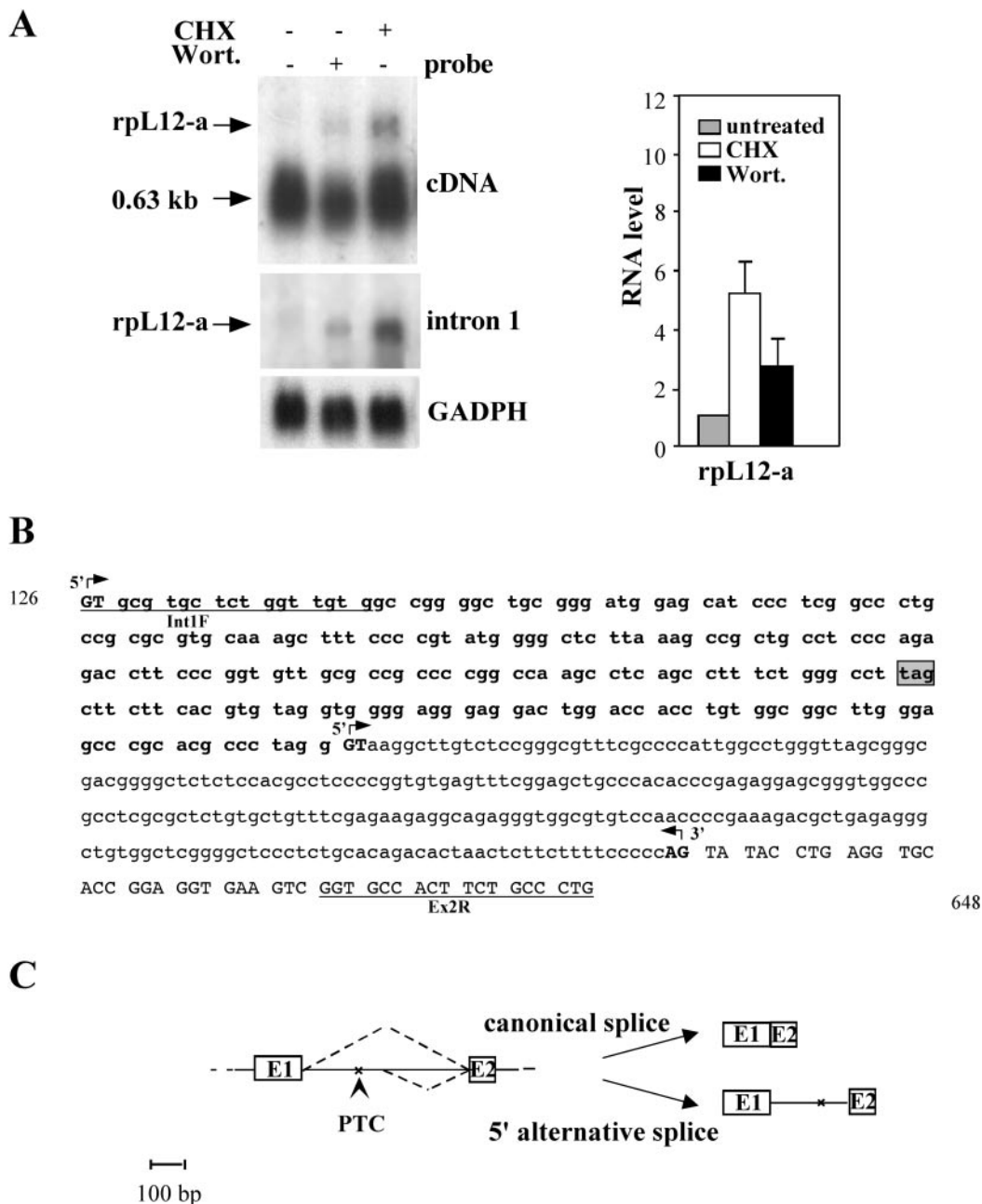
Figure 3. Identification and expression of an alternatively spliced isoform of human *rpL12* gene. (**A**) Left panel: northern blot analysis of *rpL12* expression in Calu-6 cells after incubation with CHX (4 h) or Wort. (2 h). Total RNA purified from treated or untreated cells was hybridized with the cDNA specific probes as indicated. The size of canonical mRNA is indicated; rpL12-a indicates the newly identified isoform. The quantity of RNA was normalized using a GAPDH-specific probe. A representative result of three independent experiments is shown. Right panel: rpL12-a mRNA was quantified by PhosphorImager, normalized to GAPDH levels and expressed in a graph as a ratio to endogenous levels in untreated cells. Numerical values are the average of three independent experiments, reported with SDs. (**B**) Nucleotide sequence of *rpL12* intron 1 with 5′ or 3′ flanking exonic sequences. Lower case letters refer to intronic nucleotides; upper case letters refer to exonic nucleotides. The intronic sequence retained by alternative splicing is indicated in bold. The primers used for RT–PCR are underlined. Canonical and alternative splice sites (GT/AG), as determined by sequence analysis of RT–PCR products, are shown in bold upper case letters and indicated by 5′ or 3′ arrows. The ORF of exonic and retained intronic sequences is shown. Nucleotides encoding the PTC are boxed and shaded in gray. The nucleotide positions according to the corresponding NCBI/GenBank sequences (accession no. NT_008470) is indicated. (**C**) Schematic drawing of alternative splicing of *rpL12*; the two first exons (E1 and E2) and the first intron are shown.

