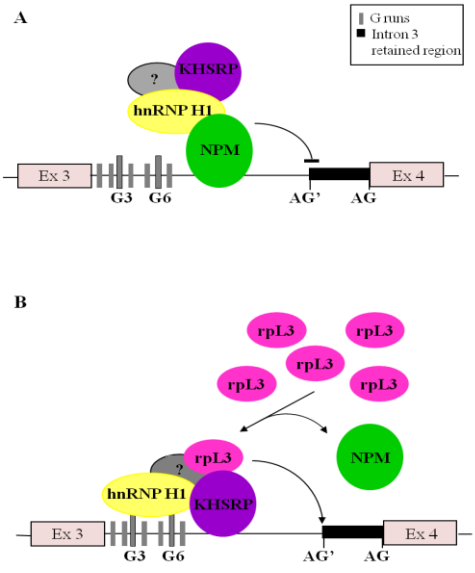
UNIVERSITA' DI NAPOLI "FEDERICO II"

DOTTORATO DI RICERCA
BIOCHIMICA E BIOLOGIA CELLULARE E MOLECOLARE
XXIV CICLO

Morena Catillo

Post-transcriptional regulatory strategies and extraribosomal functions of human rpL3



Academic Year 2010/2011

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Post-transcriptional regulatory strategies and extraribosomal functions of human rpL3

Morena Catillo

Tutor
Prof. Concetta Pietropaolo

Coordinator
Prof. Paolo Arcari

Co-Tutor
Prof. Giulia Russo

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Riassunto

Il gene *rpL3* produce, oltre all'isoforma canonica di mRNA che verrà tradotta in proteina, un'isoforma alternativa, sottoposta alla degradazione da parte dell'NMD (*Nonsense mediated mRNA decay*), in quanto presenta un PTC (*premature termination codon*). Inoltre, l'iperespressione della proteina rpL3 determina un incremento del livello dell'isoforma alternativa, non produttiva, che ha come conseguenza la diminuzione di rpL3 (Cuccurese et al. 2005). Tale *feedback* negativo innescato dall'accumulo della proteina ribosomale, rappresenta una strategia che regola finemente la quantità di proteina ribosomale ad un livello appropriato, soprattutto rilevante per eventuali funzioni extra-ribosomali della proteina rpL3.

L' attività da me svolta durante il dottorato è stata indirizzata alla comprensione del ruolo di alcuni putativi interattori proteici di rpL3 e del suo trascritto primario identificati mediante spettrometria di massa sui prodotti di esperimenti di "GST Pull Down" e "RNA Pull Down", quali hnRNP H1, NPM e KHSRP, nella regolazione post-trascrizionale del gene di rpL3.

Con l'ausilio di approcci bioinformatici, sono stati individuati degli elementi *cis*-acting, detti elementi G-run, nell'introne 3 del gene *rpL3* coinvolto nello *splicing* alternativo. Tali G-run contribuiscono ad indirizzare la scelta di siti di *splicing* alternativi (Marcucci et al. 2007), interagendo con fattori proteici appropriati. Io ho dimostrato *in vitro* la capacità di legame di hnRNP H1 al trascritto primario del gene *rpL3*, utilizzando il saggio "REMSA" (RNA Electrophoretic Mobility Shift Assay).

Poiché era stato dimostrato che gli elementi G3 e G6 (Napolitano, tesi di dottorato 2010) sono importanti per innescare lo *splicing* alternativo, ho studiato in seguito a transfezioni in transiente l'effetto di iperespressione di hnRNP H1 in presenza di tali elementi G-run mutagenizzati.

Successivamente attraverso esperimenti *in vivo* di immunoprecipitazione ed *in vitro* di "GST Pull Down" ho definito le relazioni che intercorrono tra le proteine hnRNP H1, NPM, KHSRP e rpL3, e il loro ruolo nel promuovere la selezione del sito di *splicing* (canonico o alternativo) sul trascritto del gene *rpL3*.

In cloni stabili di cellule PC12 (cellule di feocromocitoma di ratto) che esprimono in maniera inducibile rpL3 mediante sistema tet-off, ho effettuato esperimenti di iperespressione delle proteine e valutato tramite RT-PCR l'effetto di ciascuna di esse sulla produzione della quota di trascritto alternativo del gene di rpL3 (rpL3-a), sia in condizioni basali sia di iperespressione di rpL3. Analogamente, ho stimato l'effetto su rpL3-a anche in seguito a silenziamento delle stesse proteine, mediante RNA-interference e successiva analisi per RT-PCR.

Gli esperimenti effettuati hanno dimostrato che hnRNP H1, NPM e KHSRP sono coinvolte con ruoli diversi nella regolazione post-trascrizionale del gene di rpL3 e ci hanno permesso di ipotizzare un modello di regolazione (Russo et al. 2011) che prevede una sequenza di interazioni tra queste proteine e il trascritto.

Tale regolazione post-trascrizionale che vede coinvolta l'associazione dello *splicing* alternativo con l'NMD può essere molto rilevante per modulare in maniera fine la quota della proteina ribosomale rpL3 disponibile per assolvere a funzioni extra-ribosomali. Ci sono infatti sempre

più evidenze di proteine ribosomali che hanno ruoli extra-ribosomali; in particolare noi abbiamo dimostrato la capacità di rpL3 di regolare la propria produzione innescando lo *splicing* alternativo. Inoltre dati preliminari ottenuti in laboratorio indicano una seconda funzione extra-ribosomale di rpL3 che appare coinvolta nel meccanismo di regolazione dell'espressione di p21 indipendente da p53.

Summary

In addition to canonical mRNA isoform normally translated in to protein, rpL3 gene produces an alternative mRNA isoform, containing a PTC (premature termination codon) degraded by NMD (Nonsense mediated mRNA decay).

Overexpression of rpL3 causes an increase in the level of alternative unproductive, mRNA isoform, which results in a decrease of rpL3 production (Cuccurese et al. 2005). This negative feedback loop triggered by the accumulation of the ribosomal protein, represents a strategy that finely regulates the amount of ribosomal protein to an appropriate level, especially relevant to extra-ribosomal functions of rpL3.

For my PhD project I worked to understand the role of some putative protein interactors (hnRNP H1, NPM and KHSRP) of rpL3 and its primary transcript. The protein factors were identified by mass spectrometry on the products of "GST Pull-Down" and "RNA Pull Down" experiments, and my work aimed to clarify the role of these proteins in the post-transcriptional regulation of *rpL3* gene.

Through bioinformatics approaches, we have identified cis-acting elements, called G-run elements, within intron 3 of *rpL3* gene involved in the alternative splicing. These G-run elements cooperate to direct the choice of alternative splice sites (Marcucci et al. 2007), through interactions with appropriate protein factors. I have shown, in vitro, the ability of hnRNP H1 to bind rpL3 primary transcript, using "REMSA" (RNA electrophoretic mobility shift assay).

Because it was demonstrated that the elements G3 and G6 (Napolitano, Ph.D. thesis 2010) are relevant to trigger alternative splicing, I have studied with transient transfections the effect of hnRNP H1 overexpression in presence of mutated G-run elements.

Then, through in vivo experiments of immunoprecipitation and in vitro experiments of "GST Pull Down", I have defined the relationships between hnRNP H1, NPM, KHSRP and rpL3, and their role in promoting the selection of splice site (canonical or alternative) on the *rpL3* gene transcript. In stable clones of PC12 cells (rat pheochromocytoma cells) expressing rpL3 in an inducible mode (tet-off system), I have carried out experiments of protein overexpression and evaluated by RT-PCR the effect of each protein on the production of the alternative transcript of *rpL3* gene (rpL3-a), both in basal conditions and in condition of rpL3 overexpression. Similarly, I have evaluated the effect on rpL3-a after silencing each protein by using RNA-interference and subsequent analysis by RT-PCR.

Experiments have shown that hnRNP H1, NPM and KHSRP are involved with different roles in the post-transcriptional regulation of *rpL3* gene and allowed us to propose a model of regulation (Russo et al. 2011) that predicts a sequence of interactions between these proteins and the transcript.

This strategy of post-transcriptional regulation, through the association of alternative splicing and NMD, can be very important to finely modulate the rpL3 amount available to extra-ribosomal functions. In fact, more and more evidences demonstrate that ribosomal proteins can exert extra-ribosomal function.

As far as rpL3 is concerned, we have demonstrated the rpL3 ability to regulate its production by triggering alternative splicing. In addition, preliminary data obtained in our laboratory indicate that rpL3 is involved in a regulatory mechanism of p21 expression independent from p53.

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1. Introduction

1.1 Ribosomal protein expression

The biosynthesis of ribosomes requires the coordinated espression of the genes coding for their structural components, rRNA and r-proteins. Control mechanisms are also necessary to maintain ribosome synthesis at the level appropriate to the requirements and growth conditions of the cell.

Early studies of mammalian r-protein gene promoters showed that transcriptional activity is approximately equivalent and there are no shared elements (Hariharan et al. 1989). More recent in silico analyses found some recurring motifs in the transcriptional control regions (Perry, 2005; Ishii et al. 2006). However, besides some variation of r-proteins transcript levels in different tissues and in neuronal differentiation, transcriptional regulation does not appear to play a major role in the control of r-protein synthesis. On the other hand the rp-genes expression depends upon many factors (Loreni et al., 1993) and is finely tuned mostly by post-transcriptional and translational regulatory mechanisms.

Although r-proteins have long been studied as ribosome components, a growing amount of data demonstrate that many r-proteins exert additional, extraribosomal functions; these findings add more relevance to the multiplicity of mechanisms discovered to finely tune the rp-genes expression. In humans, it has been shown that the interaction of mitochondrial rpL12 with mitochondrial RNA polymerase modulate gene expression in mitochondria (Wang et al., 2007). Moreover, many data indicate that in eukaryotes some r-proteins autoregulate their gene

expression through various mechanisms. Post-transcriptional regulations have been described that control the level of rp-mRNA in the cell through modulation of transcript processing, splicing and stability. In *S. cerevisiae*, rpL32 regulate both splicing and translation of its mRNA (Dabeva and Warner, 1993). In *C. elegans*, rpL12 is able to down-regulate the canonical splicing and up-regulate an alternative splicing of its pre-mRNA (Mitrovich and Anderson, 2000). In humans, available data show that some r-proteins are able to modulate their own production by regulating alternative splicing (Cuccurese et al., 2005; Malygin et al., 2007).

1.2 Regulation of processing of gene transcript

Alternative splicing (AS) is a main regulatory mechanism of gene expression by which is produced a repertoire of mRNAs, and consequently of proteins, much larger than expected from the number of genes (Grawboski and Black, 2001). The different steps leading to gene products are integrated and coordinated reaching a high efficiency, and RNA surveillance mechanisms are present to provide correct gene products (Orphanides and Reinberg, 2002).

Although the mechanisms of the AS have been extensively studied, the complex regulation of such process is still an issue. The complexity of the regulation pathways is due to an intricate and dynamic network of protein-protein and RNA-protein interactions. Changes in the activity, number, and association of splicing factors in the network can modify the selection of the splice site and the activity of the splicing machinery, and therefore the splicing pattern of the target pre-mRNA.

The regulation of pre-mRNA splicing plays an important role in human pathologies (Licatalosi and Darnell, 2010). Impairment of the AS is closely related to some disease mechanisms as in the case of spinal muscular atrophy and myotonic distrophy. Recently, new therapies aimed to correct defects arising in pre-mRNA splicing, the so-called 'splice-correction' or 'splice-modulation' therapies, have been developed (Wood et al., 2010).

Alternative splicing is modulated by cis-elements in intron or exon sequences that, associated with specific transacting factors, can negatively or positively affect splicing (Black, 2003). Exon cis-elements (ESE) differ in sequence; however, the consensus sequence (GAR)n can be drawn for the most frequently observed. The esanucleotide UGCAUG is a frequent element (Lim and Sharp, 1998, Black, 2003) in the intronic splicing enhancing sequences (ISE). ISE sequences frequently appear as a complex combination of multiple elements that mediate positive or negative effects on gene splicing (Modafferi and Black, 1997; Chan and Black, 1997). Other interesting intronic motifs include a stretch of three or four Gs (Nussinov et al., 1989); these sequence motifs have been shown to affect the splicing of chicken beta-tropomiosin gene (Sirand-Pugnet et al., 1995), of beta-globin gene (McCullough and Berget, 1997) and of human growth hormone gene (McCarthy et al., 1998).

Interaction of cis-elements with specific transacting proteins may exert positive or negative effects on gene splicing. Splicing activating factors frequently belong to the SR protein family, while different protein components of hnRNP repress splicing upon binding to cis-elements (Sun et

al, 1993; Chen et al., 1999; Expert-Bezancon et al., 2004). SR proteins share features such as one or two RNA recognizing motif (RRM) and a domain containing the serine-arginine dipeptide. SR proteins activate splicing by binding to ESE sequences and enhancing recruitment of splicing machinery factors on the 3'-splice site (Wang et al., 1995).

The accuracy of the AS depends on the stoichiometry and interactions of positive and negative regulatory proteins. Consequently, the identification of the proteins participating in the modulation of the AS is an essential step to study the many aspects of gene expression.

AS can also control gene expression quantitatively by generating unproductive mRNAs that are targeted for decay by nonsense-mediated decay (NMD) (McGlincy and Smith 2008).

1.3 Nonsense-mediated mRNA decay (NMD)

NMD is a surveillance pathway that detects and selectively degrades aberrant mRNAs harboring a premature termination codon (PTC), thus preventing the production of truncated polypeptides potentially deleterious to the cell (Fig. 1.1).

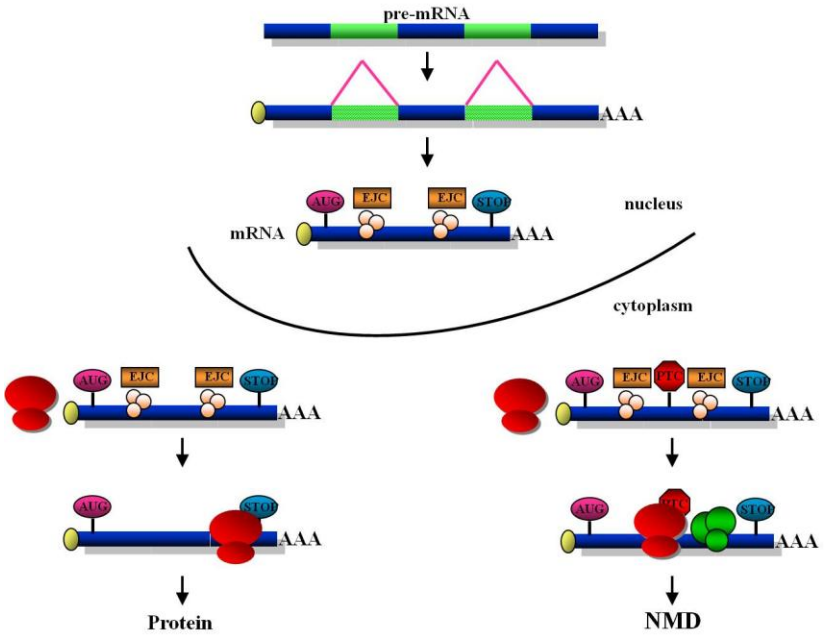

Figure 1.1: **Nonsense-mediated mRNA decay (NMD)**.
Normally, an EJC is removed from the ribosome during the pioneer round. If upstream from an EJC a nonsense codon (PTC) is present, the EJC will not be removed from the ribosome and thus will trigger NMD-mediated degradation.

PTC may arise through a nonsense or frameshift mutation of DNA, as a consequence of DNA rearrangement, or, more often PTCs are a consequence of splicing errors producing aberrant mRNAs (Wagner and Lykke-Andersen, 2002). NMD is highly conserved in eukaryotes; many studies have dissected machinery components, and the issue of its localization in a cell compartment has been thoroughly discussed (Chang et al, 2007; Nicholson et al., 2010; and references therein). In the nucleus, several proteins take part in the assembly of a large molecular complex deposited at each exon-exon junction (EJC). Such mRNP complex, rearranged, and including other transiently associated proteins moves to the

cytoplasm where a mRNA scanning by the translational apparatus will take place by mean of a pioneer round of translation. EJCs will be removed during the pioneer round of translation, which stops at the PTC thus leaving unremoved EJC sitting on exon-exon junction downstream from the PTC. The up-frameshift protein 1 (UPF1) is recruited on the unremoved EJC, and, following interactions between UPF1 and NMD factors, the decay process initiates. UPF1 shows elicase activity, and undergoes phoshorylation and dephosphorylation rounds; although it has been shown that UPF1 is essential to initiate NMD, its role in the process is still unknown.

1.4 AS-NMD association

Alternative splicing can quantitatively control gene expression by producing PTC containing mRNAs, unproductive, degraded by NMD. The association AS-NMD which results in a quantitative regulation of the productive RNA abundance has been shown to be effective in the expression of many genes (Green 2003). In fact, the process regulating gene expression through AS-NMD association has been defined RUST (Regulated Unproductive Splicing and Translation; Lewis et al., 2003).

Data from several laboratories demonstrate that some alternative isoforms of mRNA encoding ribosomal proteins (rp) are NMD substrates. In Caenorhabditis elegans, it has been shown that the AS of genes rpL3, rpL12, rpL10 and rpL7 gives raise to aberrant mRNA isoforms, which contain a PTC resulting from an incomplete intron removal, and are natural substrate of NMD (Pulak and Anderson, 1993). The AS event appears conserved in humans and in other mammals on rpL3 and rpL12 genes. In

fact, previous data from the laboratory where I carried out my thesis studies demonstrated that human rpL3 gene transcript gives rise to a canonical mRNA and to an alternative mRNA isoform containing a PTC targeted to decay by NMD. rpL3 is able to modulate its own production via a negative feedback loop. In fact, rpL3 overexpression results in a decreased level of the canonically spliced mRNA, and an increased production of the alternatively spliced isoform (Fig. 1.2; Cuccurese, 2005).

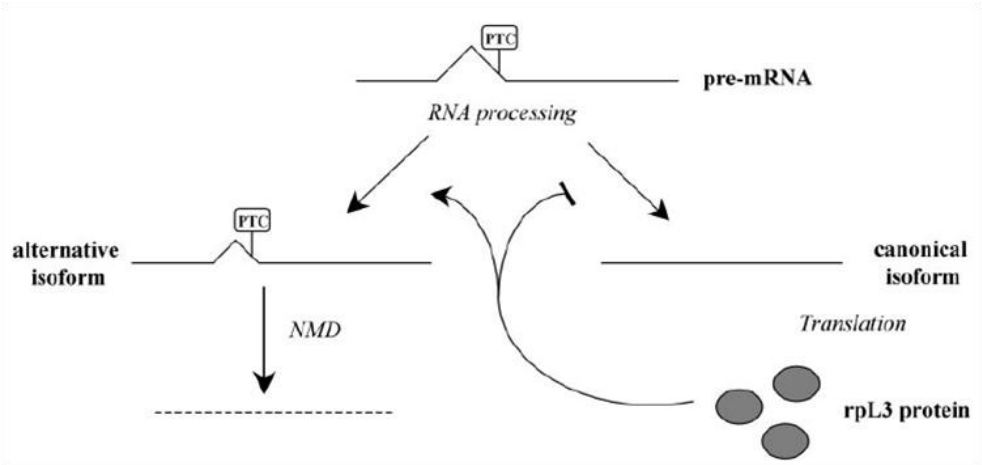

Figure 1.2: **Model for rpL3 feedback regulation**.
A canonically spliced isoform will be translated to produce rpL3 protein. The alternative mRNA isoform is unproductive and will be removed by NMD. When present in excess, rpL3 down-regulates canonical splicing and up-regulates the alternative splicing of its own pre-mRNA.

1.5 Aim of the studies

These results raised the issue of identifying molecular partners of rpL3 involved in the splicing event. Data showing that rpL3 is unable to bind its own gene transcript, led to the working hypothesis that rpL3 modulates the splicing of its gene by interacting with one or more regulatory proteins. Since the accuracy of the AS depends on the stoichiometry and interactions of positive and negative regulatory proteins, the identification of proteins participating in the modulation of the AS of rpL3 gene appeared an essential step to understand the regulation of this gene. In fact GST-pull down and RNA-pull down assays followed by Mass Spectrometry analysis confirmed that many putative rpL3 and intron 3 interactors were present in human cell extracts (Fig. 1.3; Russo A. et al., 2010).

Among others, the heterogeneous nuclear ribonucleoprotein hnRNP H1 was identified as a transacting factor able to interact in vitro and in vivo with rpL3 and with intron 3 transcript of the rpL3 gene. Further data demonstrated that hnRNP H1 is involved in promoting the AS of human rpL3 pre-mRNA. In addition, the same group identified and characterized the cis-acting regulatory elements, G runs, involved in hnRNP H1-mediated regulation of splicing (Russo A. et al., 2010). For my thesis project, I have analyzed the role of hnRNP H1 in the rpL3 autoregulatory loop, and identified two new regulatory proteins, KHSRP (K-homology splicing regulatory protein, also known as KSRP) and NPM (B23, numatrin or NO38), which exhibit opposite effects on the splicing reaction of rpL3 pre-mRNA. The data reported here contribute to the laboratory effort to shed

light on protein-protein and RNA-protein interactions within putative RNP complexes involved in the modulation of splicing of the rpL3 gene.

GST-pull down

M kDa	Protein	Accession n°
130	RNA helicase A	Q08211
110	RNA helicase GU	Q9NR30
	E1B-AP	Q9BUJ2
	Nucleolin	P19338
50	hnRNP H1	P31943
	ZBTB4	Q9P1Z0
35	Nucleophosmin	P06748
30	NRG1	B0FYA9
	Histon cluster 1, H1c	A8K4I2
	RPS6	P62753
	RPS2	P15880
	SF2p32	Q07021
26	RPS8	P62241
22	RPS5	P46782
	RPS9	P46781
19	RPS11	P62280
	RPS17	P08708
17	RPS16	Q61PX4
	RPSL22 proprotein	P35268
	RPS19	P39019
15	RPS15a	P62244
14	4DE4DIP	Q5VU43

RNA-pull down

M kDa	Protein	Accession n°
130	SAP 130	Q08211
	Importin 4	
120	Importin 7	Q95373
	KIAA0079	P53992
110	Nucleolin	P19338
	Beta2 - adaptin	P63010
	Importin beta- 3	O00410
	RNA helicase Gu	Q9NR30
80	KHSRP	Q92945
77	moesin	P26038
70	FBP1	Q96AE4
	hnRNPM4	P52272
	nucleoporin 85	Q9BW27
65	Lamin A/C isoform2	P02545
60	FBP3	Q96I24
55	Neuroleukin	P06744
	FBP2	Q92945
	PTBP1	Q9BUQ0
	Gps1	QI3098
50	hnRNP H1	P31943
	RuvB - like2	Q9Y230
47	MBP-1	P06733
	eIF3e	P60228
45	Musclebind like protein 1	Q9NR56
40	SGN4	Q9BT78

Figure 1.3: Mass-spectrometry analysis

rpL3 and intron 3 RNA interacting proteins identified by mass-spectrometry analysis of products of GST- and RNA-pulldown experiments

2. Materials and Methods

2.1 Cell cultures, transfections and drug treatment

Human cell line HeLa was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax, supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and penicillin–streptomycin 50 U/mL.

