ARTICLE
Journal of Cellular Biochemistry 110:294–303 (2010)

Journal of Cellular Biochemistry

RpS3 Translation Is Repressed by Interaction With Its Own mRNA

Hag Dong Kim,[1] Tae-Sung Kim,[1] Yoo Jin Joo,[1] Hyun-Seock Shin,[1] Sang-Hwa Kim,[1] Chang-Young Jang,[1] Cheol Eui Lee,[2] and Joon Kim[1*]

[1] *Laboratory of Biochemistry, School of Life Sciences and Biotechnology, and BioInstitute, Korea University, Seoul 136-701, South Korea*

[2] *Department of Physics, Korea University, Seoul 136-701, South Korea*

ABSTRACT

Ribosomal protein S3 (RpS3) is a well-known multi-functional protein mainly involved in protein biosynthesis as a member of the small ribosomal subunit. It also plays a role in repairing various DNA damage acting as a repair UV endonuclease. Most of the rpS3 pool is located in the ribosome while the minority exists in free form in the cytoplasm. We here report an additional function of rpS3 in which it represses its own translation by binding to its cognate mRNA. Through RT-PCR of the RNAs co-immunoprecipitated with ectopically expressed rpS3, rpS3 protein was found to interact with various RNAs—endogenous rpS3, 18S rRNA. The S3-C terminal domain was shown to be the major mRNA binding domain of rpS3, independent of the KH domain. This interaction was shown to occur in cytoplasmic fractions rather than ribosomal fractions, and then is involved in its own mRNA translational inhibition by in vitro translation. Furthermore, when Flag-tagged rpS3 was transiently transfected into 293T cells, the level of endogenous rpS3 gradually decreased regardless of transcription. These results suggest that free rpS3 regulates its own translation via a feedback mechanism. J. Cell. Biochem. 110: 294–303, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: rpS3; RIBOSOME; POLYSOME; MONOSOME; KH DOMAIN

The ribosome, a complex of four rRNAs and approximately 80 ribosomal proteins, plays a pivotal role in protein biosynthesis in the cell [Mager, 1988; Wool et al., 1995]. Ribosomes in the cell constitute approximately 80% of total RNA and 10% of total protein. Recently, numerous ribosomal proteins have been reported to play other roles, so-called extra-ribosomal functions [Wool, 1996; Chen and Ioannou, 1999; Naora, 1999; Oh et al., 2002; Zimmermann, 2003]. For example, ribosomal protein L11 acts as a negative regulator of HDM2, thereby establishing an L11-HDM2-p53 pathway for monitoring ribosomal integrity in vivo [Zhang et al., 2003]. Ribosomal protein S3a is vital in cell transformation and death [Naora et al., 1998] while ribosomal protein P0 possesses putative DNA repair activity [Grabowski et al., 1991].

RpS3, a 26.7 kDa protein, is a member of the small ribosomal subunit known to be involved in the initiation of translation, supported by its cross-links to the eukaryotic initiation factors, eIF-2 [Westermann et al., 1979], and eIF-3 [Tolan et al., 1983; Polakiewicz et al., 1995]. Interestingly, it was reported that this protein possesses an extra-ribosomal function in which DNA damage caused by UV irradiation is repaired [Kim et al., 1995; Yacoub et al., 1996; Jung et al., 2001; Lee et al., 2002]. Apparently, this enzyme cleaves UV-irradiated DNA and AP (apyrimidic/apurinic) DNA by breaking via a lyase mechanism the phosphodiester bond between the cyclobutane pyrimidine dimer and 3' site of the AP DNA [Kim et al., 1995; Kanno et al., 1999; Hegde et al., 2001; Lee et al., 2002]. DNA repair endonuclease and the activity and ribosome incorporation ability appear to be regulated by phosphorylation and methylation of the protein [Kim et al., 2009a,b; Shin et al., 2009]. In addition, cells from Xeroderma pigmentosum group D (XPD) patients are subject to high incidence of skin cancer show abnormal activity of this enzyme. Since the column profile of rpS3 endonuclease activity appears to differ in XPD cells compared to normal cells [Kim et al., 1995], there is a probability that the defective function of rpS3 is related with XP disease. Recently, it was demonstrated that rpS3 is located on the beak portion of the small ribosomal subunit, slightly changing its conformation during the translation initiation step [Passmore et al.,

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: KRF; Grant numbers: 07-314-C00129, 07-005-J03603; Grant sponsor: Proteomics; Grant number: FPR08B1-230.

