

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

Annapina Russo^{1,2}, Morena Catillo¹, Davide Esposito¹, Paola Briata³, Concetta Pietropaolo¹ and Giulia Russo^{1,*}

¹Dipartimento di Biochimica e Biotecnologie Mediche, Università Federico II, Via Sergio Pansini 5, Napoli 80131, ²CEINGE Biotecnologie Avanzate S.C.a.r.l., Via Gaetano Salvatore 482, Napoli 80145 and ³Istituto 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 dystrophy. 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'-TTAAAGAGACTTCCTCC A-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'-GGAAATAGCTGAAAAGGC T-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% glycerol, 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 pelleted, washed twice with 1 ml of 2 M NaCl and equilibrated in washing buffer (5 mM HEPES pH 7.9, 1 mM MgCl₂, 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 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).

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 Transcriptase (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'-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).

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

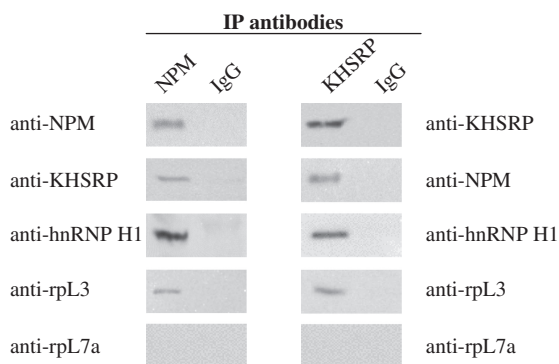


Figure 1. *In vivo* binding of NPM, KHSRP, hnRNP H1 and rpL3. NPM or KHSRP were specifically immunoprecipitated from HeLa cells extracts with antibodies against the endogenous NPM and KHSRP. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with antibodies versus the indicated proteins.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

in 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.

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

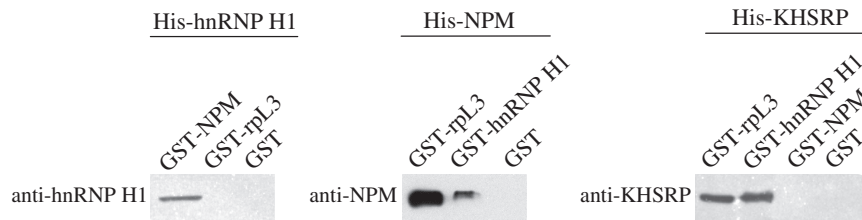


Figure 2. Analysis of the interactions between hnRNP H1, NPM, KHSRP and rpL3. Western blotting (WB) of GST pull-down experiments. Fifty microgram of GST-tagged proteins (GST-NPM, GST-rpL3 and GST-hnRNP H1) or GST (control) were immobilized on glutathione-sepharose beads and incubated with 20 μ g of His-hnRNP 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.

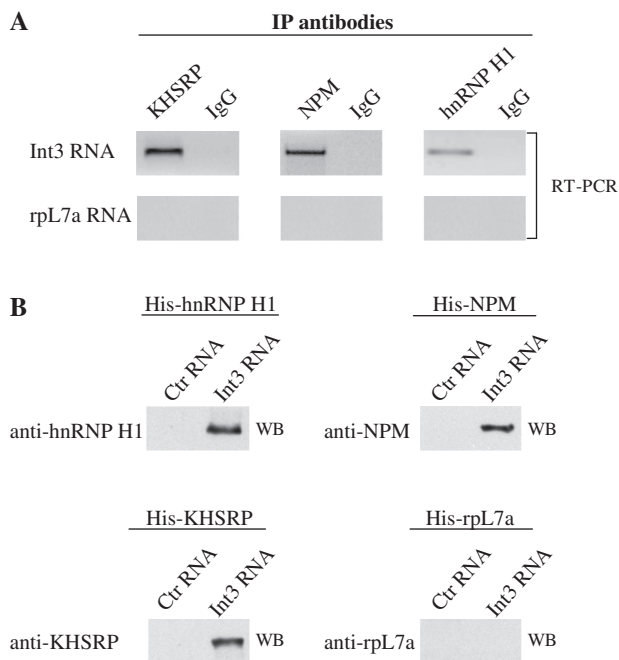


Figure 3. Analysis of the interactions of intron 3 of rpL3 pre-mRNA with NPM and KHSRP, *in vivo* and *in vitro*. (A) *In vivo* binding of intron 3 transcript to NPM and KHSRP. RT-PCR analysis, by using primers against rpL3 intron 3 transcript and rpL7a RNA transcript, of RNA extracted from the NPM, KHSRP, hnRNP H1 (control) and IgG immunocomplexes. Note the absence of signal in IgG immunocomplexes. (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 (Ctrl 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).