L3–8 cell line, derived from rat PC12 Tet-Off cell line and conditionally overexpressing rpL3 upon doxycyclin removal, was grown in DMEM supplemented with 5% fetal calf serum (FCS), 10% horse serum, 2mM L-glutamine and 100 mg/mL G418, hygromycin 200 mg/mL and doxycyclin 10 ng/mL. The expression of HA-rpL3 was induced upon removal of doxycyclin. siRNA transfections were performed in HeLa cells (1 x 10^6 cells, 6mm well plate) at a concentration of 150nM by using Oligofectamine Reagent (Invitrogen) according to the manufacturer's instructions. Plasmids were transfected in L3–8 cell line or in HeLa cells (2.5 x 10^6 cells, 6mm well plate) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after DNA or siRNA transfections, cells were treated with 100 mg/ml cycloheximide (CHX) for 4 h to block NMD. Then, RNA and proteins were extracted by using the Trizol procedure (Invitrogen) for RT–PCR analysis and western blot, respectively.

2.2 DNA constructs and production of recombinant proteins

The intron 3, regions A, B and C of intron 3 of rpL3 pre-mRNA were obtained by RT-PCR using specific primers and cloned into the pGEM-4Z

vector to obtain constructs pGEM4Z-Int3, pGEM4Z-IntA, pGEM4Z-IntB and pGEM4Z-IntC, respectively. The primers used were: Int3 5'-AATTGGTAAGGGAGGAG-3' and 5'-TATGCCTTCAGGAGCAGA-3'; IntA 5'-AAGAATTGGTAAGGGAGG-3' and 5'-GGAAGCCCACTCAGTGAT-3'; IntB 5'-CCAGGGCAAAAGGTTTG-3' and 5'-GTAAAGGCCTTCTTCTTAG-3'; IntC 5'-AAGAATTGGTAAGGGAGG-3' and 5'-TCAGAATGAGGGTGTTAGC-3'. The cDNAs of hnRNP H1, rpL3 and NPM were obtained by RT-PCR from HeLa cells using specific primers and cloned into the prokaryotic expression vector pGEX4T3. The primers used were: GST-hnRNP H1 5'-ATGATGTTGGGCACGGAA-3' and 5'-CTATGCAATGTTTGATTG-3'; GST-rpL3 5'-ATGTCTCACAGAAAGTTC-3' and 5'-AGCTCCTTCTTCCTTTGC-3'; GST-NPM 5'-ATGGAAGATTCGATGGAC-3' and 5'-TTAAAGAGACTTCCTCCA-3'.

The cDNA of hnRNP H1 and NPM were also cloned into a version of the eukaryotic expression vector pcDNA4/HisMax C (Invitrogen) containing the HA epitope, and into the prokaryotic expression vector pRSET-A (Invitrogen) containing the Histidine tag. Flag-KHSRP and His-KHSRP were already available (Gherzi 2004). The recombinant proteins GST-hnRNP H1, GST-rpL3 and GST were expressed in *Escherichia coli* and purified by using glutathione Sepharose 4B beads according to the manufacturer's instructions (GE Healthcare). The recombinant proteins His-NPM, His-hnRNP H1 were expressed in *E. coli* and purified by the nickel–nitrilotriacetic acid (Ni–NTA)-Agarose chromatography according to the manufacturer's instructions (Qiagen, Valencia, California). His-tagged

KHSRP was expressed in Sf9 cells using the *Baculovirus* system (Baculogold, BD Biosciences) and purified by Ni–NTA-Agarose chromatography (Gherzi, 2004).

2.3 RNA electromobility shift assay (REMSA)

RNA probes were transcribed in vitro using ^{32}P-NTPs and SP6 polymerase according to the manufacturer's instructions (Roche), and construct pGEM4Z-IntA, pGEM4Z-IntB, or PGEM4Z-IntC as template.

The probes were purified using Sephadex G-25 columns. 5×10^5 counts/min of each RNA probe were incubated in binding buffer containing 20 mM Hepes, pH 7.9, 150 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton, 1 mg/mL tRNA, and 0.4 U/μL RNase Inhibitor in the presence or absence of the recombinant protein GST-hnRNP H1 or GST for 1 h at 4 °C. The complexes formed were then resolved onto a 5% polyacrylamide (37.5:1 acrylamide:bisacrylamide ratio) RNA native gel. The gel was dried at 80 °C for 30 min and the results visualized by autoradiography.

2.4 RNA interference

The target sequences of small interfering RNAs (siRNA) in hnRNP H1 were: 5’-GGAAATAGCTGAAAAGGCT-3’ and 5’-CCACGAAAGCTTATGGCCA-3’. The siRNAs targeting NPM and KHSRP were purchased from Santa Cruz Biotechnology.

2.5 GST and RNA pull-down assay

For GST pull-down assay, 50 µg of the fusion protein or GST control, as bait were immobilized on glutathione-Sepharose beads and incubated with 20 µg of the recombinant protein of interest in pull-down buffer (50mM Tris–HCl, pH 7.5, 0.4mM EDTA, 150mM NaCl, 10% glicerol, 1% NP-40, 1mM sodium-ortovanadate, 50mM NaF, 5mM DTT and Protease Inhibitor Mix 1x) at 4 °C for 1.5 h. The beads were washed extensively and boiled in SDS sample buffer. The eluted proteins were loaded on 12% SDS–PAGE and analyzed by western blotting.

RNA pull-down assay was carried out by using adipic acid dehydrazide beads. 20 µg of intron 3 RNA, transcribed in vitro from pGEM4Z-Int3, were placed in a 400 µL reaction mixture containing 100 mM NaOAc pH 5.2 and 5mM sodium m-periodate (Sigma), incubated for 1 h in the dark at room temperature, ethanol precipitated and resuspended in 100 µL of 100 mM NaOAc, pH 5.2. Then, 300 µL of adipic acid dehydrazide agarose beads 50% slurry (Sigma) equilibrated in 100 mM NaOAc pH 5.2 were added to this mixture, which was then incubated for 12 h at 4 °C on a rotator. The beads with the bound RNA were pelletted, washed twice with 1 mL of 2M NaCl and equilibrated in washing buffer (5mM HEPES pH 7.9, 1mM MgCl2, 0.8mM magnesium acetate). The intron 3 RNA was then incubated with 50 µg of each recombinant protein for 30 min at room temperature in a final volume of 0.6 mL. The beads were then washed four times in 1.5 mL of washing buffer. Bound proteins were eluted in SDS sample buffer loaded on a 12% gel for SDS–PAGE and analyzed by western blotting.

2.6 Immunoprecipitation and western blotting

For immunoprecipitation assay, 1 mg of HeLa whole-cell lysate was incubated with 30 µL of protein A/G agarose beads coated with 5 µg of anti-NPM or anti-KHSRP (Santa Cruz Biotechnology) at 4 °C for 12 h. The beads were washed and boiled in the SDS sample buffer. The eluted proteins were loaded on 12% SDS–PAGE and detected by western blotting. Aliquots of protein samples (30 µg) were resolved by 12% SDS–gel electrophoresis and transferred into nitrocellulose filters. The membranes were blocked in PBS, 0.1% Triton and 5% dry milk for 2 h, and then challenged with anti-NPM, anti-KHSRP, anti-hnRNP H1, anti-HA, anti-Flag (Santa Cruz Biotechnology) anti-rpL3 and anti-rpL7a (Primm). The proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Pierce).

2.7 RNP immunoprecipitation assay

For RNP immunoprecipitation assay (RIPA), HeLa cells (2 x 10^6 cells) were lysed in 600 µL RIPA buffer 1x (10mM Tris–HCl pH 7.5, 150mM NaCl, 0.1mM EDTA, 1mM Na ortovanadate, 0.05M NaF, 0.5% NP-40) with protease inhibitors mix 1x (Roche) for 60 min on ice, and then centrifuged at 10 000 g at 4 °C for 15 min. The supernatant was subjected to a pre-clearing step in which it was incubated with 50 µL of protein A/G plus agarose for 1 h at 4 °C. The pre-cleared cell extracts were then incubated with antibodies specific for each protein (Santa Cruz Biotechnology) overnight at 4 °C. Protein A/G plus agarose beads (50 µL of 50% slurry) were then added and the mix was incubated for 1 h at 4 °C with gentle

shaking and centrifuged. The immunoprecipitates were suspended in 100 µL TES buffer (10mM Tris–HCl pH 7.5, 0.5mM EDTA, 0.5% SDS), incubated at 65 °C for 10 min and centrifuged for 1 min at 10 000g. Ten microliters of the supernatant were stored as immunoprecipitated samples and subsequently fractionated by SDS–PAGE to be analyzed by western blotting. RNA was extracted from 90 µL using the Trizol procedure (Invitrogen).

2.8 RT-PCR

For RT–PCR analysis, 1 µg of total RNA was reverse transcribed into cDNA with the random hexamers technique using 200U of Superscript II RNAse H^- Reverse Trancriptase (Invitrogen). The reaction was carried out at 42 °C for 50 min and was terminated by heating to 75 °C for 15 min. Ten of the 40 µL of reaction mix were PCR amplified in a final volume of 50 µL, using 5 mM of each specific primer, 10mM dNTPs and 0.5U of Taq DNA polymerase (Invitrogen). Typically, 25–30 cycles of amplification were performed. The primers were: rpL3-a 5'-CTCCGCTGGGCTCTGCCC-3'(forward) and 5'-CTTCAGGAGCAGAGCAGA-3' (reverse); rpL3-c 5'-GGGCATTGTGGGCTACGT-3' (forward) and 5'-GTAAAGGCCTTCTTCTTAG-3' (reverse); and β-actin 5'-GGCACCACCTTCTACA-3' (forward) and 5'-CAGGAGGACAATGAT-3' (reverse).

3 Results

3.1 hnRNP H1 binds specifically intron 3 of rpL3 pre-mRNA

To identify the cis-acting elements in the intron 3 that participate in the regulation of rpL3 pre-mRNA splicing, we used a computational analysis to look for hnRNP H1-binding sequence motifs in the intron 3 of rpL3 pre-mRNA. The search revealed the presence of seven G-rich sequences known as G-run elements that are able to bind the hnRNP H1 protein. Because of experimental evidence that hnRNP H1 activity in the splicing event is mediated by the binding of the protein to G runs, we performed REMSA experiments to assess whether the putative binding sites are necessary for the interaction of hnRNP H1 with intron 3 in the pre-mRNA transcript. Intron 3 was divided in three regions: region A spanning 365 nt downstream from the 5′ donor splice site containing seven G-run elements, region B (289 nt) constituted by the 3′ moiety of intron 3 and 27 nt of exon 4, and region C (209 nt), a more restricted region encompassing the seven G runs (Fig. 3.1A). These regions were transcribed *in vitro* with ^{32}P-NTPs and incubated with recombinant protein GST-hnRNP H1 or GST. The resulting RNA–protein complexes were analyzed on a native polyacrylamide gel and the results visualized by autoradiography. As shown in the figure, GST-hnRNP H1 was able to interact specifically with RNA transcribed on regions A (Fig. 3.1B) and C (Fig. 3.1C) of intron 3, but failed to do so when challenged with the B region transcript (Fig. 3.1B). No mobility shift was observed when recombinant GST was challenged with a transcript constituted by the three regions of intron 3 (Fig. 3.1B and 3.1C).

These results indicate that hnRNP H1 binds in vitro to the region of the intron 3 transcript that contains seven G run elements.

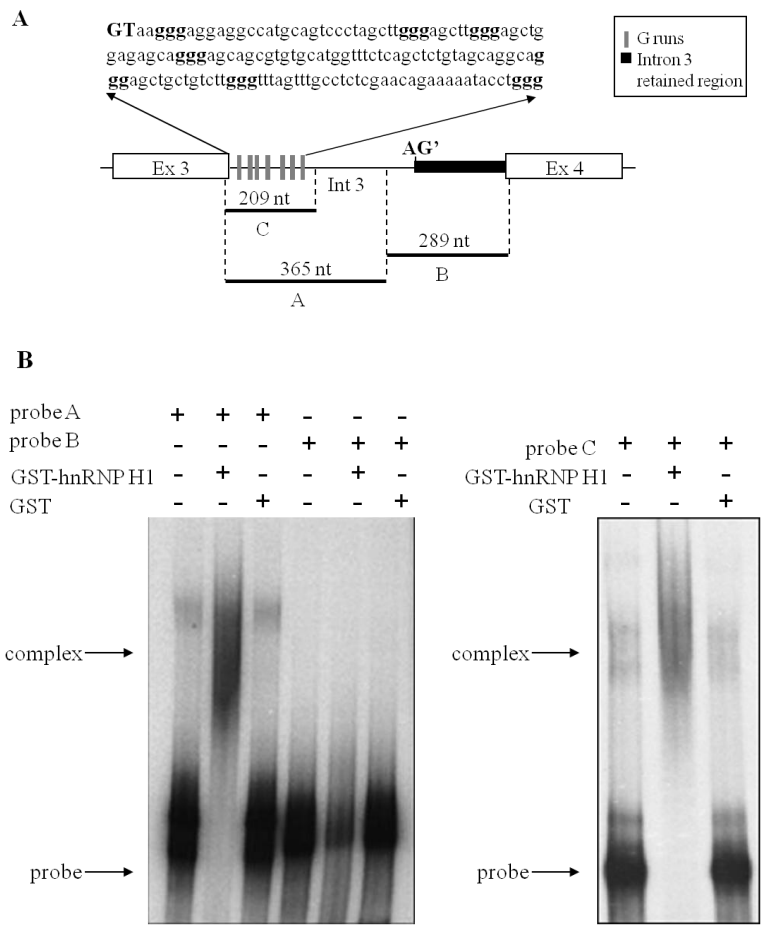

Figure 3.1 hnRNP H1 binds region A of rpL3 pre-mRNA intron 3. **(A)** schematic representation of the regions A, B and C of intron 3. **(B)** REMSA of hnRNP H1 with regions A and B. **(C)** Analysis with region C.

3.2 hnRNP H1 binds G3 and G6 regulating AS

To evaluate the role of each G run in the regulation of splicing of rpL3 pre-mRNA, we produced a series of wt-rpL3 minigene mutants (see Napolitano PhD thesis 2010); the results strongly suggest that elements G3 and G6 are directly involved in the regulation of the splicing of rpL3 transcript intron 3 (Fig. 3.2A).

These results together with data from hnRNP H1 silencing (see Napolitano PhD thesis 2010) provide strong evidence that the effects on the splicing pattern of rpL3 pre-mRNA could be mediated directly by the interaction of hnRNP H1 with G3 and G6 elements. To verify these findings, we overexpressed hnRNP H1 in HeLa cells transfected with the wt-rpL3 (minigene containing wild type G-run elements) , G[2,3,5,6]* (where G-run element number 2, 3, 5 and 6 were mutated in GAT) or G[3,6]* (where only G3 and G6 elements were mutated in GAT) minigenes under conditions that could lead to a larger than three-fold increase of the rpL3-a transcript level. Despite hnRNP H1 overexpression, the splicing pattern of the double mutant G[3,6]* showed that the level of alternative transcript was 60% lower than the level of the control wt-rpL3 minigene (Fig. 3.2B).

These data indicate that the G3 and G6 elements represent the consensus binding motifs of hnRNP H1 and are required for the hnRNP H1-mediated regulation of rpL3 pre-mRNA splicing.

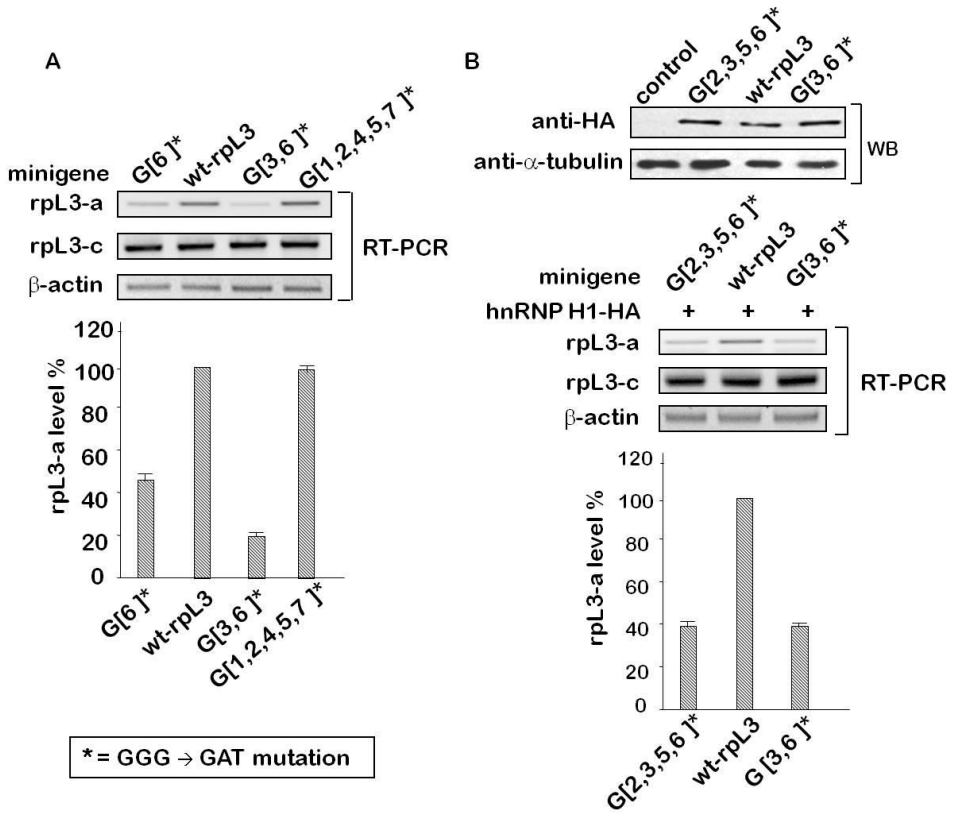

Figure 3.2 hnRNP H1 binds G3 and G6 regulating AS (A) Representative RT-PCR of total RNA extracted from CHX-treated HeLa cells transfected with the wt-rpL3 minigene or mutated constructs (see Napolitano PhD thesis) **(B)** hnRNP H1-HA was detected by western blotting (WB) using antibodies against the HA epitope. Loading in the lanes was controlled by detection of tubulin protein (upper panel). Representative RT-PCR of total RNA extracted from CHX-treated HeLa cells transfected with hnRNP H1-HA or co-transfected with G[2,3,5,6]* or G[3,6]* or the wt-rpL3 minigene and hnRNP H1-HA (lower panel).

3.3 NPM, KHSRP, hnRNP H1 and rpL3: interaction in vivo and in vitro

As discussed previously, we have identified by a proteomic analysis a number of proteins associated to rpL3 and/or to intron 3 transcript of the rpL3 gene, and we focused our studies on proteins involved in RNA processing as putative components of a RNP complex including rpL3 and mediating alternative splicing of the rpL3 pre-mRNA.

Particularly, we asked whether hnRNP H1, NPM and KHSRP could be part of a RNP complex including rpL3 through a direct or indirect interaction in vivo, thus we have performed a co-immunoprecipitation assays showed in figure 3.3A in which NPM and KHSRP were immunoprecipitated with specific antibody from HeLa cells extracts.

Immunoprecipitated proteins were separated by SDS–PAGE and the presence of NPM and KHSRP was investigated by western blotting in the reciprocally immunoprecipitated complexes. The results of these experiments showed that NPM and KHSRP were coimmunoprecipitated, thus indicating that they can associate in vivo. Furthermore, a specific signal for rpL3 appeared both in NPM as well as in KHSRP immunocomplexes.

These data suggest that rpL3 is able to interact in vivo with both proteins. The presence of signal for hnRNP H1 in both immunoprecipitates was consistent with a specific association between NPM, KHSRP and hnRNP H1. A control immunoprecipitate obtained with anti-IgG antibodies did not give any signal, when probed with anti-NPM, anti-KHSRP, anti-rpL3 or anti-hnRNP H1.

In an effort to define a RNP complex including rpL3, we investigated protein–protein interactions that rpL3 protein partners may establish within the complex. To this aim, we performed GST pull-down assays by using purified recombinant proteins. His-tagged proteins were tested for their ability to bind to immobilized GST-tagged proteins. GST-tagged proteins, and GST as control, were immobilized using GSH-sepharose beads and incubated with purified His-tagged proteins. Western blot analysis was performed on each pull-down sample. Figure 3.3B shows that His-hnRNP H1 was present in the pull-down preparation of GST-NPM, but not in those of GST-rpL3 or GST; His-NPM was detected in the pull-down preparations of GST-rpL3, GST-hnRNP H1, but not in that of GST; and His-KHSRP was revealed in the pull-down preparations of GST-rpL3 and GST-hnRNP H1, but not in those of GST-NPM or GST.

These results indicate that although a direct interaction between rpL3 and hnRNP H1 has not been observed, rpL3 and hnRNP H1 are able to interact directly with NPM and KHSRP, while NPM and KHSRP are not able to interact directly in the in vitro assay.

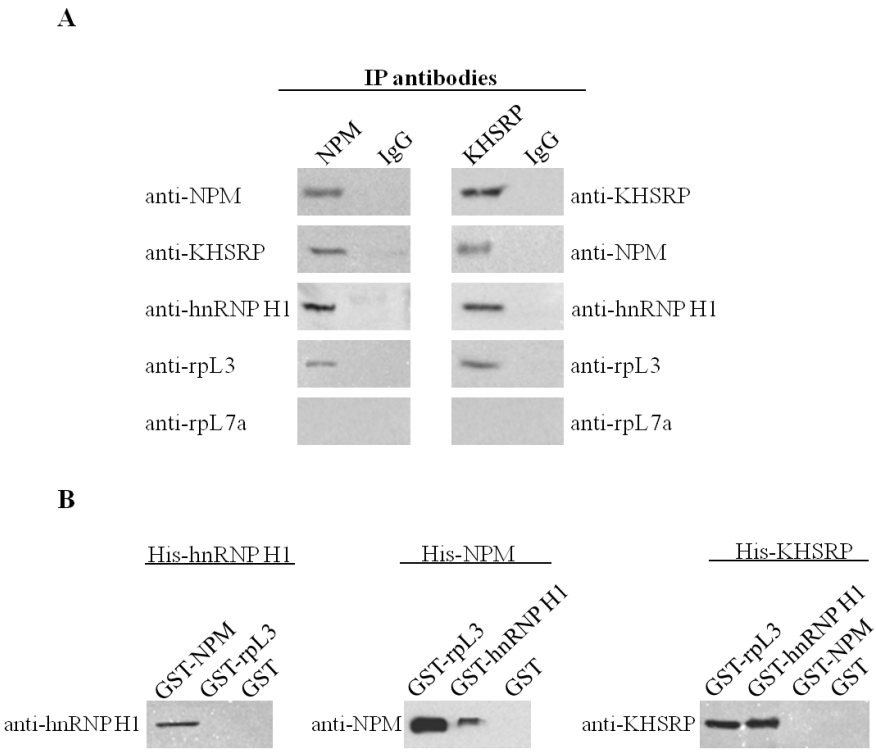

Figure 3.3 (A) In vivo binding of NPM, KHSRP, hnRNP H1 and rpL3. NPM or KHSRP were specifically immunoprecipitated from HeLa cells extracts with antibodies against the endogenous NPM and KHSRP. Immunoprecipitates were separated by SDS–PAGE and immunoblotted with antibodies versus the indicated proteins. Note the absence of signal in IgG immunocomplex. **(B) Analysis of the interactions between hnRNP H1, NPM, KHSRP and rpL3**. Western blotting (WB) of GST pull-down experiments. Fifty microgram of GST-tagged proteins (GST-NPM, GST-rpL3 and GST-hnRNP H1) or GST (control) were immobilized on glutathione-sepharose beads and incubated with 20 mg of His-hnRNP H1, His-NPM or His-KHSRP. The eluted proteins were then analyzed by immunoblot with antibodies anti-hnRNP H1, anti-NPM and anti-KHSRP. Note the absence of signal in control GST pull-down preparations.