*Correspondence to: Joon Kim, Laboratory of Biochemistry, School of Life Sciences & Biotechnology, and BioInstitute, Korea University, Seoul 136-701, South Korea. E-mail: joonkim@korea.ac.kr

Received 4 April 2009; Accepted 15 January 2010 • DOI 10.1002/jcb.22537 • © 2010 Wiley-Liss, Inc.
Published online 9 March 2010 in Wiley InterScience (www.interscience.wiley.com).

2007]. Wan et al. [2007] demonstrated that rpS3 is a component of the NF-κB complex, and that its interaction with other components in complex increases the DNA binding activity of NF-κB. Furthermore, it was reported that interaction between rpS3 and Hsp90 is necessary for ribosomal protection from degradation [Kim et al., 2006]. RpS3 proteins from various species are also conserved at several motifs: the nuclear localization signal—KKRK, KH (hnRNP K homology) domain—RNA binding domain and S3-C terminal domain are all well-conserved among eukaryotes.

Recent reports have suggested that many ribonucleoproteins (RNPs) play a variety of roles by binding to their target mRNAs. For example, heterogeneous nuclear RNPs (hnRNPs) [Mili et al., 2001] and Sam68 [Matter et al., 2002] splice and transport mRNA out of the nucleus. HnRNPs are also responsible for controlling mRNA activity in the cytoplasm [Loflin et al., 1999]. An additional function is the maintenance of mRNA stability. By binding to their mRNAs, RNPs are involved in the degradation of mRNA either directly by nuclease or by restricting the access of degradative enzymes. Yet another function is the regulation of mRNA translation. Here, dihydrofolate reductase (DHFR) binds to its own mRNA and results in its own translational inhibition [Standart and Jackson, 1994; Tai et al., 2002].

Previous studies have shown that some ribosomal proteins in *Escherichia coli* and yeast interact with their own mRNA causing translational repression. For example, a number of *E. coli* ribosomal proteins such as S7, S8, S15, and L1 repress the translation of their own mRNA in vitro [Zengel and Lindahl, 1994] by interacting with the mRNA leader sequence, similar to rRNA [Serganov et al., 2003]. This phenomenon has been observed in mammalian ribosomal proteins as well [Neumann et al., 1995; Tasheva and Roufa, 1995]. Upon overexpression, rpS13 [Malygin et al., 2007] and rpS26 [Ivanov et al., 2005] bind the introns of their own pre-mRNA to suppress splicing.

In this report, it was observed that an ectopically expressed rpS3 protein interacts with its own mRNA via UV-crosslinking and RNP-immunoprecipitation experiments. When we separated the cytosolic and ribosomal fractions by ultracentrifugation, the majority of ectopically expressed rpS3 was found in the cytosolic fraction where it interacted with its own endogenous mRNA. In this article, we will discuss how this binding affects the translational repression of rpS3.

MATERIALS AND METHODS

PLASMID CONSTRUCTION

The full-length human rpS3 gene was cloned in-frame with a sequence coding for Flag (pcDNA3-Flag, Invitrogen), His (pET21a, Novagen) and Glutathione *S*-transferase (GST, pGEX5x-1, Amersham Biosciences). Deletion mutant sequences of human rpS3 were amplified by PCR using five pairs of primers. The resulting PCR products were cut with *Eco*RI and *Xho*I and cloned in-frame into pGEX5x-1.