sequences have a more complex pattern of conservation (Figure 4B). In fact, the average sequence identity shared by human and bovine introns is 50%; this identity reaches 60% for the snoRNA-encoding introns 1, 5 and 7. A putative snoRNA coding sequence has also been identified in intron 3

of *rpL3*; however, it does not appear to be expressed and, moreover, the highly conserved region is significantly longer than the putative snoRNA sequence (27). Thus, no other intron sequence appears to be as conserved as the intron 3 region that is affected by alternative splicing. The pattern of conservation

A

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hInt3  GTAAGGGAG--GAGGCCATGCAGTCCCTAGCTTGGGAGCTTGGGAGCTGGAGAGCAGGGAGCAGCGTGTG-CATGGTTTCTC  2222
bInt3  GTAAGGGAGCCGCGGCTCGGCTCACCCTGGGCTGGG---CCAGGGG--AAAGGGCTGTGTGCTTTGTGGGGCCTCAGCTCCG
mInt3  GTAAGAGGCTCTTGGGTT--CCCTTAGAAGTAAGGGACACTCTGAGCTGGGATGTAAAGTGAAGGAAGTTGGATCAAAGGTT

hInt3  AGCTCTGTAGCAGGCAGGGAGCTGCTGTCTTGGGTT--TAGTTTGCCTCTCGAACAGAAAAATACCTGGGATCAGACTTGGC  2302
bInt3  TATTCGGCGGCAGGGCAGCTGCTTTGGCGTCACATT--TAGTC-GCCTCTTGGGCAGAACAGTTCTCAGGAACA-ATGGGAG
mInt3  TTTTGAACCAAAAGCTGACTGTTAACCCTTGAGGGTGCTGGTCAG--TCCTGGTTAGCAGAAGTGTTAGAGTTG--CTTTGA

hInt3  CAGCCATCTGGTTTCAGCCGGAAGCTAACACCCTCATTCTGAGTCCAGGCTAGTGGGTGGTGGCTGAGGACCCAGTTGGTTGA  2385
bInt3  CAGCGTCCCGG--------------GACATTTCCTTT---GGTGTTGACCT-CGGGTGCTGCCTCAG----------TTCG
mInt3  CACTGAAGTGGC-----------AGGGAAGCTGGAAAACAAGACAT---CCTGCATCTGG-GCCTTAGT---------GTGAG

hInt3  GGAGTCCAACTAGAAACCAGGAGAGGTAGCAGAATAATTAAACCCCTATAGGGGTGGCAAGGGCCTCTGGCCCTGGGTGGAAT  2468
bInt3  GCAATCCTTTAGGTTGCTGACAAGTATGGGGGTCTGTTTAGA--------GTGGTAACTGATACCCCCAGCCC-ACCCCAGGT
mInt3  GTAATCCTTTGAG------GCTTGGATGCTGGCCTTGTCT----------GTACTAGATAGGTCCCCTGCCTCTGGCCTGAGC

hInt3  GTCCCATTTCCTTCATCACTGAGTGGGCTTCCCCAGGGCAGAAAGGTT----TGTCCCGGAGCAAGAATTTTGTTT---GAAT  2544
bInt3  GTGTGGTCTGGTTAATGGCTAA--------TTTGGAGCAAGAA--TT----TATTTGTGTGTCCGAATGTTGT--------
mInt3  CCCTGACTTCCACTACTATGAGATGTGCC-CCCAGATAAAGGGATGTTCTGGTGCCCTGGAGCAAGAGTTGTGTGTTGTGTAT

hInt3  GCGTGTAACTTAAATTAACCTTGTGGACCTCTGCTCAGCTCCGCTCGGCTCTGCCCGATGAGCTCCATCCAGGCTCCGCTTGC  2627
bInt3  --GTTTCATT---ATTAACCTTGTGGACCTCTGCTCAGCTCCGCTCGGCTCTGCCCGATGAGCTCCATCCAGGCTCCGCTTGC
mInt3  ATTTGTGGCTTATATTAACCT-GTGGACCTCTGCTCAGCTCCGCTCGGCTCTGCCCGATGAGCTTCATCCAGGCTCCGCTTGC

hInt3  TGGTGGAAAAGGCTCCTTAGAAGCCGGCAATGAGCTCCATCCCCACGCGGTGCCAGTGTGCCTTCCGCTCACCCCTCGGAGGG  2710
bInt3  CGGTGGAAAAGGCTCCTTAGAAGCCGGCAATGAGCCCCGTCCCCACACGGTGCCTGTGTGCCTCCCCCTCACCCGTTGGAGGG
mInt3  CGGTGGAAAAGGCTCCTTAGAAGCCGGCAATGAGCCCCATCCCCAAATGGTGCCAGTCTGCCTTCCTCTCACCTGTTGCAAGG