3.4 NPM, KHSRP, hnRNP H1 and intron 3 transcript: interaction in vivo and in vitro

Mass spectral analysis of RNA pull-down experiments shown previously (see Introduction, Fig. 1.3), revealed that KHSRP was able to interact with intron 3 transcript of rpL3 gene in vitro. To confirm this interaction in vivo, we performed RNA affinity immunoprecipitation experiments. We specifically immunoprecipitated KHSRP, and hnRNP H1 as control, from HeLa cell extracts by using monoclonal antibodies against the endogenous proteins and searched for the intron 3 transcript in the RNA–protein immunoprecipitate complex (Figure 3.4A). Amplification of the signal corresponding to the intron 3 of rpL3 transcript, but not of rpL7a gene transcript, by RT–PCR indicated that KHSRP was able to bind with rpL3 pre-mRNA. The absence of signal in the immunoprecipitate with anti-IgG confirmed the validity of this assay. Although NPM was not found associated to the intron 3 transcript in vitro previously, we wondered whether this interaction could occur in vivo. To this aim, we specifically immunoprecipitated NPM from HeLa cell extracts by using monoclonal antibodies against the endogenous NPM. Analysis of RNA extracted from the immunoprecipitate complex demonstrates that NPM is able to precipitate the intron 3 transcript (Figure 3.4A).

Next, in order to establish whether one or more among the identified proteins was able to interact directly with intron 3 RNA, or whether additional factors were required, we performed RNA pull-down experiments by using purified recombinant proteins. For this purpose, a transcript corresponding to the entire intron 3 of the rpL3 gene was used as bait and

incubated with the purified recombinant proteins His-hnRNP H1 (control), His-NPM or His-KHSRP and His-rpL7a as a control RNA binding protein. Then, RNA-associated proteins were eluted and analyzed by western blotting with antibodies against the endogenous hnRNP H1, NPM, KHSRP and rpL7a. The presence of signals specific for NPM and KHSRP indicated that these two proteins are able to recognize and bind to sequences in the rpL3 intron 3 transcript (Figure 3.4B).

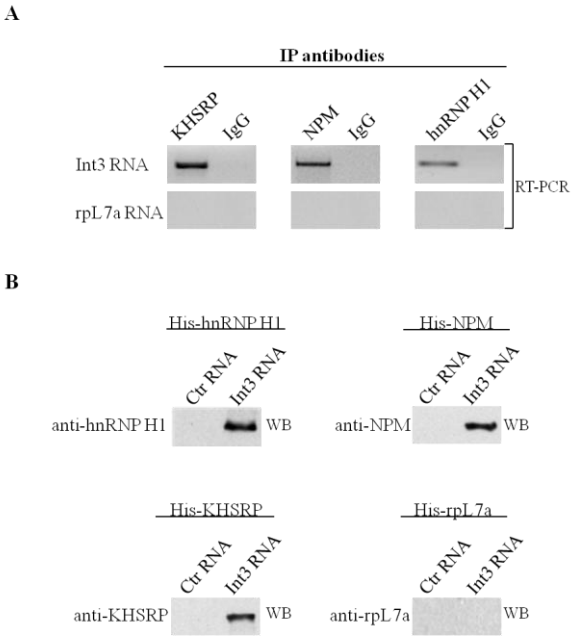

Figure 3.4 Analysis of interaction between intron 3 and NPM, KHSRP *in vivo* and *in vitro*. (A) RT–PCR analysis, by using primers against rpL3 intron 3 transcript and rpL7a RNA transcript, of RNA extracted from the NPM, KHSRP, hnRNP H1 (control) and IgG immunocomplexes. Note the absence of signal in IgG immunocomplex. **(B)** WB of RNA pull-down experiments, using adipic acid dehydrazide agarose beads coated with intron 3 transcript or unrelated RNA (Ctr RNA) incubated with purified proteins His-hnRNP H1, His-NPM, His-KHSRP and His-rpL7a.

3.5 KHSRP regulates alternative splicing of rpL3 pre-mRNA

The identification of a specific interaction of KHSRP with rpL3 and intron 3 transcript, prompted us to investigate a possible role of KHSRP in the rpL3 gene splicing. To this aim, we analyzed the effects of the alteration in the expression levels of KHSRP on the rpL3 RNA splicing pattern.

We used L3-8 cells, a PC12 Tet-Off cell line stably transfected with a vector containing the human rpL3 coding sequence fused to the hemagglutinin (HA) epitope coding sequence. L3-8 cells were transiently transfected with the DNA construct expressing Flag-KHSRP. We chose a dose of Flag-KHSRP that would result in about a 50% increase of the rpL3 alternative isoform mRNA (see Napolitano PhD thesis 2010). 24 hours after transfection, we treated cells with CHX and induced the expression of HA-rpL3 by removing doxycycline from medium. Cells were then lysed and analyzed for the production of Flag-KHSRP and HA-rpL3 by western blotting using anti-Flag and anti-HA antibodies, respectively (Fig. 3.5A, left panel). Total RNA was analyzed by RT-PCR using specific primers to amplify canonical (rpL3-c) or alternative (rpL3-a) isoform of rpL3 mRNA (Fig. 3.5A, right panel). The expression of exogenous KHSRP caused an increase in rpL3-a mRNA level. Of interest, the increase in the rpL3-a mRNA caused by overexpression of KHSRP was greater after the induction of HA-rpL3 (Fig. 3.5A, right panel).

The effects of KHSRP on rpL3 splicing were also investigated in condition of mRNA silencing. L3-8 cells were treated with siRNA specific for KHSRP and, 24 hours after transfection, cells were treated with CHX and HA-rpL3 production was induced by removing doxycycline from

culture medium. Cells were then lysed and protein extracts were analyzed for the expression of KHSRP and HA-rpL3 by western blotting using anti-KHSRP and anti-HA antibodies, respectively. As shown in Figure 3.5B (left panel), the decrease of KHSRP level was about 70% compared to the protein amount detected in the control lysates. To examine the effects of the reduced production of KHSRP on the splicing pattern of rpL3 pre-mRNA, we analyzed total RNA extracted from the same samples by RT-PCR using specific primers to amplify rpL3-c and rpL3-a mRNAs. As expected, in cells overexpressing rpL3, we observed an increased level of rpL3-a mRNA isoform. However, in cells in which KHSRP had been silenced, rpL3 induced overexpression was less effective in promoting the production of rpL3-a mRNA isoform (Fig. 3.5B, right panel).

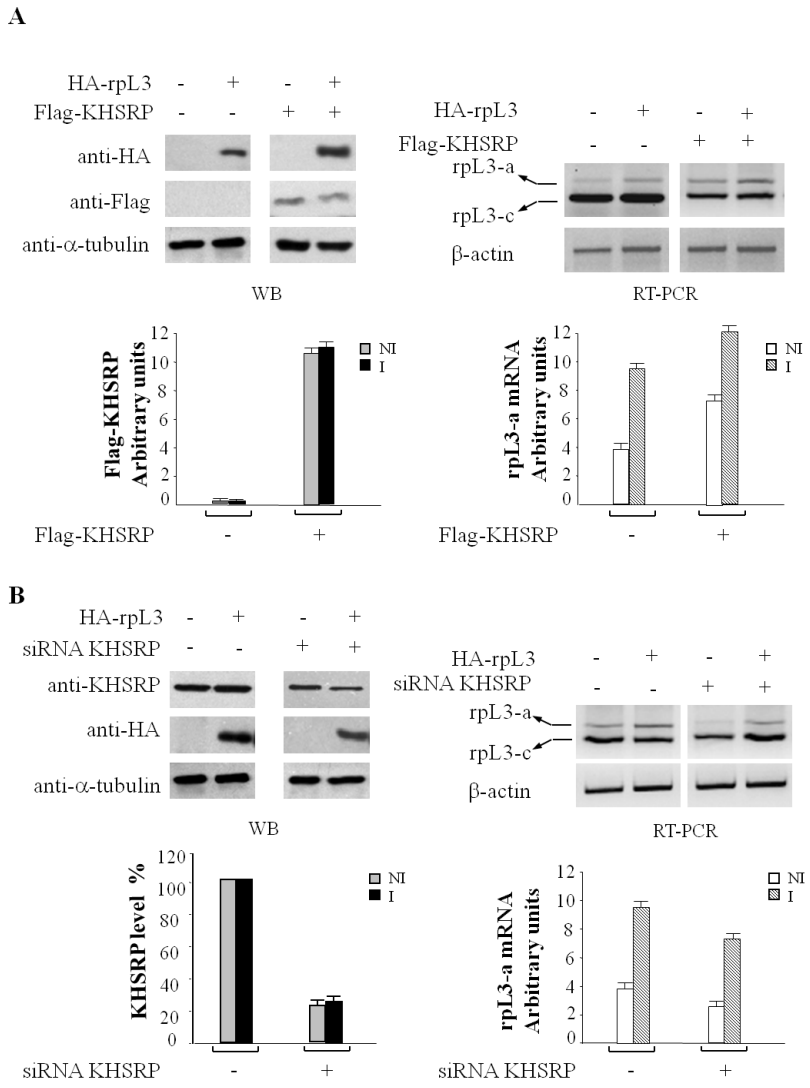

Figure 3.5 (A) Effect of KHSRP over expression and (B) Effect of RNAi-mediated depletion of KHSRP on rpL3-a mRNA level in L3-8 cells.

3.6 Coupling of hnRNP H1and KHSRP in rpL3 pre-mRNA splicing

Both hnRNP H1 and KHSRP promote 3' cryptic splicing site selection of rpL3 pre-mRNA, so we asked whether a cooperation between these two proteins would occur, or whether their functions were redundant. To clarify this issue, we switched off the expression of the gene encoding hnRNP H1 by using RNA interference. To this purpose, siRNAs against hnRNP H1 were transiently transfected in L3-8 cells. 24 hours after transfection, cells were treated with CHX, induced for HA-rpL3 production and harvested; RNA and proteins were extracted. Lysates from cells transfected with siRNA or untransfected were probed with hnRNP H1 antibodies. As shown in Figure 3.6A (left panel), the residual level of hnRNP H1 was about 20% of the protein detected in the control lysates. To investigate the effects of the reduced production of hnRNP H1 on the splicing pattern of rpL3 pre-mRNA, we examined the level of rpL3 mRNA isoforms using RT-PCR. Under normal conditions of rpL3 expression, the depletion of hnRNP H1 resulted in a relevant decrease (about 80%) of the alternative mRNA (rpL3-a) level compared to controls. In conditions of hnRNP H1 removal, rpL3, although overexpressed, failed to activate the selection of the 3' cryptic splicing site (Fig. 3.6A, right panel). These data indicate clearly that hnRNP H1 plays a crucial role in triggering the alternative splicing reaction of rpL3 pre-mRNA.

Next, we investigated a possible involvement of hnRNP H1 in KHSRP-mediated activity. To this aim, we analyzed the effects of hnRNP H1 depletion and KHSRP overexpression on rpL3 splicing reaction. L3-8 cells were transiently co-transfected with siRNA specific for hnRNP H1 and

a DNA construct expressing Flag-KHSRP. 24 hours after transfection, cells were treated with CHX, induced for HA-rpL3 expression, and harvested. hnRNP H1 and KHSRP protein levels were detected by western-blotting (Fig. 3.6B, left panel). RNA extracted from the same lysates was analyzed by RT-PCR. The figure 3.6B (right panel) shows that when hnRNP H1 was depleted, the enforced expression of KHSRP did not cause an increase of rpL3-a mRNA level, either in normal conditions of rpL3 expression, or in rpL3 overproduction. These findings indicate that the ability of KHSRP to influence the splicing of rpL3 pre-mRNA is mediated by hnRNP H1.

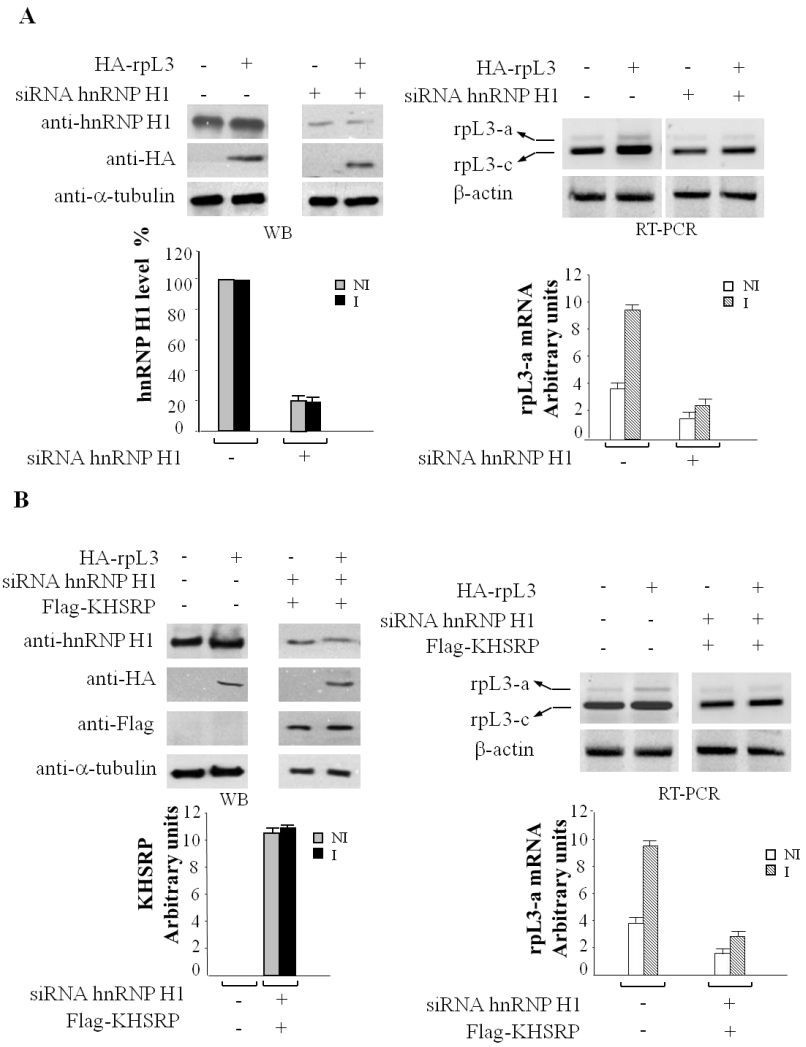

Figure 3.6 (A) Effect of RNAi-mediated depletion of hnRNP H1 on rpL3-a mRNA level in L3-8 cells. (B) Effect of KHSRP over expression in condition of hnRNP H1 silencing on rpL3-a mRNA level in L3-8 cells.

3.7 NPM regulates alternative splicing of rpL3 pre-mRNA

To investigate the role of NPM in the context of rpL3 autoregolatory loop, we performed experiments of overexpression and RNAi-mediated silencing of NPM in L3-8 cell line. Cells were transiently transfected with a DNA vector expressing HA-NPM.. 24 hours after transfection, cells were treated with CHX and induced to express HA-rpL3. Cells were then lysed and analyzed for the production of HA-NPM and HA-rpL3 by western blotting by using anti-HA antibody (Fig. 3.7A, left panel). Total RNA from the same samples was analyzed by RT-PCR by using specific primers to amplify rpL3-c and rpL3-a isoforms of rpL3 mRNA. The overexpression of NPM resulted in a reduction in the rpL3-a mRNA amount in normal conditions of rpL3 expression, and prevented the increasing of rpL3-a mRNA level when rpL3 protein was overexpressed (Fig. 3.7A, right panel).

To investigate the effects of NPM removal on rpL3 gene splicing, NPM expression levels were reduced by transfecting L3-8 cells with specific siRNA. 24 hours after transfection, cells were treated with CHX and induced to express HA-rpL3. Protein extracts from cells were tested for the production of HA-NPM and HA-rpL3 by western blotting (Fig. 3.7B, left panel). Total RNA was analyzed by RT-PCR by using specific primers to amplify rpL3-c and rpL3-a mRNA isoforms. In normal conditions of rpL3 expression, the depletion of NPM did not affect the level of the rpL3-a mRNA. Of interest, upon NPM silencing, HA-rpL3 expression caused a significant increase of rpL3-a mRNA levels (Fig. 3.7B, right panel).

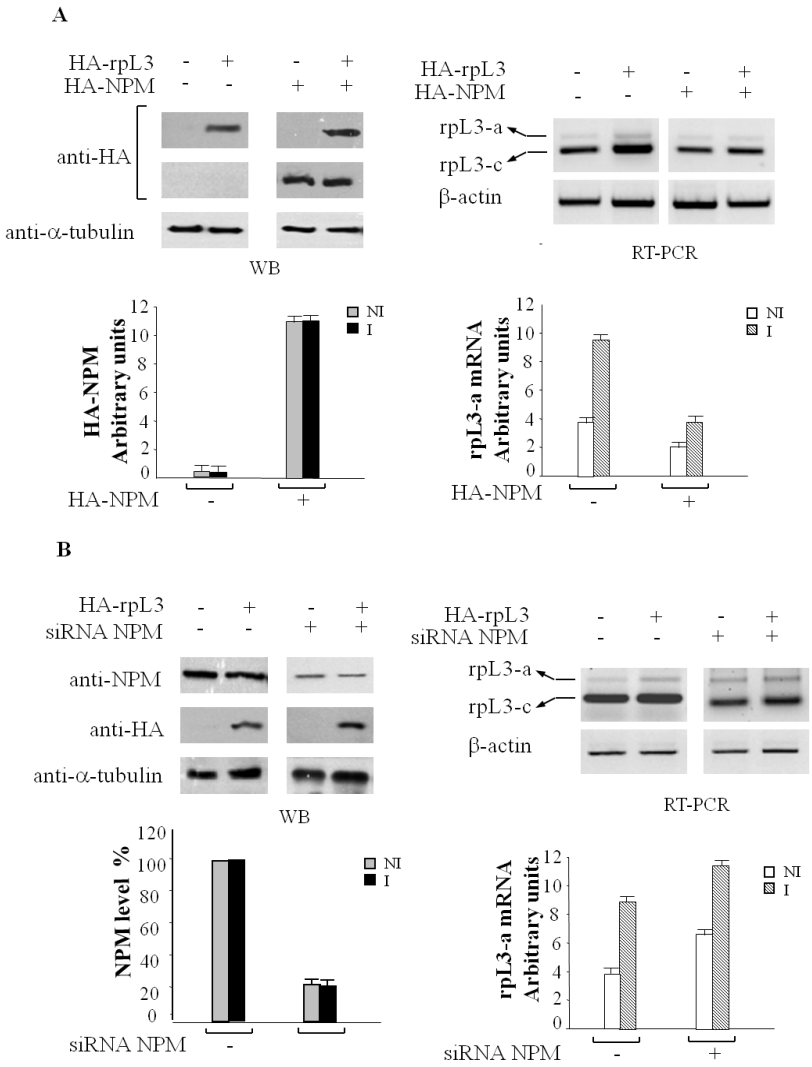

Figure 3.7 Effect on rpL3-a mRNA level in L3-8 cells after NPM over expression (A) or RNAi-mediated depletion (B).

4. Discussion

In eukaryotes, in order to maintain ribosome biosynthesis at the level appropriate to growth conditions and requirements of the cell, the expression of ribosomal protein is regulated by multiple control mechanisms, mostly at post-transcriptional and translational level (Cuccurese et al. 2005, Russo A. et al. 2010, Russo A. et al. 2011, Loreni et al. 1993); moreover, several studies prove that ribosomal proteins, in addition to the role as components of the translation machinery, exert a variety of extraribosomal functions, for which additional, specific regulatory strategies are required (Wang et al. 2007, Chaudhuri et al. 2007).

More specifically, in our laboratory was previously demonstrated a role of AS-NMD in the regulation of the rpL3 gene expression, promoted by rpL3 protein itself as part of an auto-regulatory negative-feedback loop (Cuccurese et al. 2005). In order to investigate the molecular mechanism of this auto-regulation, were identified several proteins able to bind in vitro rpL3, the intron 3 of rpL3 gene, or both (see Introduction, Fig. 1.3), since in most cases, the choice of a splicing site is made by a dynamic and complex combination of different splicing regulators; also, the function of an individual splicing factor may be different depending on interacting partners present in the regulatory network. In particular, I reported in this thesis the analysis of the role of hnRNP H1, and of two new regulatory proteins, identified, KHSRP (K-homology splicing regulatory protein, also known as KSRP) and NPM (B23, numatrin or NO38) in the rpL3 auto-regulatory loop.

REMSA experiments showed that the splicing factor hnRNP H1binds rpL3 intron 3, through a region containing seven G-run elements (Fig. 3.1).

Previous results demonstrated that rpL3 protein, within a RNP complex including the constitutive splicing factor hnRNP H1, promotes the alternative splicing reaction of its own gene (see Napolitano PhD thesis 2010) and mutational analysis of the seven G-run elements in rpL3 intron 3 showed that G3 and G6 affected splicing. In fact, when both elements were mutated (G[3,6]*), the level of the alternative mRNA isoform was reduced by 80%, while when wild-type G3 and G6 were conserved and all other G runs mutated (see G[1,2,4,5,7]* in Fig. 3.2A), the amount of alternative rpL3 mRNA isoform was the same as the amount obtained from the wt-rpL3 primary transcript. Moreover, when G3 and G6 motifs were mutated, hnRNP H1 overexpression did not affect the level of the alternatively spliced mRNA isoform (Fig. 3.2B).

These results prove that G3 and G6 form a functional unit, and that its interaction with hnRNP H1 promotes the selection of the alternative 3′ splice site of intron 3.

Thereafter, analysis of immunoprecipitate of NPM and KHSRP in HeLa cell extracts showed that NPM and KHSRP coimmunoprecipitate together with rpL3 and hnRNP H1, indicating that these proteins associate in vivo, although the occurrence of multiple complexes including different combination of protein interactions cannot be excluded (Fig. 3.3A). In addition, in vitro GST pull-down experiments (Fig. 3.3B) demonstrated that NPM and KHSRP are able to recognize and bind rpL3 and hnRNPH1,

whereas a direct binding between NPM and KHSRP, or rpL3 and hnRNPH1 has not been observed.