For cloning each rpS3 mRNA fragment, 2 μg of 293T total RNA was incubated with 50 pmol of reverse primer (5′-GGTCTTTGTACAAAATTTTATTAAAGG-3′), 25 mM dNTPs, 40 U of recombinant RNasin (Promega), 2 U of AMV reverse transcriptase (Promega) and 0.5× AMV reverse transcriptase buffer at 37°C for 120 min. PCR was performed with a primer pair set (5′-AAAGAATTCCCTTTCCTTTCAGCG-3′ and 5′-AAAGTCGACGGTCTTTGTACAAAA-3′), followed by cloning of PCR products into the pGEM-T Easy vector (Promega). Sub-fragments were also prepared by PCR amplification with their respective primers; for K1, 5′-AAAGAATTCCCTTTCCTTTCAGCG-3′ and 5′-AAAGTCGACCCCTCCTAGGAGTTT-3′; for K2, 5′-AAAGAATTCCTTGCTGTGCGGAGG-3′ and 5′-AAAGTCGACAGTTGGGTCCAAGGG-3′; for K3, 5′-AAAGAATTCGGTAAGATTGGCCCT-3′ and 5′-AAAGTCGACGGTCTTTGTACAAAA-3′. Sub-fragments were then cloned in pGEM-T Easy vector for transcription.

PROTEIN EXPRESSION AND PURIFICATION

The pET21a and pGEX5x-1 based plasmids were used to transform *E. coli* strain BL21(DE3)/pLysS (Novagen) cells. Transformed cells were grown in LB broth containing 50 μg/ml ampicillin overnight at 37°C. Next, one-hundredth of the cells were grown in LB broth containing 50 μg/ml ampicillin and 0.4% dextrose at 30°C until cell density reached $A_{600} = 0.6–0.8$. The recombinant proteins were induced with 0.5 mM isopropyl-1-β-D-galactopyranoside (IPTG) and purified with glutathione-Sepharose 4B (Pharmacia) and Ni^{2+}-NTA beads (Quiagen) according to manufacturer's instructions.

CELL CULTURE AND TRANSFECTIONS

Human embryonic kidney epithelial 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. These cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Transfections were performed with Lipofectamine (Gibco-BRL) as instructed by the manufacturer.

ANTIBODIES AND IMMUNOBLOTTING

Monoclonal anti-actin, anti-Erk, and anti-p53 along with polyclonal anti-rpS6, anti-Rack1, and anti-p53 antibodies were obtained from Santa-Cruz Biotechnology Inc., where as monoclonal anti-FLAG antibody was obtained from Sigma–Aldrich Co. Rabbit polyclonal anti-rpS3 antibody was raised against a polypeptide corresponding to C-terminal 146 residues of rpS3.

Transfected 293T cells were lysed by lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin) for 30 min on ice. Supernatants were collected by centrifugation at 12,000*g* for 10 min at 4°C and protein concentration was determined by Bradford protein assay. The lysates were boiled in SDS–PAGE sample buffer and separated by SDS–PAGE, transferred to PVDF membranes, probed with antibodies as indicated and illuminated with an enhanced chemiluminescence (ECL) system (Roche).

RNP IMMUNOPRECIPITATION AND RT-PCR

Transfected 293T cells were lysed by cold RNP immunoprecipitation buffer (100 mM Tris–HCl (pH 7.4), 150 mM Nacl, 1 mM EDTA, 1% NP-40, 0.25 U/μl recombinant RNasin, 5 mM DTT) in the presence of protease inhibitors (2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin) for 30 min on ice. Supernatant was collected by centrifugation at 12,000*g* for 10 min at 4°C and was pre-cleared by