hInt3  GTGATGAAGGCCTGCACCTGG-TCCCCTCCCCAACTCTGCTCTGCTCCTGAAG  2762
bInt3  CTGATGAAGGCTGGCACCTGGGCCCCCTCCCCAACTCTGCTCTGCTCCTGAAG
mInt3  GTGATGAAGGCCTGCACCGGG-CCCCTCCCCAACTGTGCTCTGCTCCTGAAG
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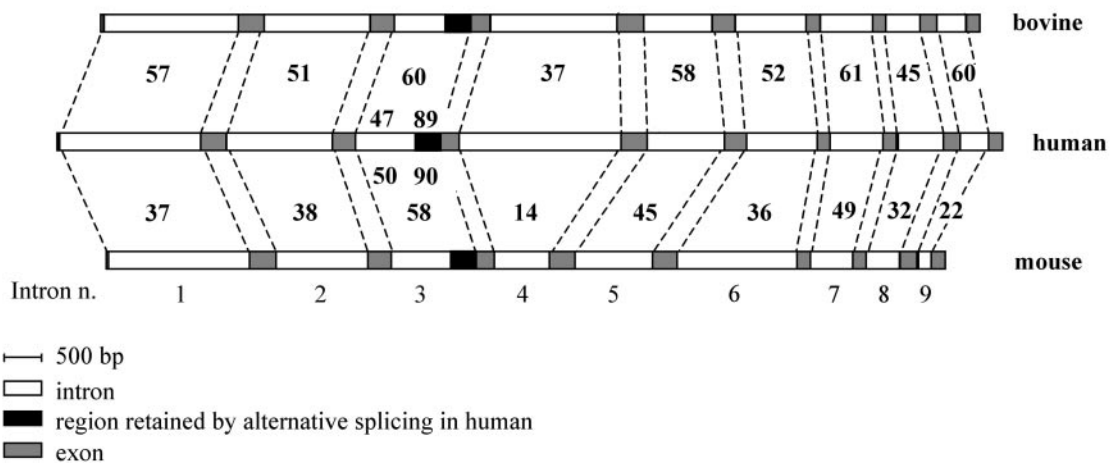
B

Figure 4. (**A**) Sequence comparison of the *rpL3* alternatively spliced intron among mammals. Nucleotide sequences of human, bovine and mouse intron 3 (hInt3, bInt3 and mInt3, respectively) are aligned. Conserved residues are shaded in gray; the region retained by alternative splicing is underlined. The numbers on right of sequences indicate the nucleotide positions of the human *rpL3* gene according to the corresponding GenBank sequences. Accession nos of sequences (NCBI/GenBank): AJ238851 and NT_039621 for bovine and mouse genes, respectively; for the human gene, refer to the legend to Figure 1 (the arrowhead indicates the alternative splice site). (**B**) Comparative analysis of rpL3 introns among mammals. The numbers indicate the percent nucleotide identity of human sequences compared to the mouse and bovine genes. For intron 3, the nucleotide identity of the portions retained and excised by alternative splicing is indicated.

is even more interesting when we compare the human and mouse genes. In fact, although the average sequence identity of introns is lower (30%), reaching ∼50% only for introns 5 and 7, which, in analogy with the human and bovine *rpL3* genes, could contain snoRNAs, the region of intron 3 retained after alternative splicing is, again, highly conserved (90% identity). Moreover, it is noteworthy that the whole mouse intron 3 appears to be more conserved than any other intron (58%).

We next searched EST databases for cDNA sequences containing the region of interest. We found that several cDNA clones of *Mus musculus* and *Bos taurus* EST (e.g. accession nos CA574552, AW761943, AV612606 etc.) contain sequences spanning 2583–2762 nt of the human intron 3.

Taken together, the foregoing results indicate that: (i) the sequence of alternatively spliced *rpL3* intron is under strong selective pressure, the retained portion being conserved as well as the exons; (ii) the type of alternative splicing described in the human gene appears to be conserved among mammals.

We conducted a similar sequence analysis of alternatively spliced intron 1 of the human *rpL12* gene. The exon–intron organization of this gene is known for fewer organisms. However, while the bovine and human sequences of *rpL12* intron 1 share a 70% identity, the mouse sequence appears much less conserved. In fact, we found that the human intron 1 is larger than the corresponding intron in the mouse gene, and sequence conservation is restricted to a few short regions (Figure 5A). However, a BLAST search in the mouse EST database performed with the mouse intron sequence as a query revealed that several expressed sequences contain the 5′-most 121 nt of intron 1. Furthermore, using a RT–PCR strategy (Figure 5B) similar to that described above for human rp genes, we identified a mouse alternative cDNA containing this region. Thus, alternative splicing occurs in the 5′ region of intron 1 in the mouse *rpL12* gene as well as in the human counterpart. Sequence analysis of the alternative transcript demonstrated that, unlike the human *rpL12* and *rpL3* alternative isoforms in which PTCs occur in the retained intron sequences, in the mouse alternative *rpL12* transcript the partial retention of intron 1 results in a frame-shift that introduces a PTC in the downstream exon 3 (Figure 5B). Notably, the 5′ alternative splice site in the mouse *rpL12* gene is located at exactly the same position as in the human gene, and in a highly conserved region (Figure 5A).