Likewise, analysis of RNA immunoprecipitation assays showed the presence of intron 3 RNA in the immunoprecipitate of NPM and KHSRP (Fig. 3.4A), and RNA pull-down experiments showed that NPM and KHSRP are able to contact directly the intron 3 RNA (Fig. 3.4B) as previously shown for hnRNP H1 by REMSA. Taken together, these data strongly suggest that NPM and KHSRP with hnRNP H1 are involved in the control of the splicing of rpL3.

Analysis of the effects of individual expression of NPM, hnRNP H1 or KHSRP provided an insight also on the specific role of each protein factor on the rpL3 gene splicing. In the context of rpL3 autoregulatory circuit, protein overexpression data indicated that KHSRP represents a positive regulator of the alternative splicing that cooperates with rpL3 and hnRNP H1 in the activation of 3'-cryptic splice site (Fig. 3.5A); instead, silencing of KHSRP caused about 20% decrease of the alternative mRNA isoform produced when rpL3 is overexpressed, suggesting that KHSRP is not a crucial player in the rpL3 autoregulatory loop (Fig. 3.5B).

Moreover, when hnRNPH1 expression was silenced, rpL3 overexpression failed to result in the activation of cryptic the 3'-splice site (Fig. 3.6A), and KHSRP, even in excess, was unable to increase the alternative mRNA isoform in the absence of hnRNP H1 (Fig. 3.6B).

These results strongly indicate that hnRNP H1 is an essential component of rpL3 auto-regulatory loop, while KHSRP might play a role as

an enhancer of hnRNP H1-mediated activation of rpL3 gene alternative splicing.

It is known that the RNA binding activity of KHSRP is mediated by four KH domains. Among these, KH1 and KH3 domains recognize and bind specifically a G-rich target (UGGG and GGGU, respectively) (Garcia-Mayoral et al. 2007). The analysis of the human intron 3 transcript sequence showed that G6 motif, an essential element for the splicing regulatory activity of hnRNP H1, could represent also a binding site for KH1 or KH3.

In addition, the UGCAUG element, essential to the binding of KHRSP to src-DCS (Downstream Control Sequence), is also present in the sequence of intron 3 RNA and it is located close to the hnRNP H1 binding site G3 and G6 motifs (Markovtsov et al. 2000). Consequently, it is possible to hypothesize that KHSRP might interact with these sequences within intron 3 transcript as well as with hnRNP H1 making more stable and efficient the association of hnRNP H1with intron 3 RNA.

Differently, NPM acts as a negative regulatory factor of rpL3 gene alternative splicing (Fig. 3.7); thus, based on these data, we propose a working model, which overcomes the model proposed previously (Russo A. et al. 2010).

The model predicts the existence of, at least, two complexes whose protein composition depends on rpL3 protein levels. When cell functions require an efficient production of rpL3, the interaction of NPM with intron 3 pre-mRNA might affect its secondary structure and mask the binding sites of hnRNP H1. At the same time, repression of AS might be enforced by a concomitant direct binding of NPM to the positive regulator factor hnRNP

H1 (Fig. 4.1A). When rpL3 levels exceed cell requirements for rpL3 ribosomal as well as extraribosomal functions, the protein could interact with NPM; consequently, NPM would be released from the RNP complex to which rpL3 could be associated by interacting with KHSRP. The removal of NPM, and the rearrangement of the interactions within the RNP complex, could make available the enhancer unit G3+G6 within intron 3 transcript and, at the same time, the presence of rpL3 in the remodeled complex could induce conformational changes that favor the interaction of hnRNP H1 with G3+G6. Finally, such complex could promote the activation of the alternative mode of splicing reaction (Fig. 4.1B).

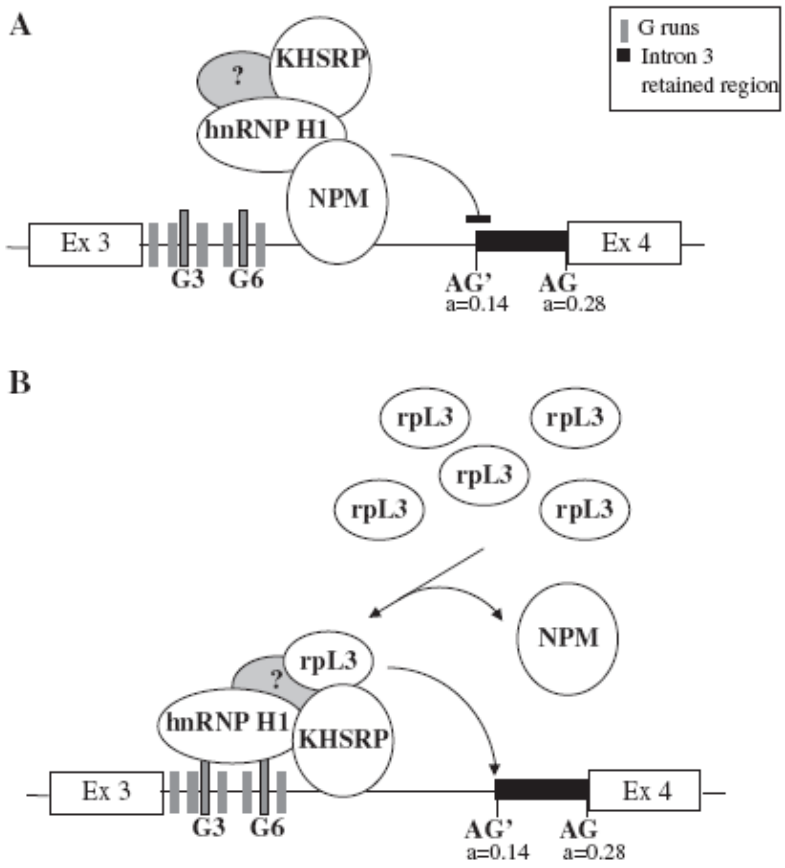

Figure 4.1: Schematic representation of proposed rpL3 feedback regulation.

Concluding, AS-NMD may be most relevant to finely modulate production of rpL3 available for extraribosomal functions; rpL3 indeed has been involved so far in two extraribosomal functions: a) in the splicing regulation of its own pre-mRNA and, b) Preliminary results obtained in our lab indicate that rpL3 is involved in a regulatory mechanism of p21 expression independent from p53.

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hnRNP H1 and intronic G runs in the splicing control of the human rpL3 gene

Annapina Russo [a,c], Gabriella Siciliano [a], Morena Catillo [a], Chiara Giangrande [b], Angela Amoresano [b], Pietro Pucci [b,c], Concetta Pietropaolo [a,*], Giulia Russo [a,*]

[a] Dipartimento di Biochimica e Biotecnologie Mediche, Università Federico II, Via Sergio Pansini 5, Napoli 80131, Italy
[b] Dipartimento di Chimica Organica e Biologica, Università Federico II, Complesso Universitario Monte S. Angelo, Via Cinthia 4, Napoli 80126, Italy
[c] CEINGE Biotecnologie Avanzate S.C.a.r.l., Via Comunale Margherita 482, Napoli 80145, Italy

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ABSTRACT

By generating mRNA containing a premature termination codon (PTC), alternative splicing (AS) can quantitatively regulate the expression of genes that are degraded by nonsense-mediated mRNA decay (NMD). We previously demonstrated that AS-induced retention of part of intron 3 of rpL3 pre-mRNA produces an mRNA isoform that contains a PTC and is targeted for decay by NMD. We also demonstrated that overexpression of rpL3 downregulates canonical splicing and upregulates the alternative splicing of its pre-mRNA. We are currently investigating the molecular mechanism underlying rpL3 autoregulation. Here we report that the heterogeneous nuclear ribonucleoprotein (hnRNP) H1 is a transacting factor able to interact in vitro and in vivo with rpL3 and with intron 3 of the rpL3 gene. We investigated the role played by hnRNP H1 in the regulation of splicing of rpL3 pre-mRNA by manipulating its expression level. Depletion of hnRNP H1 reduced the level of the PTC-containing mRNA isoform, whereas its overexpression favored the selection of the cryptic 3′ splice site of intron 3. We also identified and characterized the cis-acting regulatory elements involved in hnRNP H1-mediated regulation of splicing. RNA electromobility shift assay demonstrated that hnRNP H1 specifically recognizes and binds directly to the intron 3 region that contains seven copies of G-rich elements. Site-directed mutagenesis analysis and in vivo studies showed that the G3 and G6 elements are required for hnRNP H1-mediated regulation of rpL3 pre-mRNA splicing. We propose a working model in which rpL3 recruits hnRNP H1 and, through cooperation with other splicing factors, promotes selection of the alternative splice site.

1. Introduction

Alternative splicing (AS) is one of the main regulatory mechanisms of gene expression; it results in a repertoire of mRNAs, and consequently of proteins, much larger than expected from the number of genes. This process contributes substantially to cell-specific and tissue-specific gene expression; it is estimated that over 60% of human genes are alternatively spliced [1].

Splicing is modulated by cis elements in intron or exon sequences that, associated with specific transacting factors, can negatively (intronic or exonic splicing silencer sequences, ISS or ESS) or positively (intronic or exonic splicing enhancing sequences, ISE or ESE) affect splicing [2]. Although ESE elements differ in sequence, most share a consensus sequence (GAR)n. The UGCAUG esanucleotide is a frequent element in ISE sequences [3]. Most regulatory sequences are a complex combination of multiple elements that mediate positive or negative effects on gene splicing [4–6]. Intronic stretches of three or four Gs called "G runs" are involved in the splicing of a number of genes [7–13].

Among the factors found to control the selection of the splice site, components of the SR protein family have been shown to antagonize the activity of protein components of heterogeneous nuclear ribonucleoproteins (hnRNPs) [14,15]. hnRNPs are a large group of nuclear RNA-binding proteins that share a common structural domain denoted "qRRM" and are implicated in a variety of processes including RNA stability and translation. Accumulating evidence indicates that these proteins may play an important role in the control of splice site selection [16,17]. hnRNP H1 is a member of the hnRNPH/F family. It is able to bind the splicing regulatory cis elements, i.e. ESE, ISE, ESS and ISS. Depending on the interacting cis element and on the gene context, hnRNP H1 can affect the selection of the splice site and consequently promote either constitutive or alternative splicing [9–13,18].

Data from several laboratories demonstrate that some evolutionarily conserved AS events give rise to aberrant transcripts, which are substrate of nonsense-mediated mRNA decay (NMD). NMD is an RNA

Abbreviations: AS, alternative splicing; hnRNP, heterogeneous nuclear ribonucleoprotein; NMD, nonsense-mediated mRNA decay; PTC, premature termination codon; r-protein, ribosomal protein; rp, ribosomal protein; rp-mRNA, mRNA for ribosomal protein; RT-PCR, reverse transcriptase-PCR; SH-PTP1, SH-protein-tyrosine phosphatase 1; siRNA, small interfering RNA; SR, serine-rich

* Corresponding authors. G. Russo is to be contacted at tel.: +39 0817463061; fax: +39 0817463074. C. Pietropaolo, tel.: +39 0817463065; fax: +39 0817463074.
E-mail addresses: pietropaolo@dbbm.unina.it (C. Pietropaolo), russog@dbbm.unina.it (G. Russo).

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surveillance mechanism that selectively degrades aberrant mRNAs that contain a premature termination codon (PTC), thus preventing the production of truncated polypeptides potentially deleterious to the cell. This process is highly conserved in eukaryotes. A PTC may arise through a nonsense or frameshift mutation of DNA, as a consequence of DNA rearrangement, or through splicing errors that produce aberrant mRNAs [19]. A PTC could also arise from alternative splicing that produces an intron-derived nonsense codon or a shift in the ORF that generates a downstream nonsense codon. The resulting aberrant mRNA is targeted for NMD rather than being translated into protein. Thus, the AS–NMD association results in quantitative post-transcriptional regulation of gene expression [20].

Genes that encode ribosomal proteins (rp) are regulated by AS–NMD. This regulatory strategy appears to be evolutionarily conserved in nematodes [21] and mammals [22]. We recently demonstrated that AS of genes for human proteins rpL3 and rpL12 generates alternative RNA isoforms consequent to the removal of part of intron 3 and of intron 1, respectively. The resulting mRNAs include intronic sequences that contain a PTC and are targeted for decay by NMD. We also demonstrated that overexpression of rpL3 results in the down-regulation of canonical splicing and upregulation of the alternative splicing of rpL3 pre-mRNA [22]. Here we report that hnRNP H1 is an rpL3 partner and plays an important role in the splicing regulation of rpL3 pre-mRNA. We also report that G-rich sequences in intron 3 are cis-regulatory elements involved in hnRNP H1-mediated regulation.

2. Materials and methods

2.1. Cell cultures, transfections and drug treatment

Human cell lines HeLa and Calu6 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax (Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS). In addition, Calu6 culture medium was supplemented with 0.1 mM non-essential amino acids (Euroclone, West York, UK). siRNA transfections were performed in HeLa cells (1×10^6 cells, 6 mm-well plate) at a concentration of 150 nM by using Oligofectamine Reagent (Invitrogen) according to the manufacturer's instructions.

Plasmids were transfected in HeLa cells (2.5×10^6 cells, 6 mm-well plate) by using Lipofectamine 2000 (Invitrogen) or Fugene6 (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. 24 h after DNA or siRNA transfections, cells were treated with 100 μg/ml cycloheximide for 4 h to block NMD. Then, RNA and proteins were extracted by using the Trizol procedure (Invitrogen) for RT-PCR analysis and western blot, respectively. Transfection efficiency was assessed by co-transfecting a GFP-expressing vector and normalizing RNA levels against GFP mRNA levels (data not shown).

2.2. Constructs, mutagenesis, and RNA interference

The cDNAs of hnRNP H1 and rpL3 were obtained by RT-PCR from HeLa cells using specific primers (Table 1) and cloned into the prokaryotic expression vector pGEX4T3 (GE Healthcare, Waukesha, Wisconsin). The cDNA of hnRNP H1 was also cloned into a version of the eukaryotic expression vector pcDNA4/HisMax C (Invitrogen) containing the HA epitope. The full intron 3 of rpL3 gene, or regions A, B and C (Fig. 3A) were obtained by RT-PCR using specific primers (Table 1) and cloned into the pGEM-4Z vector (Promega, Madison, Wisconsin) to obtain constructs pGEM4Z-Int3, pGEM4Z-IntA, pGEM4Z-IntB and pGEM4Z-IntC, respectively. The wt-rpL3 minigene was kindly provided by Y. Tang (Dr S. Stamm's laboratory, Institute for Biochemistry, University of Erlangen—Nurenberg, Germany). It contains the genomic region of the rpL3 gene spanning from exon 3 to exon 5 flanked by insulin exon sequences (Fig. 5A) cloned in the Exontrap vector (MoBiTec, Gottingen, Germany). The constructs carrying the disruption of single or multiple G runs, i.e., GGG/GAT mutation, were obtained by PCR site-directed mutagenesis (primers in Table 1) using the QuickChange® Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California) and the wt-rpL3 minigene as template. All constructs were verified by DNA sequencing.

Table 1
Oligonucleotides sequences.

	5′-Primer (5′–3′)	3′-Primer (3′–5′)
GST-rpL3	ATGTCTCACAGAAAGTTC	AGCTCCTTCTTCCTTTGC
Int3	AATTGGTAAGGGAGGAG	TATGCCTTCAGGAGCAGA
rpL3-a	CTCCGCTGGGCTCTGCCC	CTTCAGGAGCAGAGCAGA
rpL3-c	GGGCATTGTGGGCTACGT	GTAAAGGCCTTCTTCTTAG
IntA	AAGAATTGGTAAGGGAGG	GGAAGCCCACTCAGTGAT
IntB	CCAGGGCAAAAGGTTTG	GTAAAGGCCTTCTTCTTAG
IntC	AAGAATTGGTAAGGGAGG	TCAGAATGAGGGTGTTAGC
GST-hnRNP H1	ATGATGTTGGGCACGGAA	CTATGCAATGTTTGATTG
hnRNP H1-HA	ATGATGTTGGGCACGGAA	CTATGCAATGTTTGATTG
β-Actin	GGCACCACCTTCTACA	CAGGAGGACAATGAT
Insulin	GCGAAGTGGAGGACCCACAA	ACCCAGCTCCAGTTGTGCCA

The small interfering RNAs (siRNA) targeting hnRNP H1 were: 5′-GGAAATAGCTGAAAAGGCT-3′ and 5′-CCACGAAAGCTTATGGCCA-3′. The siRNA and the Silencer® Negative Control #1 siRNA were purchased from Ambion (Foster City, California).

2.3. Nuclear extracts

Nuclear extracts were prepared from Calu6 cells. Briefly, cells from twenty-one 100-mm plates (1.4×10^8 cells) were washed with ice cold PBS and collected in 7 ml of lysis buffer containing 20 mM Hepes–KOH, pH 8.0, 5 mM $MgCl_2$, 25 mM KCl, 0.5% NP40, 0.25% sodium-deoxycholate, 5 mM DTT and Protease Inhibitor Mix 1X (Roche), and passed through a needle (23 gauge). The cell lysate was incubated for 10 min on ice, then cell nuclei were separated from the cytoplasmic fraction by centrifugation for 5 min at $1000\times g$ at 4 °C. Nuclei from twenty-one 100-mm plates were resuspended in 2 ml of nuclear lysis buffer (20 mM Hepes–KOH, 5 mM $MgCl_2$, 0.5 M NaCl, 20% glicerol, 1 mM EDTA, 5 mM DTT and Protease Inhibitor Mix 1X) and incubated for 10 min on ice, then clarified by centrifugation for 10 min at $10{,}000\times g$ at 4 °C.

2.4. GST pull-down, mass spectrometry and protein identification

The recombinant proteins GST-rpL3 and GST were expressed in *Escherichia coli* and purified by using glutathione-Sepharose 4B beads according to the manufacturer's instructions (GE Healthcare). For GST pull-down assay, 100 μg of GST-rpL3 fusion protein, or GST control, was immobilized on glutathione-Sepharose beads and incubated with 10 mg of nuclear extract from Calu6 cells in pull-down buffer (50 mM Tris–HCl pH 7.5, 0.4 mM EDTA, 150 mM NaCl, 10% glicerol, 1% NP40, 1 mM sodium–ortovanadate, 50 mM NaF, 5 mM DTT and Protease Inhibitor Mix 1X) at 4 °C for 1.5 h. The beads were washed extensively and boiled in SDS sample buffer. The eluted proteins were loaded on 12% SDS-PAGE. Gels were stained with a colloidal blue staining kit (Invitrogen) and protein bands were excised from the gel and destained by repetitive washes with 0.1 M NH_4HCO_3, pH 7.5 and acetonitrile. Samples were reduced by incubation with 50 μl of 10 mM DTT in 0.1 M NH_4HCO_3 buffer, pH 7.5 and carboxyamidomethylated with 50 μl of 55 mM iodoacetamide in the same buffer. Enzymatic digestion was carried out with trypsin (12.5 ng/μl) in 10 mM ammonium bicarbonate, pH 7.8. Gel pieces were incubated at 4 °C for 2 h. Trypsin solution was then removed and a new aliquot of the digestion solution was added; samples were incubated for 18 h at 37 °C. A minimum reaction volume was used to obtain complete rehydration of the gel. Peptides were then extracted by washing the gel particles

with 10 mM ammonium bicarbonate and 1% formic acid in 50% acetonitrile at room temperature.

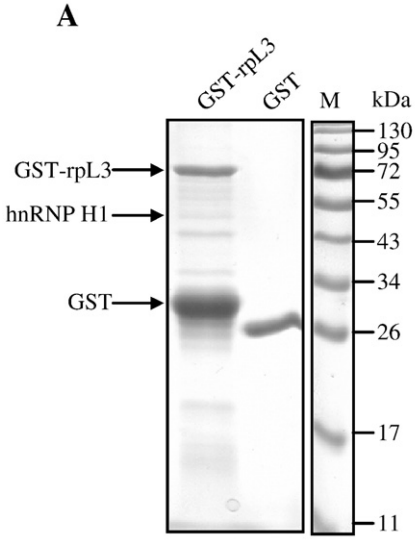
Liquid chromatography and tandem mass spectrometry (LC/MS/MS) analyses were performed on an LC/MSD Trap XCT Ultra (Agilent Technologies, Palo Alto, CA) equipped with a 1100 nanoHPLC system and a chip cube (Agilent Technologies). Peptides were analyzed using data-dependent acquisition of one MS scan (mass range from 300 to 1800 m/z) followed by MS/MS scans of the three most abundant ions in each MS scan. Raw data from nanoLC/MS/MS analyses were used to query a non-redundant protein database using in-house MASCOT software (Matrix Science, Boston, Massachusetts) [23].

2.5. Immunoprecipitation and western blotting

For immunoprecipitation assay, 1 mg of HeLa whole cell lysate was incubated with 30 µl of protein A/G agarose beads coated with 5 µg of antibody against rpL3 (Primm Milan, Italy), hnRNP H1 and SH-PTP1 (Santa Cruz Biotechnology, Santa Cruz, California) at 4 °C for 12 h. The beads were washed and boiled in the SDS sample buffer. The eluted proteins were loaded on 12% SDS-PAGE and detected by western blotting. Aliquots of protein samples (30 µg) were resolved by 12% SDS-gel electrophoresis and transferred into nitrocellulose filters. The membranes were blocked in PBS, 0.2% Tween and 5% dry milk for 2 h, and then challenged with anti-rpL3 (Primm), anti-hnRNP H1, anti-α-tubulin, anti-SH-PTP1 (Santa Cruz Biotechnology), and anti-HA (Roche). The proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Pierce, Rockford, Illinois).