adding protein A agarose, followed by incubation at 4°C for 2 h with anti-FLAG antibody. Fifty microlitres of protein A agarose was added next followed by additional incubation for 1 h at 4°C. After extensive washing, the immunoprecipitates were resuspended in RNA elution buffer (100 mM Tris–HCl (pH7.4), 150 mM NaCl, 12.5 mM EDTA, 1% SDS, 0.25 U/μl recombinant RNasin) and boiled for 3 min. The elute was then extracted with phenol/chloroform/isoamylalcohol (25:24:1) mixed with chloroform/isoamylalcohol (24:1) and then precipitated with 10 μg of glycogen (Sigma), 1/10 volumes of 3 M sodium acetate and 2.5 volumes of ethanol. For RT-PCR control, total RNA from 293T cells (5×10^5 cells) was extracted with Trizol reagent (Invitrogen). The RNAs were reverse-transcribed with oligo-dT_{15}. PCR products were visualized on a 1% TAE-agarose gel. The rpS3-specific primers for RT-PCR are rpS3-F1: 5′-AGCGGAGACCCTGTTAACTACTAC-3′ and rpS3-R: 5′-GTCTTTCTACAAAATTTTATTAAAGG-3′. The p53 specific primers for RT-PCR are p53-F: 5′-TCTGTCCCTTCCCAGAAAACC-3′ and p53-R: 5′-CGTCATGTGCTGTGACTGCTT-3′. The SHP2 specific primers for RT-PCR are SHP2-F: 5′-TCCAGATGGTGCGGTCTC-3′ and SHP2-R: 5′-TCTGCTGTTGCATCAGGC-3′.

IN VITRO TRANSCRIPTION AND CAPPING

In vitro ^{32}P-labeled RNA synthesis was performed with 1 μg of linearized pGEM-T Easy vector which cloned the rpS3 mRNA fragments, along with T7 RNA polymerase (Roche), rNTP sets (0.5 mM rATP, 0.5 mM rGTP, 0.5 mM rUTP, and 50 μM rCTP) and 50 μCi α-^{32}P CTP (NEN). For in vitro translation, unlabeled RNA was synthesized as above, substitute 0.5 mM rCTP for 50 μCi α-^{32}P CTP. Unlabeled RNA was incubated with 5 U of vaccinia virus guanylyltransferase (Ambion), S-adenosyl methionine (SAM) and 0.1 mM rGTP in 1× reaction buffer for 1 h at 37°C. Following incubation, RNA was extracted with phenol/chloroform/isoamylalcohol (25:24:1) mixed with chloroform/isoamylalcohol (24:1) and precipitated with 1/2 volumes of 7.5 M ammonium acetate and 2.5 volumes of ethanol. All RNA transcripts were resolved on a 5% polyacrylamide and 8 M urea gel to confirm their integrity and size.

IN VITRO TRANSLATION

In vitro translation was performed by incubation with 0.25 μg of unlabeled capping RNA, 35 μl rabbit reticulolysate and 20 μCi ^{35}S-methionine (NEN) for 1 h at 37°C. For the translational repression assay, 4 μg of desired proteins were added to the translation mixture. As using the control in translation experiment, 0.5 μg RNA of Firefly luciferase mRNA and 15 μg total RNA of normal 293T cells were used. Translation products were analyzed by 15% SDS–PAGE gel. The gel was dried and visualized by a PhosphorImager.

SEMI-QUANTITATIVE RT-PCR

Total RNA from transfected 293T cells (5×10^5 cells) was extracted with Trizol reagent (Invitrogen) and were reverse-transcribed with oligo-dT_{15}. PCR products were visualized on a 1% TAE-agarose gel. PCR was performed with two pairs of primer sets. RpS3 specific primers are rpS3-F2: 5′-TGTGCCATTGCCCAGGCAGAGTCT-3′ and rpS3-R: 5′-GTCTTTCTACAAAATTTTATTAAAGG-3′. GAPDH specific primers are GAPDH-F: 5′-ACCCAGAAGACTGTGGATGG-3′ and GAPDH-R: 5′-CATACCAGGAAATGAGCTTGAC-3′.

SUCROSE GRADIENT

Ribosome and non-ribosome fractions were collected as described [Wormington, 1991]. Briefly, 293T cells (7×10^6 cells) were lysed in 2 ml of lysis buffer (20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 300 mM KCl, 10 mM DTT, 100 units/ml RNasin). Following centrifugation at 10,000*g* for 15 min to remove mitochondria and cell debris, the supernatant was layered over a sucrose (20%, w/v) cushion and centrifuged in a Beckman SW41Ti rotor at 149,000*g* for 2 h. The ribosome-containing pellet and non-ribosomal supernatant were separately collected. To remove any ribosome contaminants, the supernatant was subjected to a second centrifugation at 149,000*g*. For RNA analysis, each fraction was incubated with 100 μg of proteinase K in 1% SDS and 10 mM EDTA for 1 h at 37°C. Following extraction, the RNA was resolved in a denaturing 1% agarose-formaldehyde gel. The gel was stained with ethidium bromide to visualize 18S and 28S RNA in the 40S and 60S subunits, respectively. For immunoblot analysis, ribosome pellets were resuspended in SDS–PAGE sample buffer and cytosolic fractions were precipitated with acetone and mixed with SDS–PAGE sample buffer.