The levels of rpL3 expression regulate the alternative splicing

Our demonstration that *rpL3* and *rpL12* alternative splicing is conserved among mammals strongly suggested that this event is not simply the result of a post-transcriptional processing error, but it could serve to regulate ribosomal protein expression levels. In fact, because the alternative splicing generates an unproductive mRNA isoform, the cellular level of a functional ribosomal protein might be correlated to the regulation of this event. To address this issue, we evaluated whether the amount of the *rpL3*-generated protein affects the splicing pattern of the *rpL3* gene. We used PC12 Tet-Off cells to induce overexpression of the protein. Cells were stably transfected

with a vector containing the human *rpL3* coding sequence fused to the hemagglutinin (HA) epitope coding sequence, and a hygromycin selection marker. Total cell lysates from 20 hygromycin-resistant clones were analyzed 48 h after removal of doxycyclin by western blotting with an HA-specific antibody to monitor the production of HA-L3 protein, and clones with the highest expression level were selected. Time course analysis of the two most inducible clones (L3–8 and L3–9), at both RNA and protein level, demonstrated that the expression peaks 24 h after the removal of doxycycline (data not shown).

In these experiments, total RNA was prepared from control cells (PC12) and from L3–8 and L3–9 clones, with or without doxycycline, each under drug-treatment and non-treatment conditions, and then analyzed by northern blotting (Figure 6A). The same filter was sequentially hybridized with: (i) an oligonucleotide probe specific for the HA-tag, which detects exogenous HA-L3 mRNA; (ii) the intron probe, which only detects the alternative transcript; and (iii) a probe specific for the 3′-UTR of *rpL3* cDNA, which distinguishes the endogenous canonical transcript from the exogenous transcript. The amount of each mRNA species was quantified by PosphorImager analysis (Figure 6B). The expression of the HA-L3 protein was evaluated by western blot analysis on protein extracts from the same cells using an HA antibody (αHA in Figure 6A). As expected, in both clones, exposure to CHX and the consequent block of translation reduced the amount of exogenous protein.

In control cells (Figure 6A, lanes 1–3), endogenous rpL3 mRNAs reacted in the same fashion as they did in Calu-6 cells, which indicates that the alternative splicing described for the human *rpL3* gene is conserved in rat cells. In fact, the amount of canonical mRNA was the same in treated and untreated cells, whereas the steady-state amount of alternatively spliced mRNA was very low in the absence of drugs and increased after incubation with wortmannin (3-fold) and even more so after incubation with CHX (9-fold), thus demonstrating that this transcript is stabilized by NMD inhibition. As expected, a similar pattern of expression occurred in clones L3–8 (lanes 4–6) and L3–9 (lanes 10–12) when the transcription of exogenous HA-L3 cDNA was inhibited by the addition of doxycycline.

The induction of the HA-L3 protein (lanes 7–9 and 13–15) resulted in relevant differences in the expression levels of both canonical and alternative rpL3 transcripts. In fact, the increase in the alternative isoform was greater in cells in which NMD was blocked than in control cells and in non-induced clones, which suggests that the HA-L3 protein and drugs exert an additive effect on the levels of this mRNA. Interestingly, in cells not exposed to the NMD-inhibiting drug, the overexpression of the protein was associated with accumulation of the alternative transcript. In addition, the expression of the exogenous HA-L3 protein was associated with a relevant decrease (50%) of the amount of canonical mRNA. This effect was drug-independent (lanes 8, 9, 14 and 15) and absent in non-induced clones (lanes 4–6 and 10–12). These results demonstrate that the down-regulation of canonical rpL3 mRNA is related to the expression of the exogenous protein. Moreover, clone L3–8, which expressed the highest amount of HA-L3 at both RNA and protein level, had the largest increase of the alternative transcript and the largest decrease of canonical