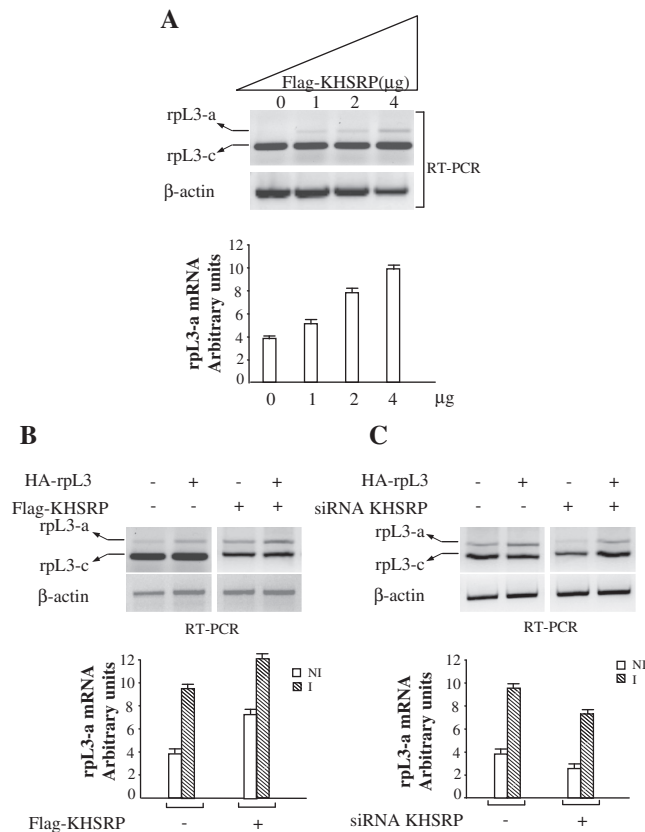


Figure 4. (A) Effects of KHSRP overexpression on rpL3-a mRNA levels in HeLa cells. Representative RT-PCR analysis of total RNA from CHX-treated HeLa cells untransfected or transfected with increasing amounts of Flag-KHSRP. (B) Effects of KHSRP overexpression on rpL3-a mRNA levels in L3-8 cells. Representative 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 $\sim 70\%$ 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 $\sim 20\%$ 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 ($\sim 80\%$) 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

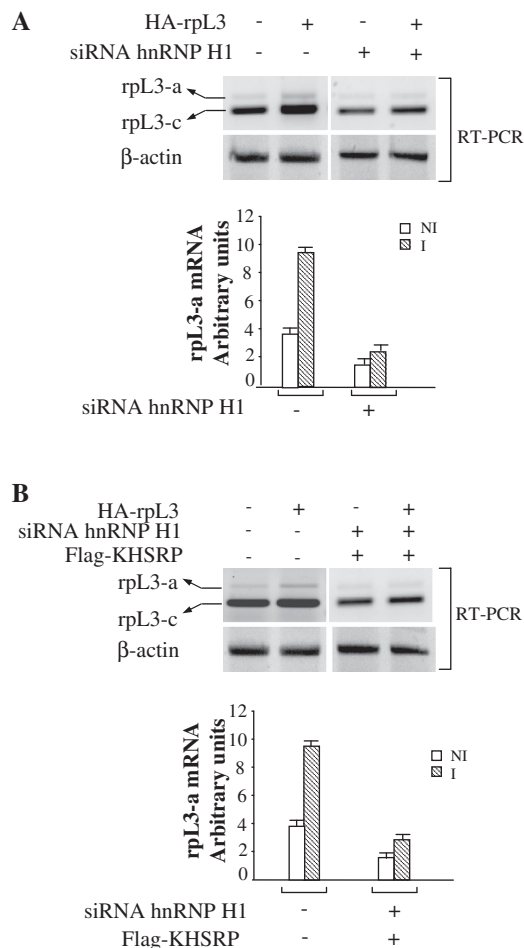


Figure 5. (A) Effects of RNAi-mediated depletion of hnRNP H1 on rpL3-a mRNA levels in L3-8 cells. Representative RT-PCR analysis of total RNA from CHX-treated L3-8 cells, untransfected or transfected with hnRNP H1-siRNA, NI or induced I for HA-rpL3 expression. (B) Effects of RNAi-mediated depletion of 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.