To construct the linearized gradient, the above-mentioned supernatants were layered on top of a 10–40% (w/v) sucrose gradient in dilution buffer (20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 300 mM KCl) and centrifuged in a Beckman SW41Ti rotor at 12,000*g* for 16 h at 4°C. After centrifugation, the absorbance of sucrose gradients was measured at 260 nm with a UV spectrophotometer equipped in Bio-Rad DuoFlow. And each fraction was collected from bottom to top of the centrifugated sample by a fraction collector.

IN VITRO BINDING ASSAY

GST fusion proteins were purified by glutathione-sepharose 4B beads. The deletion mutants of rpS3 were designated as below. GST-KH indicates the KH domain of rpS3 protein, ΔKH indicates deletion mutant that is no KH domain, and GST-C(N) indicates the fore-part of the S3-C domain, GST-P symbolizes the part that has many prolines, and GST-R symbolizes the last part of rpS3 protein. RNA interaction assays were performed by incubating immobilized GST fusion proteins with ^{32}P-labeled, in vitro transcribed RNA (160,000 cpm) in RNA binding buffer (50 mM Tris–HCl (pH 7.4), 2 mM $MgCl_2$, 100 mM KCl, 1 mM EDTA, 0.5% NP-40, 0.5 U/μl recombinant RNasin, 2 mM DTT) on ice for 15 min. After extensive washing, the labeled RNA-protein complex was counted by a scintillation counter. Binding RNA was extracted as mentioned and analyzed by dot blotting.

UV CROSS-LINKING ANALYSIS

^{32}P-radioabeled rpS3 mRNA (160,000 cpm) and 4 μg of human His-tagged rpS3 protein were incubated on ice for 15 min in RNA binding buffer. The rpS3-mRNA complexes were UV cross-linked at 100 J/cm^2 (UV-cross-linker; UVP) on ice and then incubated with 0.1 mg/ml of RNase A (Sigma) for 15 min at 37°C. The complexes were separated by 12% SDS–PAGE, and the gel was dried and visualized by a PhosphorImager.

RESULTS

RPS3 PROTEIN INTERACTS WITH ITS OWN MRNA

To investigate the RNA-binding properties of rpS3, 293T cells were transiently transfected with Flag-tagged rpS3 followed by the immunoprecipitation of RNPs from cell lysates (Fig. 1A). RNAs that interact with rpS3 protein were characterized by extraction from the immunoprecipitate, reverse-transcription with oligo-dT_{15} and PCR using specific primers for various genes. Additionally, to exclude the possibility that amplification was based on transfected DNA, we performed PCR with primers specific to the 3′-UTR of the endogenous rpS3 gene. The results did not detect p53, SHP2 or rpS6, but did find that rpS3 mRNA interacted with Flag-tagged rpS3 (Fig. 1B). To show that those mRNAs are not from the translating ribosome, we performed metabolic labeling of the protein in 293T cells. As shown in the supplemental Figure 1, the translating protein level of radio-labeled p53 is similar to that of ectopically expressed FLAG-rpS3. If the ribosome exists in the immunoprecipitate of the anti-FLAG antibody, highly translated mRNA such as p53 must have been detected. Furthermore, we demonstrated that other ribosomal proteins such as rpS6 and rack1 were not present in the immunoprecipitate of the FLAG antibody (Fig. S2). In other words, the ribosome complex was not precipitated with anti-FLAG antibody. Therefore, we concluded that exogenously expressed rpS3 specifically interacts with its own mRNA.