A

```
hInt1  GTGCGTGCTCTGGTTGTGGCCGGGGCTGCGGGATGGAGCATCCCTCGGC-------------CCTGCC  180
bInt1  GTGCGTGCTTTAGATGTGGCCGGGGCTTCGGGATGCGGCATCCCTCCCCGTATTCCGTCCGGCCCGCG
mInt1  ------------------------------------------------------------------

hInt1  GCGCGTGC----AAAGCTTTCCCCGTATGGGGCTCTTAAAGCCGCTGCCTCCCAGAGACCTTCCC---  241
bInt1  GCCCCCGCCGCTATGGCCTTTCCCACGTCGGGC-CTCAAGGCCGCTGCCTCCTAGGGGCCTGCCCTCT
mInt1  -------------------------------------------GTGCGTGCCCTCGCCGCGCCC---

hInt1  --------------GGTGTTGCGCCGCC-CCGGCCAAGCCTC--AGCCTTTCTGGGCCTTAGCTTCTT  292
bInt1  GGTGCTGCGTTCAGGGCGGTGTGCGGTC-GTGGTCGAGTCTCTTGTTCTTTCTGGGCCTCGGCTTCCC
mInt1  ------------GGGTGCTGTCCCGGGGCCTGGCGGGTCTC-------CCTTGGGCCTTGGCTGCCA
                                                               ▼
hInt1  CACGTGTAGGTGGGGGAGGGAGGACTGGACCACCTGTGGCGGCTTGGGAGCCCGCACGCCCTAGGGTAA  360
bInt1  CACGTATAGATGGGGGAGGGAGGACCGGACGGCCTGTGGCGGCTTCGCAGCCCGCACGCCTTAGGGTAA
mInt1  CGTGTGTAAGAGGGGAGGGAG-------------CCCGACGCTCTGGCGGCCCGTGCGCCTTAGGGTAA

hInt1  GGCTTGTCTCCGGGCGTTTCGCCCCATTGGCCTGGGTTAGCGGGCGACGGGGCTCTCTCCACGCCTCC  428
bInt1  GGTTTGTCTCCGGGCGCTTCTCCC---TAAACTGGGTTGGCCGGCGGCGGGGCTGCCCCCAAACCGAT
mInt1  GGCCTGGCCCGGGACATCTGAAGC------CCGACGGCAGCGCGCAGGGCGGCCTGCGCCGCAGAGGT

hInt1  CCGGTGTGAGTTTCGG-AGC--TGCCCACACCCGAGGGAGGAG---CGGGTGGCCCGCCTCGCGCTCT  490
bInt1  GTGGTGTGGGCTTCGGCAGCGGTGGCCGAACCCAAGGCCGCCGTCCCACGTGGTGCTTGGAGCGGTTC
mInt1  TCTGATTTTGCTTTTTCTTCAG--------------------------------------------

hInt1  GTGCTGTTTCGAGAAGAG-------------------GCAGAGGGT---GGCGTGTCCAACCCCGGAAA  537
bInt1  TCGCCTTGGCGGGAGGGGTGTCGTGCCTTGCTTCCCGCTGAGGCTCTTGGCATTTTTCCGCCAAGAGG
mInt1  ------------------------------------------------------------------

hInt1  GACGCTGAG--AGGGCTGTGGCTCGGG-GCTCCCTCTGCACAGACACTAACTCTTCTTTTCCCCCAG  601
bInt1  ACTGCGGAGGGAGAGCTGTGATTTGGGAGCCCCTTCTGCGGAGGCACTAATTCTTTCTTTCTTCCAG
mInt1  ------------------------------------------------------------------
```

B

Mouse *rpL12*

```
78    ATG CCG CCC AAG TTC GAC CCC AAC GAG GTC AAA GTC GGT gcg tgc cct
                                                         5'⌐►
      cgc cgc gcc cgg gtg ctg tcc cgg ggc ctg gcg ggt ctc cct tgg gcc
                                                         mInt1F
      ttg gct gcc acg tgt gta aga ggg gag gga gcc cga cgc tct ggc ggc
                                 5'⌐►
      ccg tgc gcc tta gg GTaaggcctggcccgggacatctgaagcccgacggcagcgcgcaggg
      cggcctgcgccgcagaggttctgattttgcttttttcttcAG T GTA CCT GAG GTG CAC
                                            ◄⌐ 3'
      CGG AGG CGA GGT CGG CGC CAC ATC CGC CTT GGC CCC GAA GAT CGG TCC
      TCT GGG TCT G---------------TC TCC CAA GAA AGT TGG CGA TGA      871
      mEx2R
```

Figure 5. Conservation of alternative splicing in the mammalian *rpL12* gene. (**A**) Sequence comparison between human intron 1 (hInt1) and the corresponding bovine (bInt1) and mouse intron (mInt1). The arrowhead indicates the alternative splice site. (**B**) Nucleotide sequence of mouse *rpL12* intron 1 with the 5' and 3' flanking exonic sequences. Refer to Figure 4 for alignment; accession nos of sequences (NCBI/GenBank): NT_039206 for mouse gene, NW_619058 for bovine gene; for the human gene, refer to the legend to Figure 3.

mRNA. Consequently, we conclude that the pattern of splicing is regulated by exogenous HA-L3 in a dose-dependent manner.

To determine whether this regulation is specifically mediated by rpL3, we evaluated whether alternative splicing of the *rpL3* gene is affected by overexpression of a different