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 autoregulatory 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

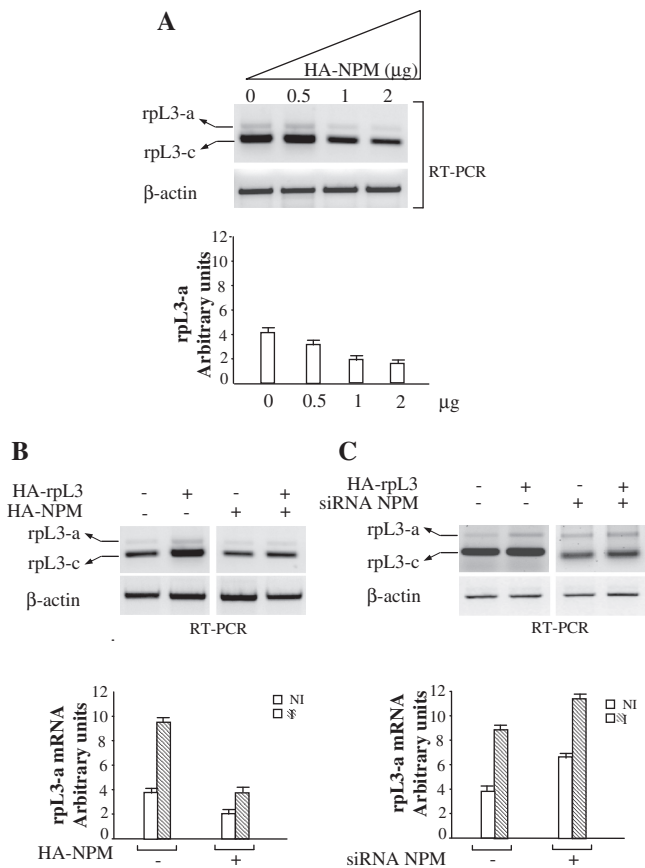


Figure 6. (A) Effects of NPM overexpression on rpL3-a mRNA levels in HeLa cells. Representative RT-PCR analysis of total RNA from CHX-treated HeLa cells untransfected or transfected with increasing amounts of HA-NPM. (B) Effects of overexpression of NPM on rpL3-a mRNA levels in L3-8 cells. Representative 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.

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).

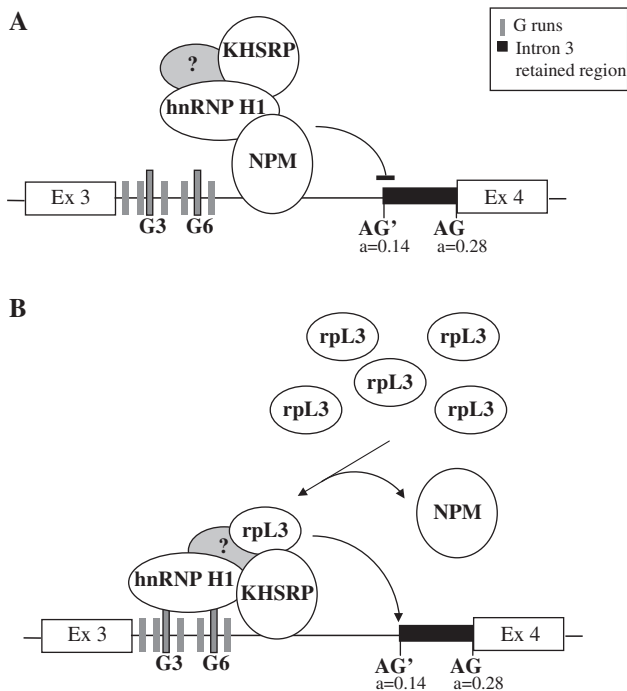


Figure 7. Schematic representation of proposed rpL3 feedback regulation. (A) In response to cell requirement for an efficient production of rpL3, NPM represses the alternative splicing of rpL3 gene. NPM, by interacting with hnRNP H1 and intron 3 pre-mRNA, prevents the binding of hnRNP H1 with G3 and 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 KHSRP 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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