MAJORITY OF FLAG-TAGGED RPS3 EXISTS IN CYTOPLASMIC AND NOT RIBOSOMAL FRACTIONS

Since most ribosomal proteins exist in the ribosome complex, except for a small portion in non-ribosomal fractions, we questioned whether or not exogenously expressed rpS3 enters into the ribosome complex. To demonstrate similar behavior between ectopically expressed FLAG-rpS3 and endogenous rpS3, we performed sucrose density gradient centrifugation. Simply, following incubation of the FLAG-rpS3 transfected 293T cells for the indicated times, cell lysates were separated on a 10–40% sucrose gradient by ultracentrifugation. The absorbance wavelength used to check the profile of the ribosome was 260 nm. As shown in supplemental Figure 3, ectopically expressed FLAG-rpS3 mainly exists in non-ribosomal fractions until 24 h. After 36 h of incubation, FLAG-rpS3 was partially translocated from non-ribosomal fractions into monosomal fractions. Therefore, ectopically expressed rpS3 also appeared to be translocated to the polysomal fractions.

Since the auto-regulation of ribosomal proteins frequently generates conditions that encourage over-production, we used 293T cells transiently transfected with Flag-rpS3 for the following experiment. Lysates from 293T cells were separated on a 10–40% sucrose gradient by centrifugation, while an absorbance of 260 nm was used for ribosome fractionations (Fig. 2A). To confirm that fractions were divided into polysome/monosome and cytosol, immunoblotting was performed for endogenous rpS3, Flag, and Erk (Fig. 2B). These results showed that 1–14 fractions contained polysomes or monosomes while 15–19 fractions were from the cytosol. As expected, the majority of transiently expressed Flag-rpS3 was found in the cytosolic fraction, as is seen in Figure 2B. From this result, it was concluded that most transiently expressed Flag-rpS3 was not ribosome-bound but present in free form. However, in time Flag-rpS3 entered into the nucleus and assembled into the ribosome.

3′-TERMINUS OF rpS3 mRNA INTERACTS WITH THE S3-C TERMINAL DOMAIN OF rpS3 AT THE N-TERMINUS

The mRNA of rpS3 consists of a 30 nt 5′-UTR, a 732 nt ORF and a 79 nt 3′-UTR. To test if any of these regions interact with the rpS3