ribosomal protein, i.e. rpL12. We selected stable transfectants inducible for the expression of the HA-tagged rpL12 and measured by northern blotting, the expression of canonical *rpL3* and of the alternatively spliced *rpL3* transcript when rpL12 was overexpressed. We found no significant change in the expression of the canonical rpL3 mRNA or in
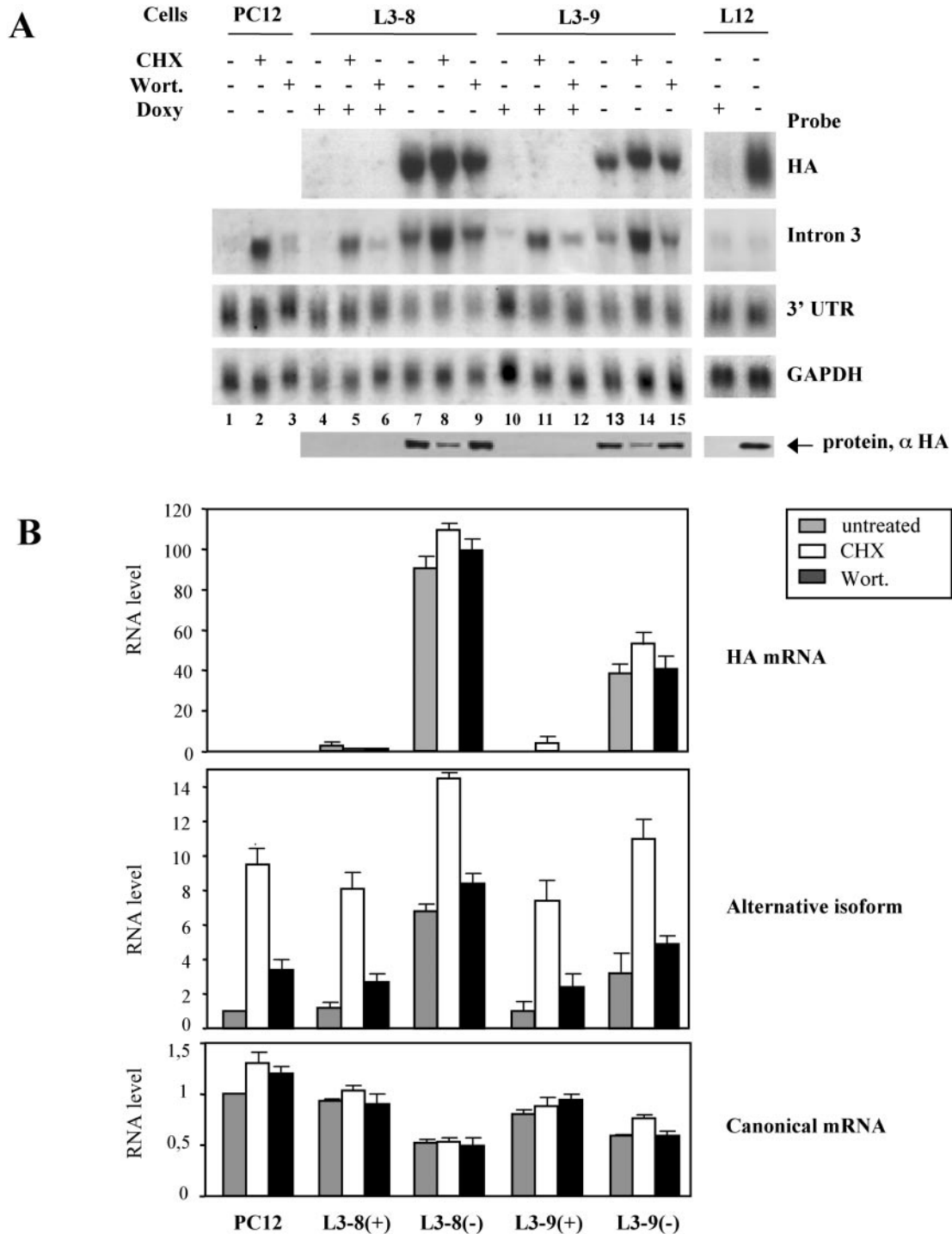
A

B

Figure 6. Effect of overexpression of r-proteins on the *rpL3* splicing pattern. (**A**) Analysis of *rpL3* expression in control cells (PC12) and in clones stably transfected with HA-tagged rpL3 cDNA (L3–8 and L3–9) or with HA-tagged rpL12 cDNA (L12). Northern blot hybridizations of total RNA were performed with the indicated probes. Protein samples purified from the same clones were analyzed by western blotting with an HA-tag specific antibody (αHA). (**B**) Quantification of mRNAs shown in panel A. The amounts of HA-mRNA are expressed in arbitrary units after normalization to GAPDH mRNA levels. The mRNA levels of endogenous isoforms were normalized to GAPDH and expressed as a ratio to endogenous levels in untreated control cells. (+), addition of doxycycline; (−), removal of doxycycline. The average of three independent experiments is shown, with SDs.

accumulation of the alternative NMD-sensitive isoform in HA-L12 overexpressing cells (Figure 6A, right panel). We thus concluded that the alternative splicing of the *rpL3* gene is regulated by its own protein product.

DISCUSSION

NMD is an mRNA surveillance mechanism that leads to selective degradation of PTC-containing mRNA transcripts arising from somatic mutations or errors in gene expression.

Considerable light has been shed on the molecular mechanism by which, *in vivo*, NMD eliminates aberrant mRNAs to prevent the production of harmful polypeptides [for reviews see (7,25,28–30)]. In contrast, little is known about the substrates of mRNA surveillance in wild-type organisms. Mitrovich and Anderson (20) demonstrated, in *C.elegans*, that ribosomal protein alternative transcripts, which are natural targets of NMD, arise not through errors but through feedback regulation of splicing triggered by the protein product. In humans, an extensive analysis by Lewis *et al.* (22) of alternatively spliced mRNA isoforms contained in the EST database demonstrated that one-third of the genes analyzed produce alternative transcripts that are candidate targets of NMD. As these authors point out, such a predicted widespread association of alternative splicing and NMD may indicate a need to eliminate a large number of aberrant mRNAs, or else, could play a functional role in regulating protein expression levels. Our data also demonstrate that the alternative transcripts of human ribosomal protein genes are natural targets of NMD and are probably regulated in levels by a negative feedback network.

We have identified alternative transcript isoforms of the human *rpL3* and *rpL12* genes. These isoforms originate from alternative splicing that causes partial removal of intron 3 in *rpL3*, and of intron 1 in *rpL12*. The resulting mRNA isoforms carry intronic sequences that contain PTCs, and are stabilized by NMD inhibition (Figures 1–3).