2.6. RNA pull-down assay

To identify proteins that bind specifically to the intron 3 transcript, we carried out an RNA pull-down assay using adipic acid dehydrazide beads. Briefly, 20 µg of intron 3 RNA, transcribed in vitro from pGEM4Z-Int3, was placed in a 400 µl reaction mixture containing 100 mM NaOAc, pH 5.2 and 5 mM sodium m-periodate (Sigma, St. Louis, Missouri), incubated for 1 h in the dark at room temperature, ethanol-precipitated, and resuspended in 100 µl of 100 mM NaOAc, pH 5.2. Then 300 µl of adipic acid dehydrazide agarose beads 50% slurry (Sigma) equilibrated in 100 mM NaOAc, pH 5.2 was added to this mixture, which was then incubated for 12 h at 4 ° C on a rotator. The beads with the bound RNA were pelletted, washed twice with 1 ml of 2 M NaCl, and equilibrated in washing buffer (5 mM Hepes, pH 7.9, 1 mM $MgCl_2$, and 0.8 mM magnesium acetate). The intron 3 RNA was then incubated with 6 mg of protein extract from HeLa cells for 30 min at room temperature in a final volume of 0.6 ml. Heparin was also added to a final concentration of 7 µg/µl. The beads were then washed four times in 1.5 ml of washing buffer. Bound proteins were



M kDa	Protein	Accession n°
130	RNA helicase A	Q08211
110	RNA Helicase Gu	Q9NR30
	E1B-AP	Q9BUJ2
	Nucleolin	P19338
50	**hnRNP H1**	**P31943**
	ZBTB4	Q9P1Z0
35	Nucleophosmin	P06748
30	NRG1	B0FYA9
	Histon cluster 1, H1c	A8K4I2
	RPS6	P62753
	RPS2	P15880
	SF2p32	Q07021
26	RPS8	P62241
22	RPS5	P46782
	RPS9	P46781
19	RPS11	P62280
	RPS17	P08708
17	RPS16	Q6IPX4
	RPSL22 proprotein	P35268
	RPS19	P39019
15	RPS15a	P62244
14	4DE4DIP	Q5VU43

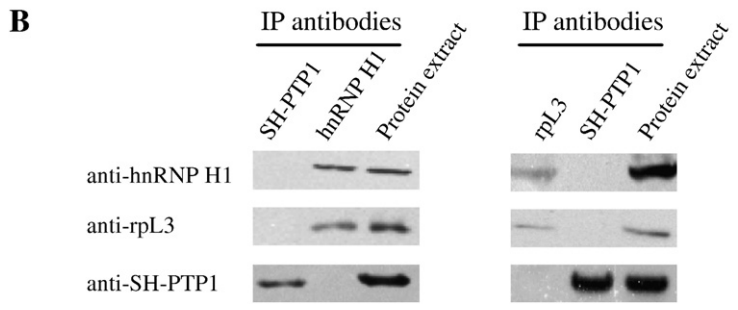


Fig. 1. In vitro and in vivo binding of hnRNP-H1 to rpL3. (A) In vitro binding of hnRNP H1 to rpL3. Coomassie Blue-stained gel of a GST pull-down experiment. The recombinant GST-rpL3 proteins and the GST control were incubated with nuclear extracts from Calu6 cells in the presence of glutathione-Sepharose beads. The eluted proteins were resolved by SDS-PAGE. M indicates the mobility of protein size markers. The arrows indicate the bands corresponding to GST-rpL3, hnRNP H1 and GST. The rpL3 partner proteins are listed. (B) In vivo binding of hnRNP H1 to rpL3. hnRNP H1 or rpL3 were immunoprecipitated from HeLa cells extracts with antibodies against hnRNP H1 and rpL3, respectively. Immunoprecipitates were separated by SDS-PAGE and immunoblotted for the proteins indicated at the left of the panel. These results are representative of three independently performed experiments.

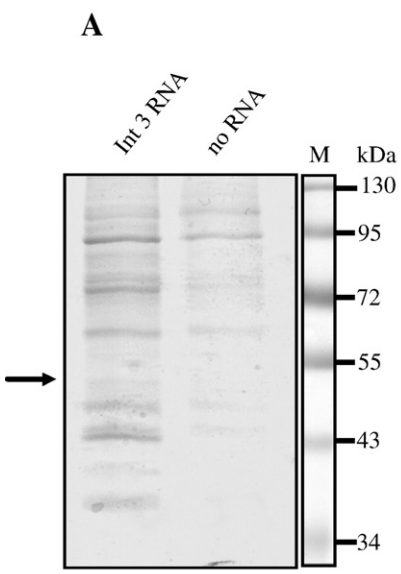
eluted in SDS sample buffer and loaded on a 12% gel for SDS-PAGE to proceed to a MS analysis (see 2.4).

2.7. RNP immunoprecipitation assay (RIPA)

For RNP immunoprecipitation assay, HeLa cells (2×10^6 cells) were lysed in 600 µl RIPA buffer 1× (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1 mM Na ortovanadate, 0.05 M NaF, and 0.5% NP40) with protease inhibitors (Roche) for 60 min on ice and then centrifuged at 10,000 ×*g* at 4 °C for 15 min. The supernatant was subjected to a preclearing step in which it was incubated with 50 µl of protein A/G plus agarose for 1 h at 4 °C. The precleared cell extracts were then incubated with antibodies specific for each protein (Santa Cruz) overnight at 4 °C. Protein G–Sepharose beads (50 µl of 50% slurry) were then added and the mix was incubated for 1 h at 4 °C with gentle shaking and centrifuged. The immunoprecipitates were suspended in 100 µl TES buffer (10 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, and 0.5% SDS), incubated at 65 °C for 10 min and centrifuged for 1 min at 10,000 ×*g*. Ten microliters was stored as immunoprecipitated samples and subsequently fractionated by SDS-PAGE to be analyzed by western blotting. RNA was extracted from 90 µl using the Trizol (Invitrogen) procedure.

2.8. RNA electromobility shift assay (REMSA)

RNA probes were transcribed in vitro using ^{32}P-NTPs and SP6 polymerase according to the manufacturer's instructions (Roche), and construct pGEM4Z-IntA, pGEM4Z-IntB, or PGEM4Z-IntC as template. The probes were purified using Sephadex G-25 columns (Roche). 5×10^5 counts/min of each RNA probe were incubated in binding buffer containing 20 mM Hepes, pH 7.9, 150 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton, 1 mg/ml tRNA, and 0.4 U/µl RNase Inhibitor (Roche) in the presence or absence of the recombinant protein GST-hnRNP H1 or GST for 1 h at 4 °C. The complexes formed were then resolved onto a 5% polyacrylamide (37.5:1 acrylamide:bis-acrylamide ratio) RNA native gel. The gel was dried at 80 °C for 30 min and the results visualized by autoradiography.



M kDa	Protein	Accession n°
130	SAP 130	Q15393
	Importin 4	Q8TEX9
120	Importin 7	O95373
	KIAA0079	P53992
110	Nucleolin	P19338
	Beta2-adaptin	P63010
	Importin beta-3	O00410
	RNA helicase Gu	Q9NR30
80	KHSRP	Q92945
77	moesin	P26038
70	FBP1	Q96AE4
	HnRNPM4	P52272
	nucleoporin 85	Q9BW27
65	Lamin A/C isoform 2	P02545
60	FBP3	Q96I24
55	Neuroleukin	P06744
	FBP2	Q92945
	PTBP1	Q9BUQ0
	Gps1	Q13098
50	**hnRNPH1**	**P31943**
	RuvB-like2	Q9Y230
47	MBP-1	P06733
	eIF3e	P60228
45	Musclebind-like protein 1	Q9NR56
40	SGN4	Q9BT78

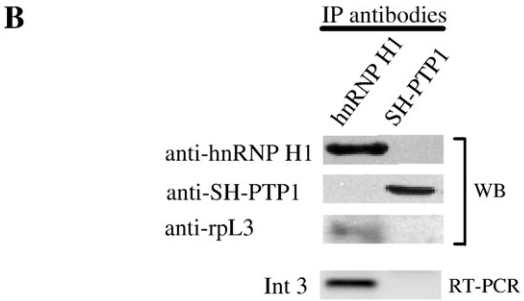


Fig. 2. Interaction of intron 3 of the rpL3 gene with hnRNP H1 in vitro and in vivo. (A) In vitro binding of the intron 3 transcript to hnRNP H1. Coomassie Blue-stained gel of a RNA pull-down experiment, using adipic acid dehydrazide agarose beads coated with intron 3 transcript was incubated with HeLa nuclear protein extract. The mobility of protein size markers is shown (M). The arrow indicates the protein band corresponding to hnRNP H1 identified by mass spectrometry analysis of eluted peptides. The intron 3 RNA interacting proteins identified are listed. (B) In vivo binding of the intron 3 transcript to hnRNP H1. Western blotting (WB) of hnRNP H1 or SH-PTP1 immunoprecipitates from HeLa cells, and RT-PCR of RNA extracted from the same immunoprecipitates by using primers against intron 3 (Table1). Note the absence of signal in SH-PTP1 immuno-complex. These results are representative of three independently performed experiments.

2.9. RT-PCR

For RT-PCR analysis, 1 μg of total RNA was reverse-transcribed into cDNA with the random hexamer technique using 200 U of Superscript II RNase H$^-$ Reverse Trancriptase (Invitrogen). The reaction was carried out at 42 °C for 50 min and was terminated by heating to 75 °C for 15 min. Ten of the 40 μl of reaction mix were PCR-amplified in a final volume of 50 μl, using 5 μM of each specific primer (Table 1), 10 mM dNTPs, and 0.5 U of Taq DNA polymerase (Invitrogen). Typically, 25–30 cycles of amplification were performed. In separate experiments, we ascertained that the cycle number was within the linear range of amplifications. PCR products were visualized on 1% agarose gel containing the fluorescent Vistra Green dye (Amersham Pharmacia Biotech). The labeling intensity of the PCR product, which is linear to the amount of DNA, was quantified using the PhosphorImager (Bio-Rad, Haercules, California).

3. Results

3.1. rpL3 interacts with hnRNP H1 in vitro and in vivo

An important issue concerning the autoregulation of rpL3 expression via AS–NMD coupling is to identify molecular partners of rpL3 involved in the splicing event. We previously found that rpL3 does not bind its pre-mRNA. Specifically, filter-binding experiments with the recombinant GST-rpL3 and GST alone as control showed that rpL3 could interact with intron 3 RNA as well as with unrelated RNA (data not shown). These findings favored the hypothesis that rpL3 modulates the splicing of its own gene by interacting with one or more regulatory proteins. Consequently, to search for rpL3 transacting factors, we carried out an in vitro GST pull-down assay. Recombinant GST-rpL3 and GST proteins were purified from *E. coli* cells, immobilized using GSH-Sepharose beads and incubated with a nuclear extract from Calu6 cells. The proteins specifically bound to GST-rpL3 and GST were pulled down, fractionated by SDS-PAGE and then visualized by Coomassie Blue staining.

The whole gel was cut in slices and protein bands, destained by repetitive washings, were excised and in situ-digested with trypsin. We also analyzed the control (proteins bound to GST) to check for nonspecific proteins. The resulting peptide mixtures were extracted from the gel and directly analyzed by LC/MS/MS to obtain sequence information on individual peptides. This information together with the peptide mass values was then used to search protein databases for corresponding proteins. The comparison of GST-rpL3 beads versus control GST beads revealed several specific proteins. Among these, sequence analysis by MS of an excised 50-kDa band yielded several peptides among which is hnRNP H1 (Fig. 1A). hnRNP H1 plays a major role in the regulation of alternative splicing of various genes. Recent studies showed that hnRNP H1 is able to bind specifically G runs on a transcript molecule [9–13,18].

To verify the interaction between rpL3 and hnRNP H1 in vivo, we performed co-immunoprecipitation experiments. hnRNP H1 (Fig. 1B,

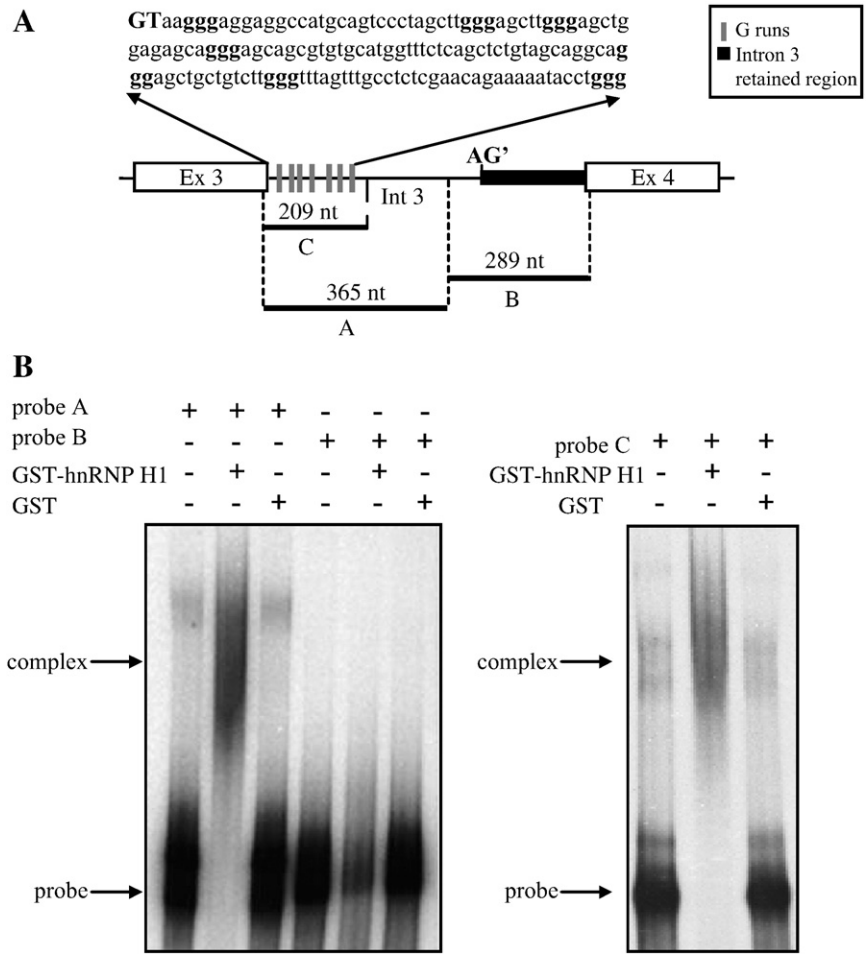


Fig. 3. hnRNP H1 binding to region A within intron 3 in vitro. (A) Schematic representation of regions A (365 nt), B (289 nt) and C (209 nt) of intron 3. The sequence of part of the region C of intron 3 including the G-run motifs is shown (boldface). (B) Gel mobility shift analysis of hnRNP H1 challenged with regions A, and B of intron 3 (left panel), or with region C of intron 3 (right panel). The results are representative of three independently performed experiments.

left panel) and rpL3 (Fig. 1B, right panel) were specifically immunoprecipitated from HeLa cell extracts and the immunoprecipitated complexes were tested by western blotting. A signal for both proteins appeared in immuno-complexes probed with antibodies against rpL3 and hnRNP H1, thus confirming that the two proteins specifically interact in vivo (Fig. 1B). A control immunoprecipitate obtained with an arbitrary antibody, anti-SH-PTP1, did not give any signal when probed with anti-hnRNP H1 or anti-rpL3 (Fig. 1B).

3.2. hnRNP H1 binds to the intron 3 of the rpL3 gene transcript in vitro and in vivo

We next performed an in vitro RNA pull-down assay to identify and isolate the RNA-binding proteins taking part in the alternative splicing event and, specifically, to determine whether hnRNP H1 was able to interact not only with rpL3 but also with its pre-mRNA. A transcript corresponding to the entire intron 3 of rpL3 pre-mRNA was used as bait for pull-down interacting proteins. The beads coated with the intron 3 transcript were incubated with a total protein extract from HeLa cells. After stringent washing, RNA-associated proteins were eluted and analyzed by SDS-PAGE. The comparison of the proteins bound to the intron 3 RNA-coated beads versus control beads with no RNA showed various specific bands (Fig. 2A). Each protein slice was cut and in situ-digested as previously described. Mass spectral analysis of the protein mixtures revealed several proteins among which is hnRNP H1.

To evaluate whether intron 3 and hnRNP H1 specifically interacted in vivo, we immunoprecipitated hnRNP H1 from HeLa cell extracts and looked for the intron 3 transcript and rpL3 in the RNA–protein immunoprecipitate complex (Fig. 2B). The specific primers, used in the RT-PCR to detect the intron 3 transcript were 18-mer oligonucleotides mapping 5 nt upstream from the 5′ splice donor site and 5 nt downstream from the 3′ splice acceptor site (Int3 in Table 1). We used western blot and anti-rpL3 antibodies to detect rpL3 in the immunocomplex. Amplification of the signal corresponding to the intron 3 transcript indicated that hnRNP H1 was able to bind rpL3 pre-mRNA in vivo. Moreover, the co-presence of rpL3 in the immuno-complex suggests that rpL3 pre-mRNA is present in a ribonucleoprotein complex that includes rpL3 and hnRNP H1, although the possibility of two independent complexes cannot be ruled out. The absence of

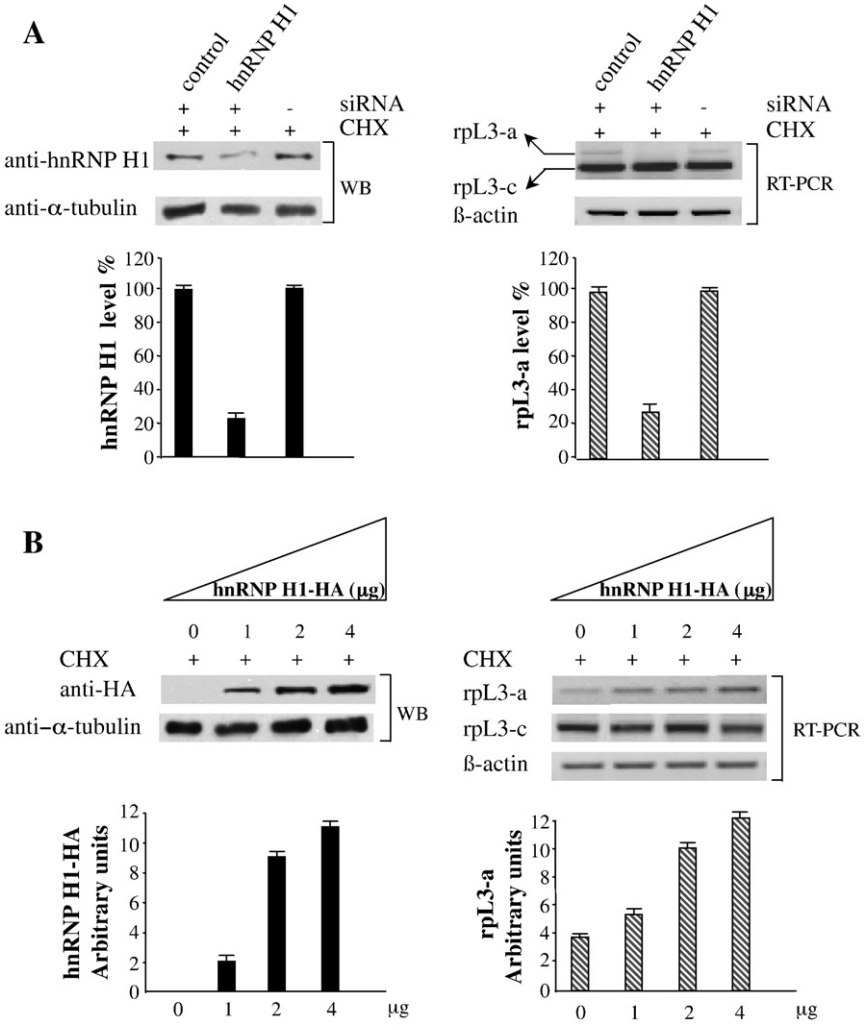


Fig. 4. (A) Effects of RNAi-mediated depletion of hnRNP H1 on rpL3-a mRNA levels. Left panel: protein samples from CHX-treated HeLa cells transfected with hnRNP H1-siRNA (hnRNP H1), with unrelated siRNA (control) or untransfected (−), were analyzed by western blotting (WB) with antibody specific for endogenous hnRNP H1. Loading in the gel lanes was controlled by detection of tubulin protein. Right panel: representative RT-PCR analysis of total RNA from the same samples. β-actin was used as a control of RNA loading. Quantification of hnRNP H1 protein and of the level of rpL3-a mRNA by PhosphorImager (Bio-Rad) is shown. (B) Effects of hnRNP H1 overexpression on rpL3-a mRNA levels. Left panel: protein overexpression detected with an antibody directed against the HA tag. Right panel: representative RT-PCR analysis of total RNA from CHX-treated HeLa cells untransfected or transfected with increasing amounts of hnRNP H1-HA. Quantification of hnRNP H1-HA protein and of the level of rpL3-a mRNA by PhosphorImager (Bio-Rad) is shown. These results are representative of three independently performed experiments.

signal in the immunoprecipitate with anti-SH-PTP1 confirmed the validity of this assay (Fig. 2B).

3.3. hnRNP H1 binds selectively to 5′ portion of intron 3

To identify the cis-acting elements of intron 3 that participate in the regulation of rpL3 pre-mRNA splicing, we used a computational analysis of this region to look for hnRNP H1-binding sequence motifs. The search revealed seven G-rich motifs that could be hnRNP H1-binding sites in the 5′ moiety on intron 3. Because of experimental evidence that hnRNP H1 activity in the splicing event is mediated by the binding of the protein to G runs, we performed REMSA experiments to assess whether the putative binding sites are necessary for the interaction of hnRNP H1 with intron 3 in the pre-mRNA transcript. We evaluated the hnRNP H1 binding of three intron 3 regions: region A spanning 365 nt downstream from the 5′ splice donor splice site containing seven G-run elements; region B (289 nt) constituted by the 3′ moiety of intron 3 and 27 nt of exon 4; and region C (209 nt), a more restricted region encompassing the seven G runs (Fig. 3A). The three regions were transcribed in vitro using ^{32}P-NTPs. The RNA probes were incubated with GST-hnRNP H1 or GST recombinant protein. The resulting RNA–protein complexes were analyzed on a native polyacrylamide gel and the results visualized by autoradiography.

As shown in Fig. 3, GST-hnRNP H1 was able to interact specifically with RNA transcribed on regions A (Fig. 3B, left panel) and C (Fig. 3B, right panel) of intron 3, but failed to do so when challenged with the B region transcript (Fig. 3B). No mobility shift was observed when recombinant GST was challenged with a transcript constituted by the three regions of intron 3 (Fig. 3B). These results indicate that hnRNP H1 binds in vitro to the region of the intron 3 transcript that contains seven G runs.

3.4. hnRNP H1 regulates the alternative splicing of rpL3 pre-mRNA

To determine the role played by hnRNP H1 in the alternative splicing of rpL3 primary transcript, we evaluated the effects of altered hnRNP H1 expression on the selection of the alternative 3′ acceptor splice site in intron 3 of the rpL3 gene. To this aim we switched-off the expression of the gene encoding hnRNP H1 by using RNA interference. siRNAs against hnRNP H1 or unrelated siRNAs were transiently transfected in HeLa cells. After transfection, cells were treated with CHX to stabilize the otherwise labile alternative splice form, and harvested; then RNA and protein were extracted. Lysates from cells transfected with unrelated siRNAs (control), specific siRNAs (hnRNP H1), or untransfected were probed with hnRNP H1 antibodies. As shown in Fig. 4A, the residual level of hnRNP H1 was about 30% of the protein detected in the control lysates. To examine the effects of the impaired production of hnRNP H1 on the splicing pattern of rpL3 pre-mRNA, we examined the rpL3 mRNA isoforms using RT-PCR. Removal of hnRNP H1 resulted in a decrease (about 70%) of the alternative mRNA (rpL3-a) level compared to controls.

To investigate the effects of hnRNP H1 overexpression we fused a cDNA encoding hnRNP H1 to an HA tag in the expression vector pcDNA3. Increasing amounts of this construct were transiently transfected in HeLa cells. 24 h after transfection, the production of hnRNP H1 was measured by western blotting (Fig. 4B). Total RNA was

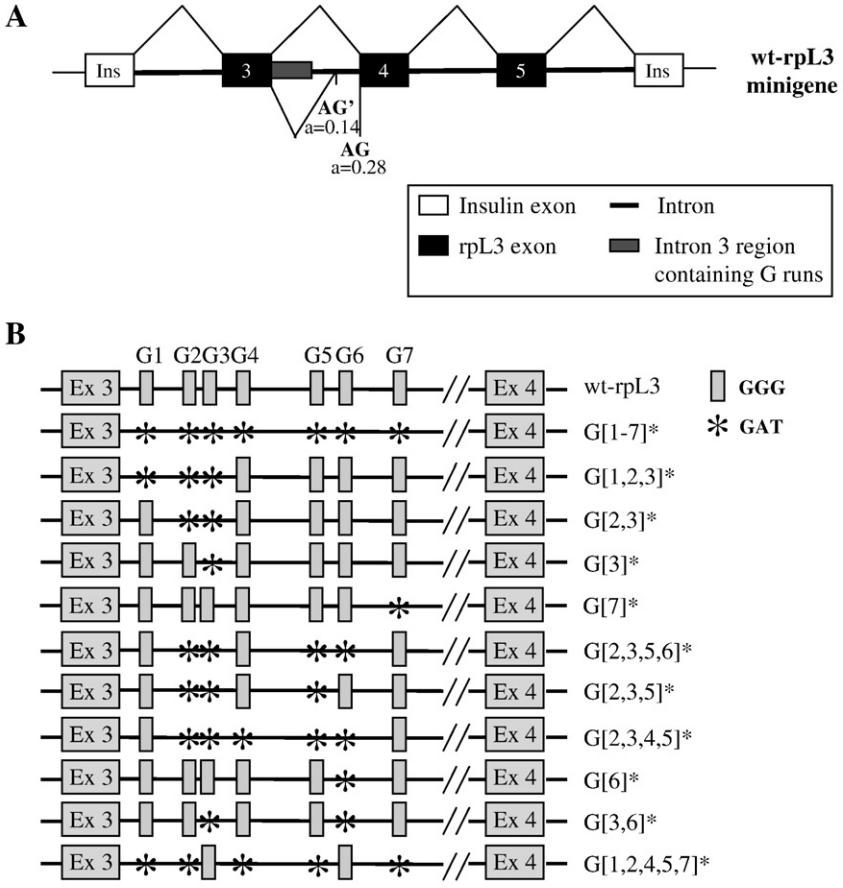


Fig. 5. (A) Schematic representation of wt-rpL3 minigene. The canonic acceptor site AG and the alternative acceptor site AG′ are indicated in boldface. The score of acceptor splice sites calculated using the Splice Site Prediction by Neural Network program (SSPNN, http://www.fruitfly.org/seq_tools/splice.html) is indicated. (B) Scheme of G-run mutants. The G runs within rpL3 intron 3 are numbered 1 to 7, and the G-run mutants are indicated by *.

analyzed by RT-PCR by using specific primers (Table 1) to amplify canonical (rpL3-c) or alternative (rpL3-a) isoforms of rpL3 mRNA. Overexpression of hnRNP H1 caused a dose-dependent increase in rpL3-a mRNA level (Fig. 4B).

3.5. G runs are involved in the control of 3′ splice site selection within rpL3 intron 3

To investigate whether guanosine stretches within intron 3 play a role in the control of splicing, we exploited a minigene containing a genomic region spanning from exon 3 to exon 5 (Fig. 5A, wt-rpL3 minigene) of the rpL3 gene. Using site-directed mutagenesis we changed GGG to GAT in all the G-run elements (G[1–7]* in Fig. 5B). After transfection of the constructs in HeLa cells, we carried out an RT-PCR analysis to determine the splicing pattern of the corresponding transcripts. Specific primers, designed on the insulin gene sequences flanking rpL3 gene sequences, were used to discriminate the minigene splicing products from the products derived from the endogenous gene (Table 1). As shown in Fig. 6A, the wt-rpL3 minigene reproduced the splicing pattern of the endogenous gene and hence it contained the information necessary for correct rpL3 splicing. Differently, pre-mRNA processing was greatly impaired in the G[1–7]* mutant, with a consequent decrease in the level of the alternative isoform of rpL3 mRNA to 20% versus the control wt-rpL3 minigene. These results show that the guanosine-rich elements within intron 3 are involved in the control of the 3′ splice site selection in rpL3 intron 3.

3.6. Functional mapping of the G motifs within intron 3

To evaluate the role of each G run in the regulation of the splicing of rpL3 pre-mRNA, we produced a series of wt-rpL3 minigene mutants. Mutation of elements G1, G2 and G3 (G[1–3]* in Fig. 5B) reduced the level of rpL3-a mRNA by 50% (Fig. 6A). The same result was obtained with mutants G[2,3]* and G[3]*. Consequently, the alteration of the splicing pattern in these mutants can be ascribed solely to disruption of the G3 element. Differently, the splicing pattern of the G[7]* mutant (Fig. 5B) shows that mutation of a single G run does not alter the level of rpL3-a mRNA. In another set of mutants, we evaluated the role of elements G3, G4, G5 and G6 in the splicing modulation of rpL3 intron 3. Mutants G[2,3,5]* and G[2–5]* yielded levels of rpL3-a mRNA similar to that of the G[2,3]* minigene mutant. On the contrary, the addition of the mutated G6 element in G[2,3,5,6]* reduced the splicing of rpL3-a mRNA to 20% of that of the control, as observed with mutant G[1–7]* (Fig. 6A). The results reported above strongly suggest that elements G3 and G6 are directly involved in the regulation of the splicing of rpL3 transcript intron 3.

To verify the role of G3 and G6 elements, we generated and analyzed another set of minigenes (see Fig. 5B): one with a mutation of element G6 (G[6]*), one with mutation of elements G3 and G6(G[3,6]*), and one

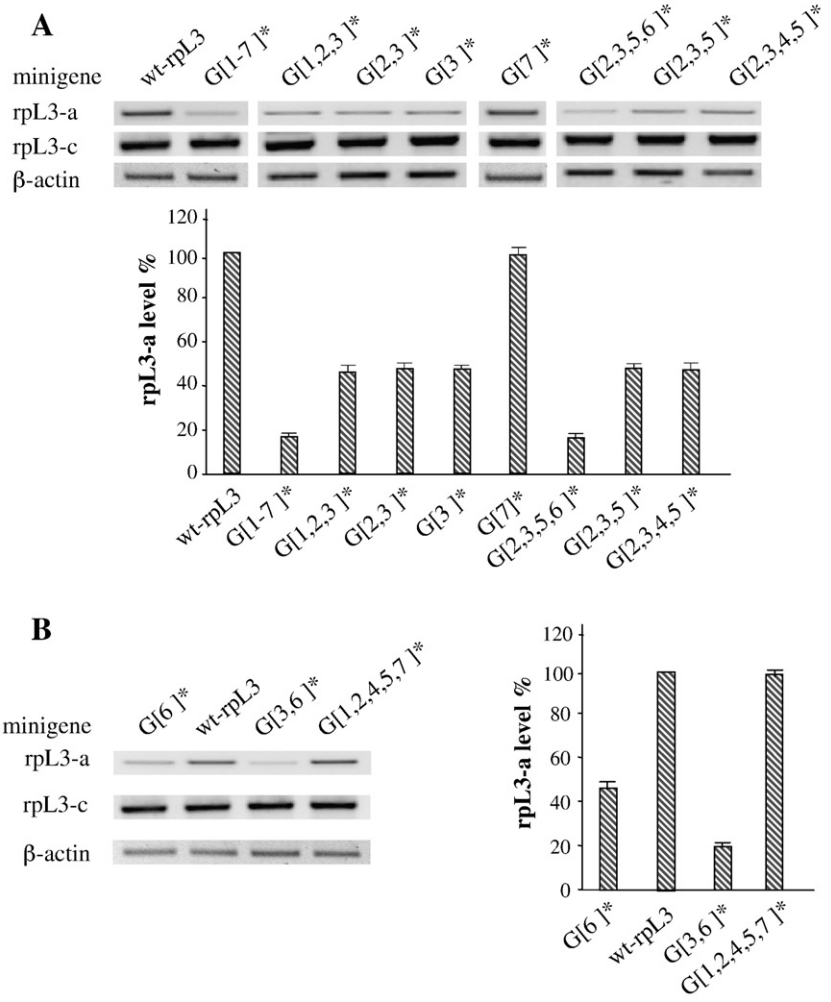


Fig. 6. (A and B) Functional mapping of the G-run elements within intron 3. Representative RT-PCR of total RNA extracted from CHX-treated HeLa cells transfected with the wt-rpL3 minigene or mutated constructs (Fig. 5) by using oligonucleotides that amplify minigene products (Table 1). β-actin was used as a control of RNA loading. Quantification of rpL3-a mRNA level using PhosphorImager (Bio-Rad) is shown.

with mutation of all G runs except elements G3 and G6 (G[1,2,4,5,7]*). Mutation of element G6 alone resulted in a splicing pattern in which the rpL3-a mRNA isoform was about 45% that of the control. A stronger effect (80% reduction of the rpL3-a mRNA isoform) was observed in the splicing pattern of the double mutant G[3,6]* (Fig. 6B); the level of the rpL3-a mRNA isoform was similar to the level obtained with mutant G [2,3,5,6]* or G[1–7]* (Fig. 6A). Thus, the G3 and G6 elements cooperate to control the selection of the alternative 3′ splice site in intron 3. Finally, mutant G[1,2,4,5,7]* (see Fig. 5B) had the same splicing pattern as the endogenous gene (Fig. 6B), thereby confirming that elements G3 and G6 play a relevant role in the selection of splicing modality of the rpL3 gene.

These data demonstrate that the G3 and G6 motifs cooperatively drive the recognition of the alternative 3′ splice site in intron 3, thereby leading to the inclusion of part of intron 3 in the rpL3-a mRNA. Furthermore, these results together with data from hnRNP H1 silencing provide strong evidence that the effects on the splicing pattern of rpL3 pre-mRNA could be mediated directly by the interaction of hnRNP H1 with G3 and G6 elements. To verify these findings, we overexpressed hnRNP H1 in HeLa cells transfected with the wt-rpL3, G[2,3,5,6]* or G[3,6]* minigenes under conditions that could lead to a larger than three-fold increase of the rpL3-a transcript level. Despite hnRNP H1 overexpression, the splicing pattern of the double mutant G[3,6]* showed that the level of alternative transcript was 60% lower than the level of the control wt-rpL3 minigene (Fig. 7). These data indicate that the G3 and G6 elements represent the consensus binding motifs of hnRNP H1 and are required for the hnRNP H1-mediated regulation of rpL3 pre-mRNA splicing.

4. Discussion

Ribosome biosynthesis requires the coordinated expression of the genes coding for the structural components, rRNA and r-proteins [24]. To maintain ribosome synthesis at the appropriate level, r-protein expression is regulated by multiple control mechanisms mostly at post-transcriptional and translational level. Moreover, r-proteins also exert a variety of extra-ribosomal functions for which additional and specific regulatory strategies are required [25–29]. In eukaryotes, several post-transcriptional regulation mechanisms have been described that control the level of rp-mRNA in the cell by modulating transcript processing, splicing, and stability. Autoregulation mechanisms appear to be a strategy by which each individual r-protein may control the level of its own expression [21,22,30–32].

We previously demonstrated that, as a consequence of a partial retention of intronic sequences, splicing of the human rpL3 transcript gives rise to a canonical mRNA and to an alternative mRNA isoform containing a PTC that is targeted to NMD. Moreover, our finding that rpL3 overexpression downregulates the level of the canonically spliced mRNA, and upregulates the production of the alternatively spliced isoform demonstrated that production of human rpL3 is regulated via a negative feedback loop [22]. Coupling of AS and NMD appears to be a tool by which to fine tune ribosomal protein levels. However, the molecular mechanism through which the process occurs is unknown.

In an attempt to shed light on this mechanism we looked for molecular partners of rpL3, and identified a number of proteins able to bind in vitro rpL3, the intron 3 of rpL3 gene, or both (Figs. 1 and 2). In vivo experiments confirmed that the splicing factor hnRNP H1 could bind rpL3 as well as its pre-mRNA, and REMSA experiments showed that this interaction occurs through a pre-mRNA region containing seven G-run elements (Fig. 3). In addition, depletion of hnRNP H1 by siRNA resulted in a significant reduction of alternative splicing of rpL3 pre-mRNA (Fig. 4A), whereas its overexpression resulted in increased production of the aberrant rpL3 mRNA (Fig. 4B), which indicates that hnRNP H1 overexpression was associated with a more efficient usage of the alternative 3′ acceptor site in intron 3.

G-run motifs are found preferentially in intronic sequences flanking a splicing site and appear to affect the recognition of an adjacent 5′ splice site or 3′ splice site of the intron. However, they can also function when located in exons [33].

Mutational analysis of the seven G-run elements in rpL3 intron 3 showed that G3 and G6 affected splicing (see Figs. 5B and 6). In fact, when both elements were mutated (G[3,6]* in Figs. 5B and 6B), the level of the alternative mRNA isoform was reduced by 80%, as occurred when all G-run motifs were mutated. When wild-type G3 and G6 were conserved and all other G runs mutated (see G[1,2,4,5,7]* in Figs. 5B and 6B), the amount of alternative rpL3 mRNA isoform was the same as the amount obtained from the wt-rpL3 primary transcript. Moreover, when G3 and G6 motifs were mutated, hnRNP H1 overexpression did not affect the level of the alternatively spliced mRNA isoform (Fig. 7). Given these results we concluded that G3 and G6 form a functional unit, and that its interaction with hnRNP H1 promotes the selection of a weak alternative 3′ splice site of intron 3. Cooperation between different G-rich motifs has been widely documented. More copies may increase the affinity of an associated factor and/or recruit more copies of an interacting factor [33]. For instance, multiple intronic G motifs regulate the splicing of the thrombopoietin gene through a complex combinatorial mechanism [11].

The molecular mechanism by which hnRNP H1 regulates AS of the rpL3 transcript remains to be clarified. Using proteomic analysis, we identified several proteins that interacted in vitro with rpL3 and with the pre-mRNA of the rpL3 gene (Figs. 1A and 2A). It is plausible that more complex protein–protein interactions between RNA-binding proteins, hnRNP H1 and rpL3 are involved in the regulation of rpL3 gene splicing. In this context, our results are consistent with a model in which the rpL3 protein directs the splicing reaction towards the alternative mode by specifically interacting with hnRNP H1 (Fig. 8).

In a splicing process leading to the exclusion of exon IIIC of the FGR2 gene, Fox2 recruits silencing factors and assists hnRNP H1 in binding an exonic splicing silencer thereby resulting in exon exclusion [34]. Regarding the rpL3 gene, the process may serve to prevent wasteful production of the protein. Binding to rpL3 could cause conformational changes in hnRNP H1 that favor interaction with the

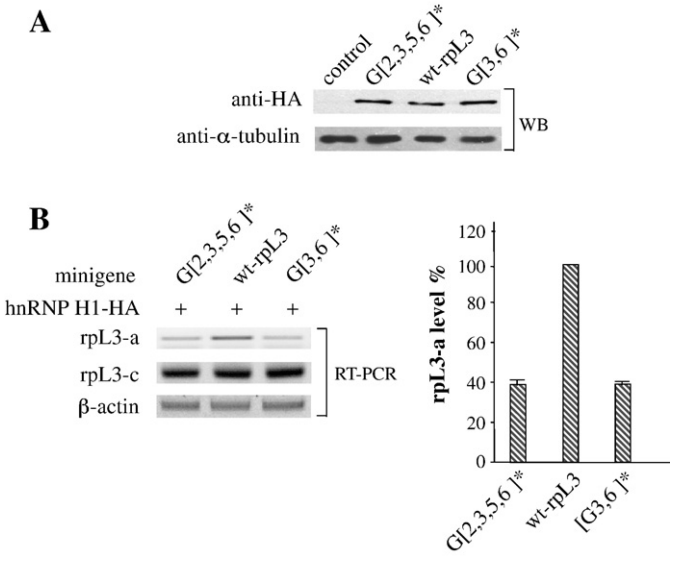


Fig. 7. hnRNP H1 regulates rpL3 pre-mRNA alternative splicing by binding to G3 and G6 elements. (A) hnRNP H1-HA was detected by western blotting (WB) using antibodies against the HA epitope. Loading in the lanes was controlled by detection of tubulin protein. (B) Representative RT-PCR of total RNA extracted from CHX-treated HeLa cells transfected with hnRNP H1-HA (4 μg, control) or co-transfected with G[2,3,5,6]* or G[3,6]* or the wt-rpL3 minigene (Fig. 5) and hnRNP H1-HA (4 μg). β-actin was used to control RNA loading. Quantification of rpL3-a mRNA level using PhosphorImager (Bio-Rad) is shown. These results are representative of three independently performed experiments.

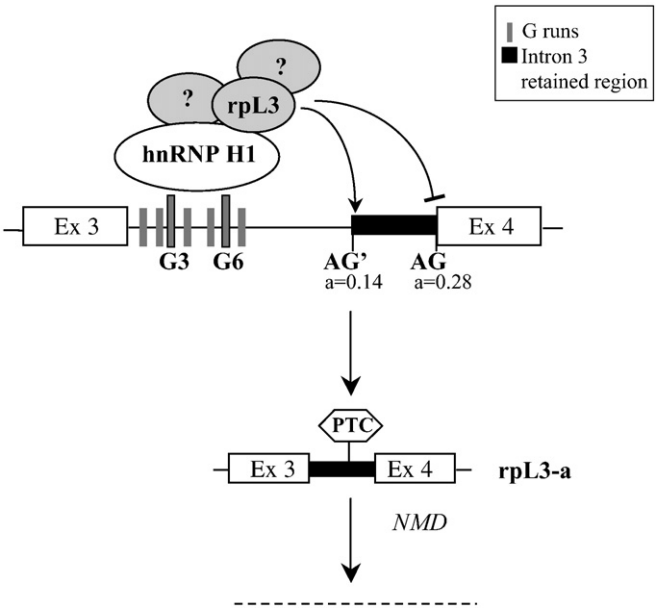


Fig. 8. Schematic representation of rpL3 feedback regulation mediated by hnRNP H1. rpL3 binds to hnRNP H1 and recruits it on intron 3. hnRNP H1 interacts with G3 + G6 units within intron 3, and, through interaction with other transacting proteins, an RNP complex promotes the selection of the weak 3′ acceptor site in intron 3.

alternative splicing enhancer unit G3 + G6 within intron 3, thereby allowing correct positioning of the spliceosome, and recruitment of additional protein factors. Ultimately, such a complex could favor the selection of the alternative 3′ splice site.

The ongoing analysis of protein components of the RNP complex involved in the selection of the alternative 3′ splice site will help to elucidate the fine autoregulatory network of rpL3 expression.

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Autoregulatory circuit of human rpL3 expression requires hnRNP H1, NPM and KHSRP

Annapina Russo[1,2], Morena Catillo[1], Davide Esposito[1], Paola Briata[3], Concetta Pietropaolo[1] and Giulia Russo[1,*]

[1]Dipartimento di Biochimica e Biotecnologie Mediche, Università Federico II, Via Sergio Pansini 5, Napoli 80131, [2]CEINGE Biotecnologie Avanzate S.C.a.r.l., Via Gaetano Salvatore 482, Napoli 80145 and [3]Istituto Nazionale per la Ricerca sul Cancro, Largo Rosanna Benzi 10, Genova 16132, Italy

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ABSTRACT

Alternative pre-mRNA splicing (AS) is a major mechanism that allows proteomic variability in eukaryotic cells. However, many AS events result in mRNAs containing a premature termination codon, which are degraded by nonsense-mediated mRNA decay (NMD) pathway. We have previously demonstrated that human rpL3 autoregulates its expression through the association of AS with NMD. In fact, overexpression of rpL3 promotes downregulation of canonical splicing and upregulation of alternative splicing that produces an NMD-targeted mRNA isoform. The result of these events is a decreased production of rpL3. We have also identified heterogeneous nuclear ribonucleoprotein (hnRNP) H1 as a splicing factor involved in the regulation of rpL3 alternative splicing and identified its regulatory *cis*-elements within intron 3 transcript. Here, we report that NPM and KHSRP are two newly identified proteins involved in the regulation of rpL3 gene expression via AS-NMD. We demonstrate that hnRNP H1, KHSRP and NPM can be found associated, and present also in ribonucleoproteins (RNPs) including rpL3 and intron 3 RNA *in vivo*, and describe protein–protein and RNA–protein interactions. Moreover, our data provide an insight on the crucial role of hnRNP H1 in the regulation of the alternative splicing of the rpL3 gene.

INTRODUCTION

Alternative splicing (AS) is an important mechanism of gene expression control, through which an individual gene gives rise to different mRNAs encoding distinct proteins, thus allowing functional and genetic variability (1). In addition to the canonical splice elements, auxiliary non-splice site RNA sequences have been identified and are located in both introns and exons. These additional elements are needed for a proper splicing and regulation of the process. They may function as enhancers or silencers of the splicing reaction, and may influence recognition and usage of the splice sites by the splicing apparatus through the binding of specific regulatory proteins (2). These proteins include members of the serine–arginine-rich (SR) proteins, the heterogeneous nuclear ribonucleoprotein (hnRNP) family and other RNA binding proteins (3). The SR proteins represent a family of highly conserved *trans*-acting factors that usually induce splicing, whereas the hnRNP proteins can affect the splicing depending on the interacting cis-elements and on the gene context (4,5). Moreover, in some case, the result of a splicing reaction is determined by the antagonistic action of hnRNP and SR proteins since they are able to recognize and bind to a composite regulatory element generated by the overlapping of enhancer and silencer elements (6).

AS can also control gene expression quantitatively by generating unproductive mRNAs that are targeted for degradation by nonsense-mediated decay (NMD) (7). NMD is a surveillance pathway that detects and selectively degrades aberrant mRNAs harboring a premature termination codon (PTC), thus preventing the production of truncated polypeptides potentially deleterious to the cell. NMD is highly conserved in eukaryotes; many studies have dissected machinery components, and the issue of its localization in a cell compartment has been thoroughly discussed (8,9 and references therein). The process regulating gene expression through AS-NMD association has been defined Regulated Unproductive Splicing and Translation (RUST) (10).

Although the mechanisms of the AS have been extensively studied, the complex regulation of such process is still an issue. The complexity of the regulation pathways is due to an intricate and dynamic network of protein–protein and RNA–protein interactions. Changes in the

*To whom correspondence should be addressed. Tel: +39 081 7463061; Fax: +39 081 7463074; Email: giulia.russo@unina.it

activity, number and association of splicing factors in the network can modify the selection of the splice site and the activity of the splicing machinery, and therefore the splicing pattern of the target pre-mRNA.

The regulation of pre-mRNA splicing plays an important role in human pathologies (11). Impairment of the AS is closely related to some disease mechanisms as in the case of spinal muscular atrophy and myotonic distrophy. Recently, new therapies aimed to correct defects arising in pre-mRNA splicing, the so-called 'splice-correction' or 'splice-modulation' therapies, have been developed (12).

The accuracy of the AS depends on the stoichiometry and interactions of positive and negative regulatory proteins. Consequently, the identification of the proteins participating in the modulation of the AS is an essential step to study the many aspects of gene expression.