In addition, we found that the *rpL3* intron responsible for introducing a PTC in the alternative mRNA isoform is conserved among mammals (human, mouse and bovine species). In fact, there is a high degree of sequence identity along the whole intron, with the highest identity occurring in the region retained consequent to alternative splicing (Figure 4). Furthermore, the presence of the alternative mRNA isoform sequence in several cDNA clones of the mouse EST database, together with the observation that the alternative transcript is also present in rat PC12 cells, strongly suggests that the splicing event identified in human cells is common to mammals.

Our analysis of aberrant mRNA production by the mouse *rpL12* provides compelling evidence that NMD target mRNA isoforms are important for gene regulation. In fact, there was no relevant similarity between human and mouse *rpL12* intron 1. However, a BLAST search in the mouse EST database and RT–PCR analysis revealed the expression of an alternatively spliced, unproductive mRNA *rpL12* isoform. Strikingly, the splicing event generating this isoform occurs in the intron of the mouse gene corresponding to the human intron 1 counterpart, and in a region where the alternative splice site is conserved not withstanding the low degree of identity.

It is noteworthy that the alternative splicing event leading to NMD-sensitive transcripts from mammalian *rpL3* and *rpL12* is conserved in lower eukaryotes. In fact, in *C.elegans* the orthologue genes produce alternatively spliced isoforms that accumulate in strains in which the NMD pathway is genetically blocked (20). Interestingly, not only the genes targeted but also the type of alternative splicing has been preserved through the evolutionary divergence of nematodes and mammals. In fact, in both, alternative isoforms are generated through the 3′ and 5′ alternative splice sites in the *rpL3* and *rpL12* genes, respectively. Given these findings, it seems highly unlikely that the unproductive mRNAs are

erroneously generated by the cell. Most probably, their expression reflects an evolutionarily conserved function that has yet to be discovered.

It is conceivable that the generation of NMD-targeted mRNA isoforms has been conserved because coupling of alternative splicing and NMD might serve to regulate *rpL3* and *rpL12* expression. In fact, a body of evidence suggests that NMD can function as a means of post-transcriptional regulation of gene expression. For example, in *C.elegans* the expression of the serin/arginine-rich (SR) proteins SRp20 and SRp30b is modulated by alternative splicing events that produce two isoforms, one of which contains a PTC and is degraded by the NMD pathway (31). In humans, the SR protein SC35 negatively autoregulates its expression via the coupling of modulated alternative splicing and NMD degradation of a PTC-containing isoform (32). A similar mechanism of regulation has been reported for the polypyrimidine tract binding protein (PTB) (33) and for the ATP-binding cassette, subfamily C, member 4 (ABBC4) (34).

A proposed model for this mechanism of protein expression down-regulation has been designated 'regulated unproductive splicing and translation' (RUST), a process through which gene expression can be regulated post-transcriptionally by the production of splice forms that will be degraded by NMD rather than translated into protein (35). However, most transcripts may not be subject to RUST. In fact, while some genes may acquire the ability to alternatively splice to turn off their expression, the presence of nonsense codons in the alternatively spliced transcript may not be functionally relevant. For example, many alternatively spliced transcripts could have acquired nonsense codons simply because there was no selection pressure to encode a functional protein product. Thus, the ability of NMD to degrade alternatively spliced transcripts may be of no regulatory significance in many circumstances.

Given our results on *rpL3* and *rpL12* mRNA expression, we asked whether the regulation of mammalian ribosomal protein expression could be explained by the RUST model. To address this issue, we investigated whether the splicing pattern was affected by the endogenous level of the functional r-protein in the cell. Using an *in vivo* overexpressing system, we demonstrate that overexpresssion of rpL3 in PC12 cells alters the *rpL3* gene splicing pattern: the alternatively spliced isoform is upregulated, whereas the canonically spliced mRNA decreases. Moreover, the total quantity of rpL3 endogenous mRNA does not change consequent to protein overexpression (data not shown). Thus, only the ratio of canonical versus noncanonical mRNA is altered. These findings suggest that the level of the L3 protein affects (directly or indirectly) a switch in splice-site selection.