Data from several laboratories demonstrate that some alternative isoforms of mRNA-encoding ribosomal proteins (rp) are NMD substrates. In *Caenorhabditis elegans*, it has been shown that the AS of genes rpL3, rpL12, rpL10 and rpL7 gives rise to aberrant mRNA isoforms, which contain a PTC resulting from an incomplete intron removal, and are natural substrate of NMD (13). The AS event appears conserved in humans and in other mammals on rpL3 and rpL12 genes. In fact, we have previously demonstrated that human rpL3 gene transcript gives rise to a canonical mRNA and to an alternative mRNA isoform containing a PTC targeted to decay by NMD. rpL3 is able to modulate its own production via a negative feedback loop. In fact, rpL3 overexpression results in a decreased level of the canonically spliced mRNA, and an increased production of the alternatively spliced isoform (14). We have also identified the hnRNP H1, as a transacting factor able to interact *in vitro* and *in vivo* with rpL3 and with intron 3 transcript of the rpL3 gene. Our data demonstrated that hnRNP H1 is involved in promoting the AS of human rpL3 pre-mRNA. In addition, we have identified and characterized the *cis*-acting regulatory elements, G runs, involved in hnRNP H1-mediated regulation of splicing (15). In the present study, we analyze the role of hnRNP H1 in the rpL3 autoregulatory loop, and we report the identification of two new regulatory proteins, KHSRP (K-homology splicing regulatory protein, also known as KSRP) and NPM (Nucleophosmin, also known as B23, numatrin or NO38), which exhibit opposite effects on the splicing reaction of rpL3 pre-mRNA. Our data contribute to shed light on protein–protein and RNA–protein interactions within putative RNP complexes involved in the modulation of splicing of the rpL3 gene.

MATERIALS AND METHODS

Cell cultures, transfections and drug treatment

Human cell line HeLa was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin–streptomycin 50 U/ml.

L3–8 cell line, derived from rat PC12 Tet-Off cell line and conditionally overexpressing rpL3 upon doxycyclin removal (14), was grown in DMEM supplemented with 5% fetal calf serum (FCS), 10% horse serum, 2 mM L-glutamine and 100 μg/ml G418 (Invitrogen), hygromycin 200 μg/ml (USB, Santa Clara, CA, USA) and doxycyclin 10 ng/ml (Sigma, St Louis, MO, USA). The expression of HA-rpL3 was induced upon removal of doxycyclin. siRNA transfections were performed in HeLa cells (1×10^6 cells, 6 mm well plate) at a concentration of 150 nM by using Oligofectamine Reagent (Invitrogen) according to the manufacturer's instructions.

Plasmids were transfected in L3–8 cell line or in HeLa cells (2.5×10^6 cells, 6 mm well plate) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after DNA or siRNA transfections, cells were treated with 100 μg/ml cycloheximide (CHX) for 4 h to block NMD. Then, RNA and proteins were extracted by using the Trizol procedure (Invitrogen) for RT–PCR analysis and western blot, respectively. Transfection efficiency was assessed by cotransfecting a GFP-expressing vector and normalizing RNA levels against GFP mRNA levels (data not shown).

DNA constructs and production of recombinant proteins

The cDNA of NPM was obtained by RT–PCR from HeLa cells using the primers 5′-ATGGAAGATTCGATGGAC-3′ (forward) and 5′-TTAAAGAGACTTCCTCCA-3′ (reverse), and cloned into a version of the eukaryotic expression vector pcDNA4/HisMax C (Invitrogen) containing the HA epitope, into the prokaryotic expression vector pRSET-A (Invitrogen) containing the Histidine tag, and in the prokaryotic expression vector pGEX4T3 (GE Healthcare, Waukesha, WI, USA). The cDNA of hnRNP H1 was obtained by RT–PCR from HeLa cells using the primers 5′-ATGATGTTGGGCACGGAA-3′ (forward) and 5′-CTATGCAATGTTTGATTGAAAA-3′ (reverse), and cloned into the prokaryotic expression vector pRSET-A. The plasmids encoding GST-hnRNP H1, GST-rpL3, HA-hnRNP H1, Flag-KHSRP and pGEM4Z-Int3 were already available (15,16).

The recombinant proteins GST-hnRNP H1, GST-rpL3, GST-rpL7a and GST were expressed in *Escherichia coli* and purified by using glutathione Sepharose 4B beads according to the manufacturer's instructions (GE Healthcare). The recombinant proteins His-NPM, His-hnRNP H1 and His-rpL7a were expressed in *E. coli* and purified by the nickel–nitrilotriacetic acid (Ni–NTA)-Agarose chromatography according to the manufacturer's instructions (Qiagen, Valencia, California). His-tagged KHSRP was expressed in Sf9 cells using the Baculovirus system (Baculogold, BD Biosciences) and purified by Ni–NTA-Agarose chromatography (16).

RNA interference

The target sequences of small interfering RNAs (siRNA) in hnRNP H1 were: 5′-GGAAATAGCTGAAAAGGCT-3′ and 5′-CCACGAAAGCTTATGGCCA-3′ (Ambion, Foster City, CA, USA). The siRNAs targeting NPM and KHSRP were purchased from Santa Cruz

Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-29771, sc-44831).

GST pull down

For GST pull-down assay, 50 μg of the fusion protein or GST control, as bait were immobilized on glutathione-Sepharose beads and incubated with 20 μg of the recombinant protein of interest in pull-down buffer (50 mM Tris–HCl, pH 7.5, 0.4 mM EDTA, 150 mM NaCl, 10% glicerol, 1% NP-40, 1 mM sodium-ortovanadate, 50 mM NaF, 5 mM DTT and Protease Inhibitor Mix 1X) at 4°C for 1.5 h. The beads were washed extensively and boiled in SDS sample buffer. The eluted proteins were loaded on 12% SDS–PAGE and analyzed by western blotting.

Immunoprecipitation and western blotting

For immunoprecipitation assay, 1 mg of HeLa whole-cell lysate was incubated with 30 μl of protein A/G agarose beads coated with 5 μg of anti-NPM or anti-KHSRP (Santa Cruz Biotechnology sc-47725, sc-33031) at 4°C for 12 h. The beads were washed and boiled in the SDS sample buffer. The eluted proteins were loaded on 12% SDS–PAGE and detected by western blotting. Aliquots of protein samples (30 μg) were resolved by 12% SDS–gel electrophoresis and transferred into nitrocellulose filters. The membranes were blocked in PBS, 0.1% Triton and 5% dry milk for 2 h, and then challenged with anti-NPM, anti-KHSRP, anti-hnRNP H1, anti-HA, anti-Flag (Santa Cruz Biotechnology sc-10042, sc-57592 and sc-807) and anti-rpL3 (Primm, Milan, Italy). The proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

RNA pull-down assay

RNA pull-down assay was carried out by using adipic acid dehydrazide beads. Briefly, 20 μg of intron 3 RNA, transcribed *in vitro* from pGEM4Z-Int3, were placed in a 400 μl reaction mixture containing 100 mM NaOAc pH 5.2 and 5 mM sodium m-periodate (Sigma), incubated for 1 h in the dark at room temperature, ethanol precipitated and resuspended in 100 μl of 100 mM NaOAc, pH 5.2. Then, 300 μl of adipic acid dehydrazide agarose beads 50% slurry (Sigma) equilibrated in 100 mM NaOAc pH 5.2 were added to this mixture, which was then incubated for 12 h at 4°C on a rotator. The beads with the bound RNA were pelletted, washed twice with 1 ml of 2 M NaCl and equilibrated in washing buffer (5 mM HEPES pH 7.9, 1 mM $MgCl_2$, 0.8 mM magnesium acetate). The intron 3 RNA was then incubated with 50 μg of each recombinant protein for 30 min at room temperature in a final volume of 0.6 ml. The beads were then washed four times in 1.5 ml of washing buffer. Bound proteins were eluted in SDS sample buffer loaded on a 12% gel for SDS–PAGE and analyzed by western blotting.

RNP immunoprecipitation assay

For RNP immunoprecipitation assay (RIPA), HeLa cells (2×10^6 cells) were lysed in 600 μl RIPA buffer 1× (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1 mM Na ortovanadate, 0.05 M NaF, 0.5% NP-40) with protease inhibitors mix 1× (Roche, Basel, Switzerland) for 60 min on ice, and then centrifuged at 10 000 *g* at 4°C for 15 min. The supernatant was subjected to a pre-clearing step in which it was incubated with 50 μl of protein A/G plus agarose for 1 h at 4°C. The pre-cleared cell extracts were then incubated with antibodies specific for each protein (Santa Cruz Biotechnology) overnight at 4°C. Protein A/G plus agarose beads (50 μl of 50% slurry) were then added and the mix was incubated for 1 h at 4°C with gentle shaking and centrifuged. The immunoprecipitates were suspended in 100 μl TES buffer (10 mM Tris–HCl pH 7.5, 0.5 mM EDTA, 0.5% SDS), incubated at 65°C for 10 min and centrifuged for 1 min at 10 000*g*. Ten microliters of the supernatant were stored as immunoprecipitated samples and subsequently fractionated by SDS–PAGE to be analyzed by western blotting. RNA was extracted from 90 μl using the Trizol procedure (Invitrogen).

RT–PCR

For RT–PCR analysis, 1 μg of total RNA was reverse transcribed into cDNA with the random hexamers technique using 200 U of Superscript II RNAse H^- Reverse Trancriptase (Invitrogen). The reaction was carried out at 42°C for 50 min and was terminated by heating to 75°C for 15 min. Ten of the 40 μl of reaction mix were PCR amplified in a final volume of 50 μl, using 5 μM of each specific primer, 10 mM dNTPs and 0.5 U of Taq DNA polymerase (Invitrogen). Typically, 25–30 cycles of amplification were performed. The primers were: rpL3-a 5′-CT CCGCTGGGCTCTGCCC-3′ (forward) and 5′-CTTCAG GAGCAGAGCAGA-3′ (reverse); rpL3-c 5′-GGGCATT GTGGGCTACGT 3′ (forward) and 5′-GTAAAGGCCT TCTTCTTAG-3′ (reverse); and β-actin 5′-GGCACCACC TTCTACA-3′ (forward) and 5′-CAGGAGGACAATGA T-3′ (reverse).

In separate experiments, we ascertained that the cycle number was within the linear range of amplifications. PCR products were visualized on 1% agarose gel containing the fluorescent Vistra Green dye (15). The labeling intensity of the PCR product, which is linear to the amount of DNA, was quantified using the PhosphorImager (Bio-Rad, Haercules, CA, USA).

RESULTS

Interactions between NPM, KHSRP, hnRNP H1 and rpL3 *in vivo* and *in vitro*

We have identified, by a proteomic analysis in a previous study, proteins associated to rpL3 and/or to intron 3 transcript of the rpL3 gene, and we focused our studies on proteins involved in RNA processing as putative components of a RNP complex including rpL3 and mediating

alternative splicing of the rpL3 pre-mRNA. Mass spectrometry results from GST pull-down experiments using GST-rpL3 as bait demonstrated that NPM is one of the proteins able to interact with rpL3 *in vitro*. Conversely, KHSRP was not found associated to rpL3. However, data obtained from RNA pull-down experiments indicate that KHSRP is able to interact with the intron 3 transcript of the rpL3 gene (15). Thus, we asked whether NPM and KHSRP could be part of a RNP complex including rpL3 through a direct or indirect interaction *in vivo*, by performing co-immunoprecipitation assays.

Figure 1 shows the results of the experiments in which NPM and KHSRP were specifically immunoprecipitated from HeLa cells extracts by using antibodies against the endogenous proteins. Immunoprecipitated proteins were separated by SDS–PAGE and the presence of NPM and KHSRP was investigated by western blotting in the reciprocally immunoprecipitated complexes. The results of these experiments showed that NPM and KHSRP were coimmunoprecipitated, thus indicating that they can associate *in vivo*. Furthermore, a specific signal for rpL3 appeared both in NPM as well as in KHSRP immunocomplexes. These data suggest that rpL3 is able to interact *in vivo* with both proteins. Since we have previously demonstrated that hnRNP H1 is found included in a complex with rpL3 and is involved in the regulation of rpL3 gene alternative splicing (15), we looked for hnRNP H1 in NPM and KHSRP immunoprecipitated complexes. The presence of signal for hnRNP H1 in both immunoprecipitates was consistent with a specific association between NPM, KHSRP and hnRNP H1. A control immunoprecipitate obtained with anti-IgG antibodies did not give any signal, when probed with anti-NPM, anti-KHSRP, anti-rpL3 or anti-hnRNP H1 (Figure 1); for a further control, see Supplementary Figure S1. In the light of these results, it is plausible to hypothesize that NPM, KHSRP, hnRNP H1 and rpL3 are associated in a single quaternary complex; alternatively, NPM and KHSRP could be part of independent protein complexes, including or not rpL3, to which they are recruited through different combination of protein–protein interactions.

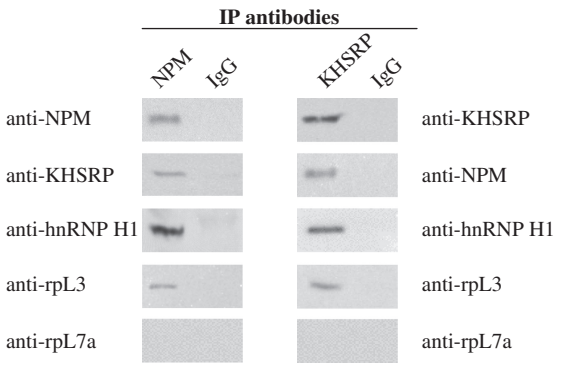

Figure 1. *In vivo* binding of NPM, KHSRP, hnRNP H1 and rpL3. NPM or KHSRP were specifically immunoprecipitated from HeLa cells extracts with antibodies against the endogenous NPM and KHSRP. Immunoprecipitates were separated by SDS–PAGE and immunoblotted with antibodies versus the indicated proteins. Note the absence of signal in IgG immunocomplex. Results illustrated in Figures 1–6 are representative of three independently performed experiments.

In an attempt to define a RNP complex including rpL3, we investigated protein–protein interactions that rpL3 protein partners may establish within the complex. To this aim, we performed GST pull-down assays by using purified recombinant proteins. His-tagged proteins were tested for their ability to bind to immobilized GST-tagged proteins. GST-tagged proteins, and GST as control, were immobilized using GSH-sepharose beads and incubated with purified His-tagged proteins. Western blot analysis was performed on each pull-down sample. Figure 2 shows that His-hnRNP H1 was present in the pull-down preparation of GST-NPM, but not in those of GST-rpL3 or GST; His-NPM was detected in the pull-down preparations of GST-rpL3, GST-hnRNP H1, but not in that of GST; and His-KHSRP was revealed in the pull-down preparations of GST-rpL3 and GST-hnRNP H1, but not in those of GST-NPM or GST. These results indicate that although a direct interaction between rpL3 and hnRNP H1 has not been observed, rpL3 and hnRNP H1 are able to interact directly with NPM and KHSRP, while NPM and KHSRP are not able to interact directly in the *in vitro* assay. No interaction whatsoever was detected with GST-rpL7a used as a further control (data not shown).

Interactions between KHSRP, NPM, hnRNP H1 and the intron 3 transcript *in vivo* and *in vitro*

Mass spectral analysis of our previous RNA pull-down experiments revealed that KHSRP was able to interact with intron 3 transcript of rpL3 gene *in vitro* (15). To confirm this interaction *in vivo*, we performed RNA affinity immunoprecipitation experiments. We specifically immunoprecipitated KHSRP, and hnRNP H1 as control, from HeLa cell extracts by using monoclonal antibodies against the endogenous proteins and searched for the intron 3 transcript in the RNA–protein immunoprecipitate complex (Figure 3A). Amplification of the signal corresponding to the intron 3 of rpL3 transcript, but not of rpL7a gene transcript, by RT–PCR indicated that KHSRP was able to bind with rpL3 pre-mRNA. The absence of signal in the immunoprecipitate with anti-IgG confirmed the validity of this assay.

Although NPM was not found associated to the intron 3 transcript *in vitro* previously (15), we wondered whether this interaction can occur *in vivo*. To this aim, we specifically immunoprecipitated NPM from HeLa cell extracts by using monoclonal antibodies against the endogenous NPM. Analysis of RNA extracted from the immunoprecipitate complex demonstrates that NPM is able to precipitate the intron 3 transcript (Figure 3A).

Next, in order to establish whether one or more among the identified proteins was able to interact directly with intron 3 RNA, or whether additional factors were required, we performed RNA pull-down experiments by

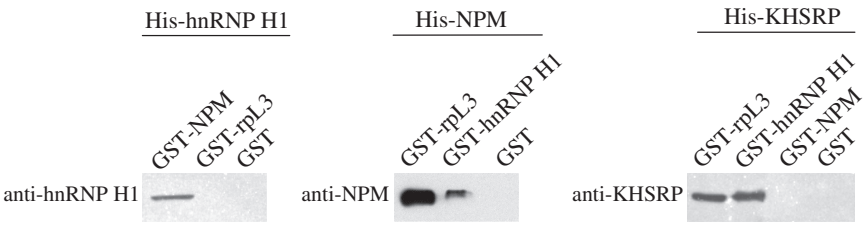

Figure 2. Analysis of the interactions between hnRNP H1, NPM, KHSRP and rpL3. Western blotting (WB) of GST pull-down experiments. Fifty microgram of GST-tagged proteins (GST-NPM, GST-rpL3 and GST-hnRNP H1) or GST (control) were immobilized on glutathione-sepharose beads and incubated with 20 µg of His-hnRNP H1, His-NPM or His-KHSRP. The eluted proteins were then analyzed by immunoblot with antibodies anti-hnRNP H1, anti-NPM and anti-KHSRP. Note the absence of signal in control GST pull-down preparations.

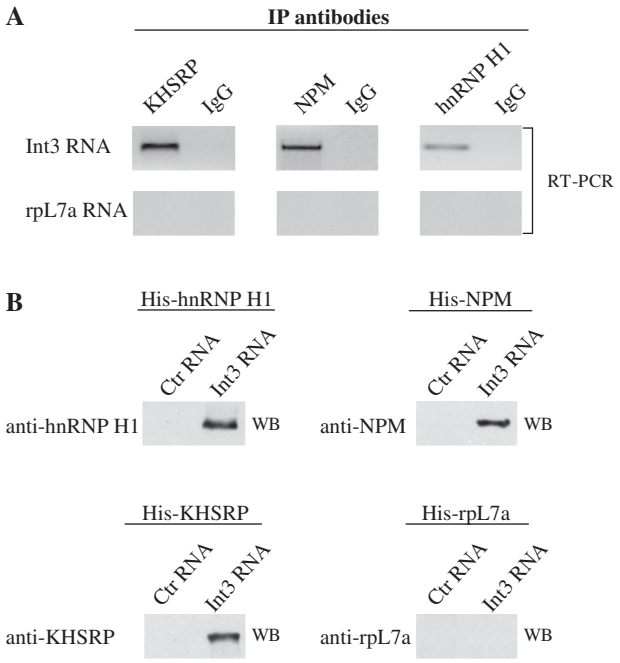

Figure 3. Analysis of the interactions of intron 3 of rpL3 pre-mRNA with NPM and KHSRP, *in vivo* and *in vitro*. (**A**) *In vivo* binding of intron 3 transcript to NPM and KHSRP. RT–PCR analysis, by using primers against rpL3 intron 3 transcript and rpL7a RNA transcript, of RNA extracted from the NPM, KHSRP, hnRNP H1 (control) and IgG immunocomplexes. Note the absence of signal in IgG immunocomplex. (**B**) *In vitro* binding of intron 3 transcript to hnRNP H1, NPM, KHSRP and rpL7a. WB of RNA pull-down experiments, using adipic acid dehydrazide agarose beads coated with intron 3 transcript or unrelated RNA (Ctr RNA) incubated with purified proteins His-hnRNP H1, His-NPM, His-KHSRP and His-rpL7a.

using purified recombinant proteins. For this purpose, a transcript corresponding to the entire intron 3 of the rpL3 gene was used as bait and incubated with the purified recombinant proteins His-hnRNP H1 (control), His-NPM or His-KHSRP and His-rpL7a as a control RNA binding protein (17). Then, RNA-associated proteins were eluted and analyzed by western blotting with antibodies against the endogenous hnRNP H1, NPM, KHSRP and rpL7a. The presence of signals specific for NPM and KHSRP indicated that these two proteins are able to recognize and bind to sequences in the rpL3 intron 3 transcript (Figure 3B).

KHSRP regulates the alternative splicing of rpL3 pre-mRNA

The identification of a specific interaction of KHSRP with rpL3 and intron 3 transcript prompted us to investigate a possible role of KHSRP in the rpL3 gene splicing. To this aim, we analyzed the effects of the alteration in the expression levels of KHSRP on the rpL3 RNA splicing pattern. Increasing amounts of a DNA construct expressing Flag-KHSRP were transiently transfected in HeLa cells. Twenty-four hours after transfection, cells were treated with CHX to stabilize the alternatively spliced isoform of rpL3 mRNA and lysed. Cells extracts were tested for the detection of Flag-KHSRP levels by western blotting with anti-Flag antibodies (Supplementary Figure S2A). Total RNA from the same cell extract was analyzed by RT–PCR using specific primers to amplify canonical (rpL3-c) or alternative (rpL3-a) isoform of rpL3 mRNA. We observed an increase in the rpL3-a mRNA level correlated, in a dose-dependent mode, with increasing amount of KHSRP (Figure 4A). These data indicate that KHSRP positively affects the selection of the 3′-cryptic splicing site within the intron 3 transcript of rpL3 gene. Thus, we investigated the possibility that KHSRP could act as a component of rpL3 autoregulatory loop. We used L3–8 cells, a PC12 Tet-Off cell line stably transfected with a vector containing the human rpL3-coding sequence fused to the hemagglutinin (HA) epitope-coding sequence (14). L3–8 cells were transiently transfected with the DNA construct expressing Flag-KHSRP. We chose a dose of Flag-KHSRP that would result in about a 50% increase of the rpL3 alternative isoform mRNA. Twenty-four hours after transfection, we treated cells with CHX and induced the expression of HA-rpL3 by removing doxycycline from medium. Cells were then lysed and analyzed for the production of Flag-KHSRP and HA-rpL3 by western blotting using anti-Flag and anti-HA antibodies, respectively (Supplementary Figure S2B). Total RNA was analyzed by RT–PCR using specific primers to amplify rpL3-c or rpL3-a mRNAs (Figure 4B). As previously demonstrated the expression of the HA-rpL3 protein resulted in an increase of the alternative isoform mRNA (14). The expression of exogenous KHSRP also caused an increase in rpL3-a mRNA level. Of interest, the increase in the rpL3-a mRNA caused by overexpression of KHSRP was greater after the induction of HA-rpL3 (Figure 4B).

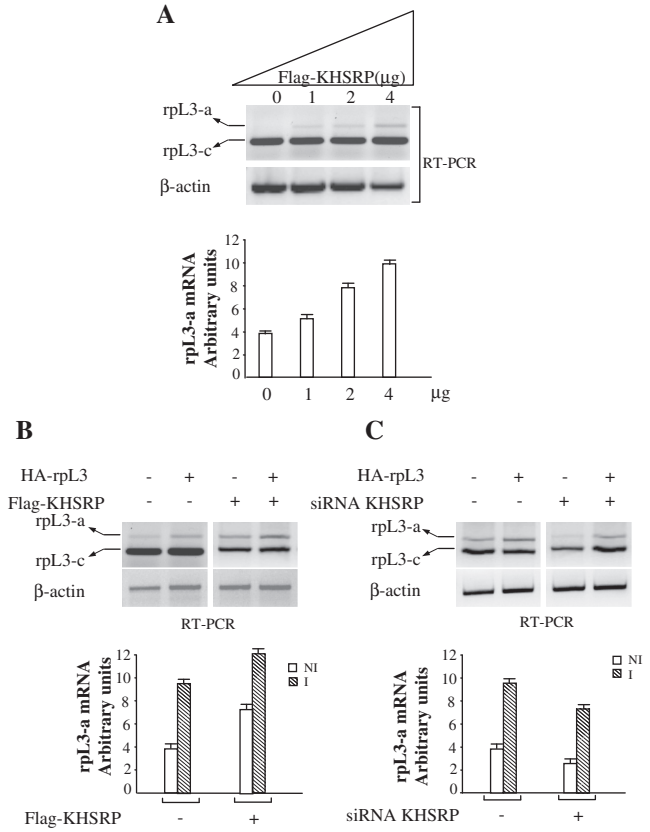

Figure 4. (**A**) Effects of KHSRP overexpression on rpL3-a mRNA levels in HeLa cells. Representative RT–PCR analysis of total RNA from CHX-treated HeLa cells untransfected or transfected with increasing amounts of Flag-KHSRP. (**B**) Effects of KHSRP overexpression on rpL3-a mRNA levels in L3–8 cells. Representative RT–PCR analysis of total RNA from CHX-treated L3–8 cells untransfected or transfected with Flag-KHSRP, non-induced (NI) or induced (I) for HA-rpL3 expression. (**C**) Effects of RNAi-mediated depletion of KHSRP on rpL3-a mRNA levels in L3–8 cells. Representative RT-PCR analysis of total RNA from CHX-treated L3–8 cells untransfected or transfected with KHSRP-siRNA, NI or induced I for HA-rpL3 expression from the same samples. The levels of rpL3-a mRNA were quantified by PhosphorImager (Bio-Rad) and normalized to β-actin levels.

The effects of KHSRP on rpL3 splicing were also investigated in condition of mRNA silencing. L3–8 cells were treated with siRNA specific for KHSRP and, 24 h after transfection, cells were treated with CHX and HA-rpL3 production was induced by removing doxycycline from culture medium. Cells were then lysed and protein extracts were analyzed for the expression of KHSRP and HA-rpL3 by western blotting using anti-KHSRP and anti-HA antibodies, respectively. As shown in Supplementary Figure S2C, the decrease of KHSRP level was ~70% compared to the protein amount detected in the control lysates. To examine the effects of the reduced production of KHSRP on the splicing pattern of rpL3 pre-mRNA, we analyzed total RNA extracted from the same samples by RT–PCR using specific primers to amplify rpL3-c and rpL3-a mRNAs. As expected, in cells overexpressing rpL3, we observed an increased level of rpL3-a mRNA isoform. However, in cells, in which KHSRP had been silenced, rpL3 induced overexpression was less effective in promoting the production of rpL3-a mRNA isoform (Figure 4C).

hnRNP H1 role in KHSRP-mediated activity

Since hnRNP H1 (15) and KHSRP have a role in promoting the alternative splicing of rpL3 gene, we asked whether a cooperation between these two proteins would occur and favor the selection of 3′-cryptic splicing site, or whether their functions were redundant. To clarify this issue, we switched off the expression of the gene encoding hnRNP H1 by using RNA interference. To this purpose, siRNAs against hnRNP H1 were transiently transfected in L3–8 cells. Twenty-four hours after transfection, cells were treated with CHX, induced for HA-rpL3 production and harvested; RNA and proteins were extracted. Lysates from cells transfected with siRNA or untransfected were probed with hnRNP H1 antibodies. As shown in Supplementary Figure S3A, the residual level of hnRNP H1 was ~20% of the protein detected in the control lysates. To investigate the effects of the reduced production of hnRNP H1 on the splicing pattern of rpL3 pre-mRNA, we examined the level of rpL3 mRNA isoforms using RT–PCR. Under normal conditions of rpL3 expression, the depletion of hnRNP H1 resulted in a relevant decrease (~80%) of the alternative mRNA (rpL3-a) level compared to controls. In conditions of hnRNP H1 removal, rpL3, although overexpressed, failed to activate the selection of the 3′-cryptic splicing site (Figure 5A). These data indicate clearly that hnRNP H1 plays a crucial role in triggering the alternative splicing reaction of rpL3 pre-mRNA.

Next, we investigated a possible involvement of hnRNP H1 in KHSRP-mediated activity. To this aim, we analyzed the effects of hnRNP H1 depletion and KHSRP overexpression on rpL3 splicing reaction. L3–8 cells were transiently co-transfected with siRNA specific for hnRNP H1 and a DNA construct expressing Flag-KHSRP. Twenty-four hours after transfection, cells were treated with CHX, induced for HA-rpL3 expression, and harvested. hnRNP H1 and KHSRP protein levels were detected by western blotting (Supplementary Figure S3B). RNA extracted from the same lysates was analyzed by RT–PCR. The Figure 5B shows that when hnRNP H1 was depleted, the enforced expression of KHSRP did not cause an increase of rpL3-a mRNA level, either in normal conditions of rpL3 expression, or in rpL3 overproduction. These findings indicate that the ability of KHSRP to influence the splicing of rpL3 pre-mRNA is mediated by hnRNP H1.

NPM controls the alternative splicing of rpL3 gene

To study the functional relevance of the interactions of NPM with rpL3 and intron 3 RNA in the control of the rpL3 pre-mRNA splicing, we overexpressed NPM in HeLa cells. Increasing amounts of a DNA construct expressing HA-NPM were transiently transfected in HeLa cells. Twenty-four hours after transfection, cells were treated with CHX and lysed. The expression levels of

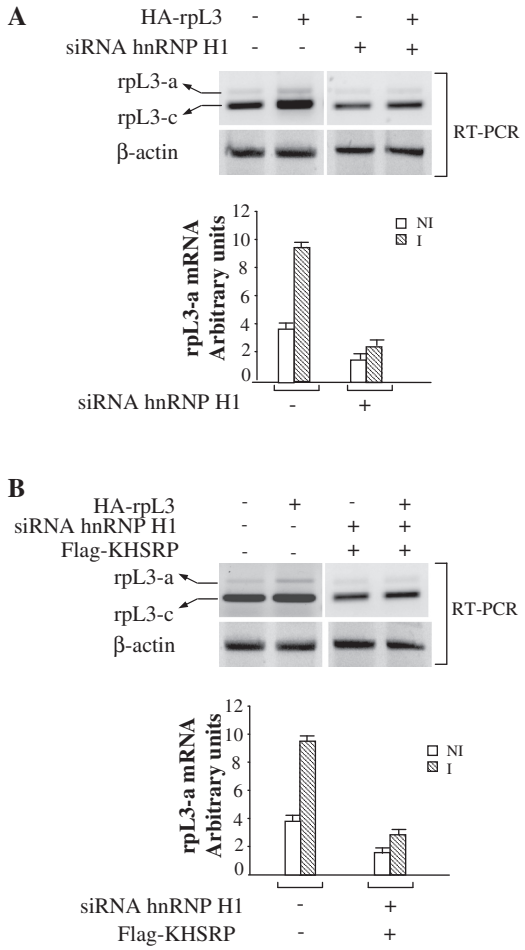

Figure 5. (**A**) Effects of RNAi-mediated depletion of hnRNP H1 on rpL3-a mRNA levels in L3–8 cells. Representative RT-PCR analysis of total RNA from CHX-treated L3–8 cells, untransfected or transfected with hnRNP H1-siRNA, NI or induced I for HA-rpL3 expression. (**B**) Effects of RNAi-mediated depletion of hnRNP H1 and KHSRP overexpression on rpL3-a mRNA levels in L3–8 cells. Representative RT–PCR analysis of total RNA from CHX-treated L3–8 cells untransfected or cotransfected with hnRNP H1-siRNA and Flag-KHSRP, NI or induced I for HA-rpL3 expression. The levels of rpL3-a mRNA were quantified by PhosphorImager (Bio-Rad) and normalized to β-actin levels.

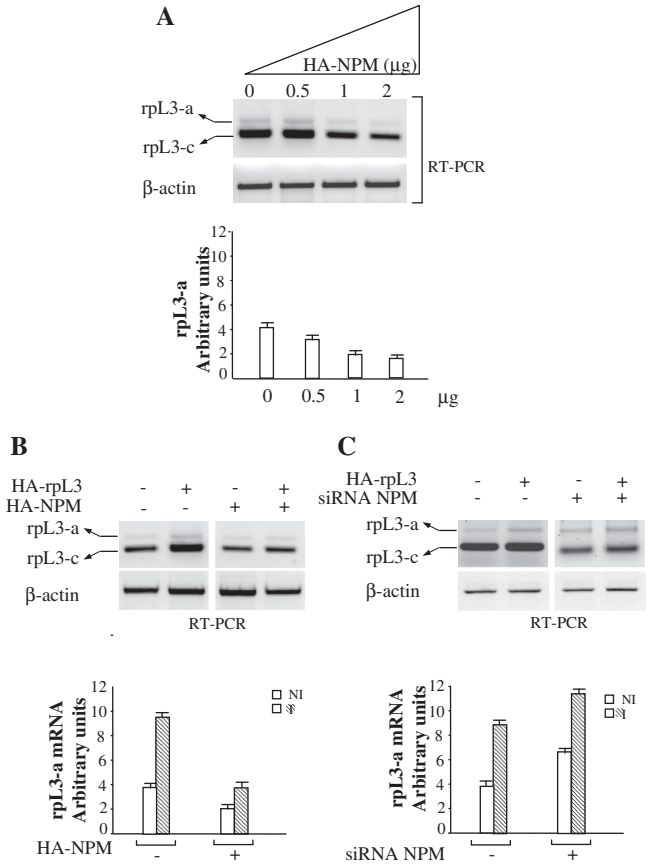

Figure 6. (**A**) Effects of NPM overexpression on rpL3-a mRNA levels in HeLa cells. Representative RT–PCR analysis of total RNA from CHX-treated HeLa cells untransfected or transfected with increasing amounts of HA-NPM. (**B**) Effects of overexpression of NPM on rpL3-a mRNA levels in L3–8 cells. Representative RT–PCR analysis of total RNA from CHX-treated L3–8 cells untransfected or transfected with HA-NPM, NI or I for HA-rpL3 expression. (**C**) Effects of silencing of NPM on rpL3-a mRNA levels in L3–8 cells. Representative RT–PCR analysis of total RNA from CHX-treated L3–8 cells untransfected or transfected with siRNA against NPM, NI or I for HA-rpL3 expression. The levels of rpL3-a mRNA were quantified by PhosphorImager (Bio-Rad) and normalized to β-actin levels.

the recombinant fusion protein were detected by western blotting using anti-HA antibodies (Supplementary Figure S4A). The pattern of the spliced rpL3 gene transcript was monitored by RT–PCR by using specific primers to amplify rpL3-c and rpL3-a isoforms of rpL3 mRNA. As shown in Figure 6A, NPM overexpression caused a dose-dependent decrease of rpL3-a mRNA amounts. Next, to investigate the role of NPM in the context of rpL3 autoregolatory loop, we performed experiments of overexpression and RNAi-mediated silencing of NPM in L3–8 cell line. Cells were transiently transfected with a DNA vector expressing HA-NPM. We chose a dose of HA-NPM that would result in about 50% decrease of the rpL3-a mRNA isoform. Twenty-four hours after transfection, cells were treated with CHX and induced to express HA-rpL3. Cells were then lysed and analyzed for the production of HA-NPM and HA-rpL3 by western blotting by using anti-HA antibody (Supplementary Figure S4B). Total RNA from the same samples was analyzed by RT–PCR by using specific primers to amplify rpL3-c and rpL3-a isoforms of rpL3 mRNA. The overexpression of NPM resulted in a reduction in the rpL3-a mRNA amount in normal conditions of rpL3 expression, and prevented the increasing of rpL3-a mRNA level when rpL3 protein was overexpressed (Figure 6B).

To investigate the effects of NPM removal on rpL3 gene splicing, NPM expression levels were reduced by transfecting L3–8 cells with specific siRNA. Twenty-four hours after transfection, cells were treated with CHX and induced to express HA-rpL3. Protein extracts from cells were tested for the production of HA-NPM and HA-rpL3 by western blotting (Supplementary Figure S4C). Total RNA was analyzed by RT–PCR by using specific primers to amplify rpL3-c and rpL3-a mRNA isoforms.

In physiological conditions of rpL3 expression, following NPM depletion, the production of rpL3-a mRNA appeared restored above the control expressing NPM. Thus, as expected, HA-rpL3 expression upon NPM silencing caused a significant increase of rpL3-a mRNA levels (Figure 6C).

DISCUSSION

AS of mRNA is mainly responsible for a variety of gene products much larger than expected from the number of genes. It has been estimated that >90% of human genes are alternatively spliced (18,19). However, a number of splicing events give rise to mRNA isoforms containing a PTC that are targeted for decay by NMD. Accumulating data indicate that association of AS and NMD (AS-NMD) may result in a quantitative post-transcriptional regulation of gene expression (7).

In eukaryotes rp expression is regulated by multiple control mechanisms, mostly at post-transcriptional and translational level in order to maintain ribosome biosynthesis at the level appropriate to growth conditions and requirements of the cell (14,15,20,21). However, several reports indicate that rp, in addition to the role as components of the translation machinery, exert a variety of extraribosomal functions, for which additional, specific regulatory strategies are required (22,23). Autoregulation may represent an efficient mechanism to control the level of a single protein; we have demonstrated previously a role of AS-NMD in the regulation of the rpL3 gene expression, promoted by rpL3 protein itself as part of an autoregulatory negative-feedback loop (14,15).

In an attempt to understand the mechanism of the regulatory strategy, we focused our studies on the identification of protein partners of the rpL3 protein, and to the analysis of their contribution to the process. Our previous results demonstrated that rpL3 protein, within a RNP complex including the constitutive splicing factor hnRNP H1, promotes the alternative splicing reaction of its own gene (15). In most cases, the choice of a splicing site is made by a dynamic and complex combination of different splicing regulators; in fact, the function of an individual splicing factor may be different depending on interacting partners present in the regulatory network. The identification of new regulatory proteins and the mapping of protein–protein interactions within the RNP complex including rpL3 are crucial steps to understand the molecular mechanism involved in the selection of the cryptic 3′-splice site within intron 3 transcript of the rpL3 gene. To this aim, we analyzed occurrence of interaction between rpL3 and putative protein partners previously identified through a proteomic analysis (15), and the role of these proteins in the autoregulatory network of rpL3 expression. Our data provide an insight in putative RNP complexes including hnRNP H1, rpL3 protein, pre-mRNA of rpL3 gene, and the newly identified protein factors, NPM and KHSRP, involved in the regulation of rpL3 gene expression via AS-NMD.

NPM is a nucleolar, ubiquitous and multifunctional phosphoprotein. NPM is involved in multiple biological functions including the ribosome biogenesis, the control of cell cycle progression and centrosome duplication. The cellular activities of NPM are tightly regulated by multiple factors that seem to be specific for each function. Post-translational modifications, oligomerization and hetero-oligomerization strongly influence the cellular functions of NPM (24). Emerging evidences indicate a functional correlation between NPM and some rp, independent from ribosome biogenesis or its assembly (25,26). It is known that NPM is involved in both positive and negative regulation of transcription, and a possible role of NPM in the splicing process has been suggested (27). To our knowledge, the present study demonstrates for the first time a role of NPM as splicing factor.

KHSRP is a multifunctional RNA-binding protein that has been mainly implicated in post-transcriptional regulation, mRNA decay and maturation of microRNA precursors (28,29). There are few data supporting an involvement of KHSRP in the splicing control (30,31).

Analysis of immunoprecipitate of NPM and KHSRP in HeLa cell extracts showed that NPM and KHSRP coimmunoprecipitate together with rpL3 and hnRNP H1, indicating that these proteins associate *in vivo*, although the occurrence of multiple complexes including different combination of protein interactions cannot be excluded (Figure 1). In addition, *in vitro* GST pull-down experiments (Figure 2) demonstrated that NPM and KHSRP are able to recognize and bind rpL3 and hnRNPH1, whereas a direct binding between NPM and KHSRP, or rpL3 and hnRNPH1 has not been observed.

On the other hand, RNA immunoprecipitation assays indicated the presence of intron 3 RNA in the immunoprecipitate of NPM and KHSRP, and RNA pull-down experiments showed that NPM and KHSRP are able to contact directly the intron 3 RNA (Figure 3) as previously shown for hnRNP H1 (15). Taken together, these data strongly suggest that NPM and KHSRP are involved in the control of the splicing of rpL3.

Our analysis of the effects of individual expression of NPM, hnRNP H1 or KHSRP provided an insight also on the specific role of each protein factor on the rpL3 gene splicing. In the context of rpL3 autoregulatory circuit, protein overexpression data indicated that KHSRP represents a positive regulator of the alternative splicing that cooperates with rpL3 and hnRNP H1 in the activation of 3′-cryptic splice site (Figure 4). However, silencing of KHSRP caused only ~20% decrease of the alternative mRNA isoform produced when rpL3 is overexpressed, suggesting that KHSRP is not a crucial player in the rpL3 autoregulatory loop (Figure 4). Instead, when hnRNPH1 expression was silenced, rpL3 overexpression failed to result in the activation of cryptic the 3′-splice site (Figure 5A). These findings together with the observation that KHSRP, even in excess, was unable to increase the alternative mRNA isoform in the absence of hnRNP H1 strongly indicate that hnRNP H1 is the key component of rpL3 autoregulatory loop, while KHSRP might play a role as an enhancer of hnRNP H1-mediated activation of rpL3 gene alternative splicing (Figure 5B).

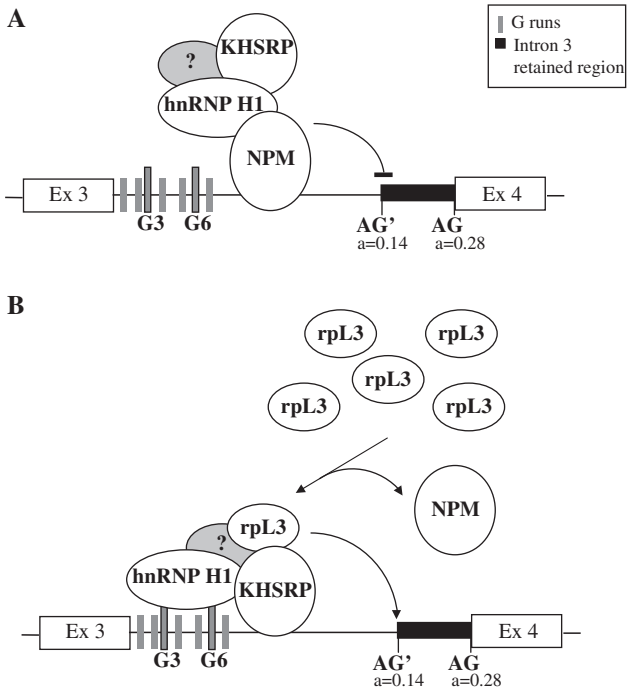

Figure 7. Schematic representation of proposed rpL3 feedback regulation. (**A**) In response to cell requirement for an efficient production of rpL3, NPM represses the alternative splicing of rpL3 gene. NPM, by interacting with hnRNP H1 and intron 3 pre-mRNA, prevents the binding of hnRNP H1 with G3 and G6 motifs. (**B**) Upon accumulation, rpL3 interacts with NPM. As consequence of this interaction, NPM is released from the RNP complex. The association of rpL3 with KHSRP and other transacting proteins induces a rearrangement of the interactions within the RNP complex that favor the interaction of hnRNP H1 with enhancer unit G3 + G6 within intron 3 transcript. Finally, the reassembled RNP complex promotes the selection of the 3′-splice site in intron 3 transcript.

It is known that the RNA binding activity of KHSRP is mediated by four KH domains. Among these, KH1 and KH3 domains recognize and bind specifically to a G-rich target (UGGG and GGGU, respectively) (32). The analysis of the human intron 3 transcript sequence showed that G6 motif, an essential element for the splicing regulatory activity of hnRNP H1, could represent also a binding site for KH1 or KH3. In addition, the UGCAUG element, essential to the binding of KHRSP to src-DCS (Downstream Control Sequence), is also present in the sequence of intron 3 RNA and it is located close to the hnRNP H1 binding site G3 and G6 motifs (31). Consequently, it seems plausible to speculate that KHSRP might interact with these sequences within intron 3 transcript as well as with hnRNP H1 making more stable and efficient the association of hnRNP H1 with intron 3 RNA.

Unlike hnRNP H1 and KHSRP, NPM behaves as a negative regulatory factor of rpL3 gene alternative splicing (Figure 6). It is possible that the inhibitory effect of NPM on alternative splicing might be due to a 'bind and block' mechanism. In fact, NPM could interact with sequences within intron 3 pre-mRNA, perhaps in the vicinity of hnRNP H1 binding sites, and sterically block the access of hnRNP H1, thus preventing its interaction with G3 and G6 elements. Since binding of hnRNP H1 to G-runs site has been demonstrated crucial, the alternative splicing of rpL3 gene would be negatively affected.

Alternatively, in the light of results demonstrating an interplay among different protein factors, possibly assembled in multiple RNP complexes, we propose a working model, which overcomes the model proposed previously (15). The model predicts the existence of, at least, two complexes whose protein composition depends on rpL3 protein levels. When cell functions require an efficient production of rpL3, the interaction of NPM with intron 3 pre-mRNA might affect its secondary structure and mask the binding sites of hnRNP H1. At the same time, repression of AS might be enforced by a concomitant direct binding of NPM to the positive regulator factor hnRNP H1 (Figure 7A).

When rpL3 levels exceed cell requirements for rpL3 ribosomal as well as extraribosomal functions, the protein could interact with NPM; consequently, NPM would be released from the RNP complex to which rpL3 could be associated by interacting with KHSRP. The removal of NPM, and the rearrangement of the interactions within the RNP complex, could make available the enhancer unit G3 + G6 within intron 3 transcript and, at the same time, the presence of rpL3 in the remodeled complex could induce conformational changes that favor the interaction of hnRNP H1 with G3 + G6. Finally, such complex could promote the activation of the alternative mode of splicing reaction (Figure 7B).

At the present, the challenge is to determine the hierarchy of interactions leading to the assembly of RNP complexes, and the dynamic nature of protein–protein interactions causing the remodeling of complexes responsible for the modulation of the splicing of the rpL3 gene.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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