Our results lend support to and extend a model proposed by Mitrovich and Anderson (20) for rpL12 in *C.elegans*. Indeed, our model predicts that the quantity of free rpL3, unassembled in ribosomal subunits, acts as a sensor of *rpL3* expression either by down-regulating canonical splicing, or by upregulating non-canonical splicing (Figure 7). Under normal growth conditions, the canonical transcript is preferentially spliced; but when conditions in the cell lead to an excess of rpL3, the canonical splicing is inhibited and the splicing is mainly directed towards the alternative mode. The result of this event is an increase in the level of alternative mRNA, which is targeted to NMD, and a consequent reduction in the
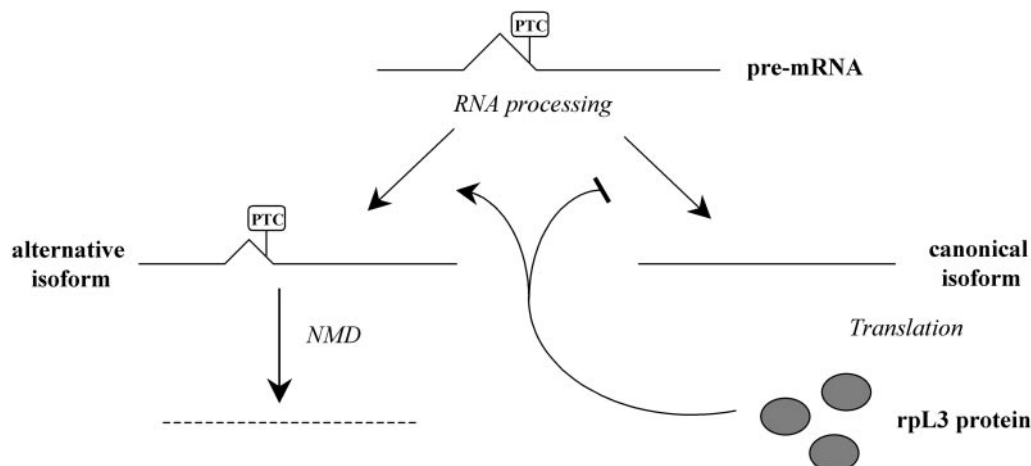
Figure 7. Model for rpL3 feedback regulation. A canonically spliced isoform encodes the rpL3 protein. When present in excess, rpL3 down-regulates canonical splicing and up-regulates the alternative splicing of its own pre-mRNA. This reduces canonical mRNA and increases alternative mRNA. This latter isoform is unproductive and is subsequently eliminated by the NMD pathway.

level of the r-protein. This negative feedback would control the levels of free rpL3 and avoid wasteful production of the protein. Thus, we propose a regulatory pathway in which the decision to splice canonically or alternatively, regulated (directly or indirectly) by the protein is followed by degradation of alternative, unproductive mRNA by NMD.

We also investigated whether this regulatory mechanism is triggered by a general signal activated by the cell upon accumulation of any other ribosomal protein, or whether it occurs only in response to the rpL3 increase. Our data show that the alternative splicing of the *rpL3* gene is not influenced by overexpression of another ribosomal protein (rpL12). Hence, we conclude that the proposed feedback process is specifically modulated by rpL3.

To our knowledge, this is the first example of post-transcriptional regulation mediated by alternative splicing that gives rise to natural NMD targets reported for mammalian ribosomal protein genes. Although we do not provide experimental evidence for a similar mechanism in the *rpL12* gene, the conserved expression pattern of the human, bovine and mouse genes indicates that the same regulation mode also applies to *rpL12*. In addition, a mechanism similar to the one we describe for mammalian rpL3 has been reported in *C.elegans* for rpL12 and inferred for rpL3 (20). The fact that the regulatory mechanism of rp gene expression is widely conserved highlights the relevance of our results.

In conclusion, NMD could be viewed as a new component of the complex cellular regulation of ribosomal protein genes. Post-transcriptional feedback loops are known to modulate the expression of other r-proteins. However, in all previous cases, excess r-protein led either to inhibition of splicing and consequent accumulation of unspliced transcript (36,37), or to destabilization of the cognate mRNA (38). Here we report that human rpL3 selects the splicing mode rather than triggering or not splicing. Moreover, we suggest that regulated splicing associated with the NMD pathway might represent a way to fine-tune the amount of an r-protein to the appropriate level. In other words, the NMD pathway might provide a means with which to lower the threshold of gene expression below what would otherwise be possible by modulating the transcription

rate alone. Alternatively, or in addition, this type of post-transcriptional regulation could provide a temporal control unattainable by transcription factors. However, in *C.elegans* (20) and in mammals, the association of splicing and NMD does not appear to be a general mechanism of regulation shared by all r-proteins. Given the global regulation network of ribosomal components, including r-proteins and rRNAs, we suppose that a mechanism like NMD, which acts only on coding RNAs, provides an additional level of modulation, perhaps specifically targeted to the r-proteins that exert extra-ribosomal functions and, as such, require ultrafine tuning.

The molecular basis of the autoregulation process identified in this study remains to be established. The striking sequence conservation of *rpL3* intron 3 may indicate that it contains *cis*-elements, recognized by a regulatory protein, that are critical for selecting the type of splicing. Perhaps, as demonstrated for other ribosomal proteins (i.e. rpL32), the free rpL3 might be directly involved in modulating splicing of the *rpL3* gene by binding the unprocessed transcript in intron 3. A similar mechanism could apply to *rpL12*. In this case, however, the critical interaction target could be a common secondary structure assumed by the loosely conserved regions of intron 1. In fact, preliminary analysis of RNA folding, performed using the M-FOLD program on human and mouse *rpL12* intron 1, has predicted similar structures for these sequences. It will be interesting to clarify the significance of the different sequence conservation in mammals of *rpL3* intron 3 and *rpL12* intron 1, and how each intron is functionally involved in the regulated splicing.

In conclusion, this report casts light on the regulation of mammalian ribosomal proteins by newly identified molecular strategies, and supports the concept that the NMD pathway plays a more prominent role in the regulation of gene expression than hitherto recognized.

ACKNOWLEDGEMENTS

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