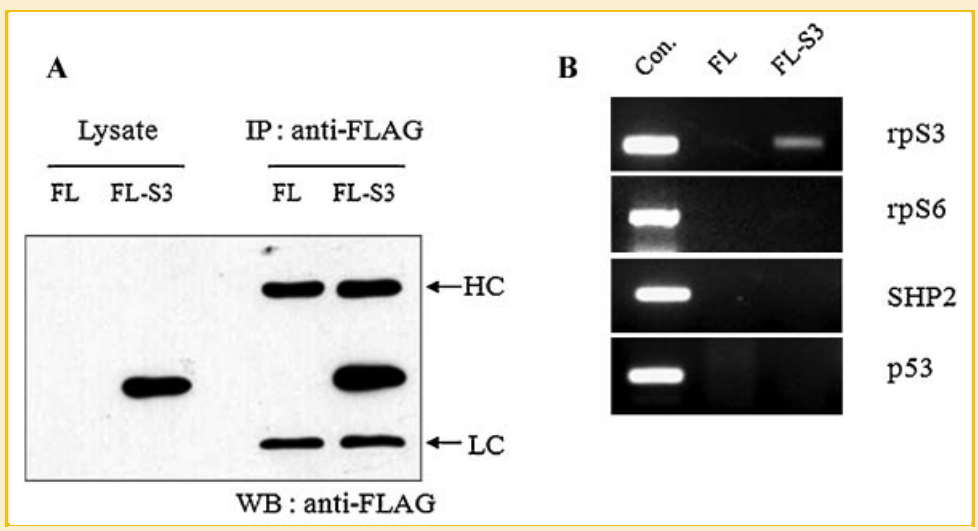

Fig. 1. Flag-tagged rpS3 binds to its own mRNA. (A): Human 293T cells were transfected with pcDNA3-Flag or pcDNA3-Flag-rpS3. Cell lysates (20 μg) were subjected to immunoblot analysis with anti-Flag antibody (left), followed by immunoprecipitation with anti-Flag antibody and immunoblot analysis with anti-Flag antibody (right). HC; IgG heavy chain, LC; IgG light chain. (B): RT-PCR amplification of mRNA associated with Flag-tagged rpS3. RNA samples were prepared as follows: For using a PCR control, total RNA (1 μg) was extracted from untransfected 293T cells (lane 1). RNAs that were immunoprecipitated with anti-Flag antibody from vector- (lane 2) and Flag-tagged S3- (lane 3) expressing 293T cells were extracted as described in Materials and Methods Section. The isolated RNAs were reverse-transcribed and amplified by PCR with rpS6-, p53-, SHP2- and rpS3-specific primers.

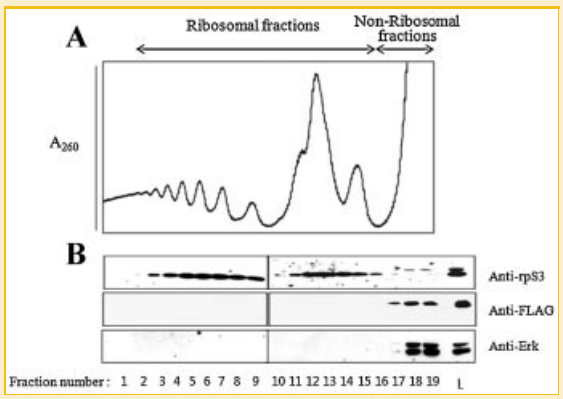

Fig. 2. Flag-tagged rpS3 exists mainly in non-ribosomal fractions. Lysates of 293T cells transiently transfected with Flag-tagged rpS3 were fractionated in a linear 10–40% sucrose gradient as described in Materials and Methods Section. Fraction 1 is from the bottom of gradient and fraction 19 is from the top of gradient. L; lysate (A) Absorbance of each fraction at 260 nm. (B): An aliquot of each fraction was analyzed by immunoblotting using anti-rpS3, anti-Flag anti-Erk antibodies.

protein, four ^{32}P-labeled transcripts were synthesized by in vitro transcription. Each transcript was subjected to the in vitro RNA binding assay with purified His-tagged rpS3 (Fig. 3A,B). The binding reactants were cross-linked by UV and resolved in SDS–PAGE. The RNA-protein complexes were then detected using a phosphor-imaging analyzer. For all constructs except vector and K1 (−30 to 336 bp) a specific band was visualized. Therefore, we concluded that the C-terminus of rpS3 mRNA (337–841 bp) interacts with its own protein (Fig. 3C).

RpS3 contains conserved amino acid sequences in its KH and S3-C terminal domains. The KH domain possesses similarity to several nucleic acid-binding proteins. For the S3-C domain, although its function in the ribosome is unclear, it possesses a hydrophobic region conserved from yeast to mammals. To investigate the binding of rpS3 protein to its mRNA, deletion mutants fused with immobilized GST were constructed (Fig. 4A,B). ^{32}P-labeled rpS3 mRNA transcribed in vitro was mixed with each deletion-GST mutant under RNA binding conditions. The binding activity was then estimated by scintillation counting and dot blot assay. As shown in Figure 4C, binding activity of the N-terminal S3-C domain was about 1.5-fold stronger than that of wild type. Except for GST-R, binding activities of the deletion mutants were around 50% of wild type whether or not mutants contained the KH domain.

Taken together, we conclude that the N-terminus of the S3-C terminal domain interacts independent of the KH domain with its own mRNA.

PROTEIN–RNA INTERACTION OCCURS IN CYTOSOLIC RATHER THAN RIBOSOMAL FRACTIONS

Since exogenously expressed rpS3 exists in free form, we investigated whether the protein–RNA interaction occurs in the ribosomal or cytosolic fraction. Lysates 293T cells transfected with Flag-tagged rpS3 were fractionated on a 20% sucrose cushion in order to separate the ribosomal fractions (R) from cytosolic fractions (C). To confirm that the ribosomal and cytosolic fractions were

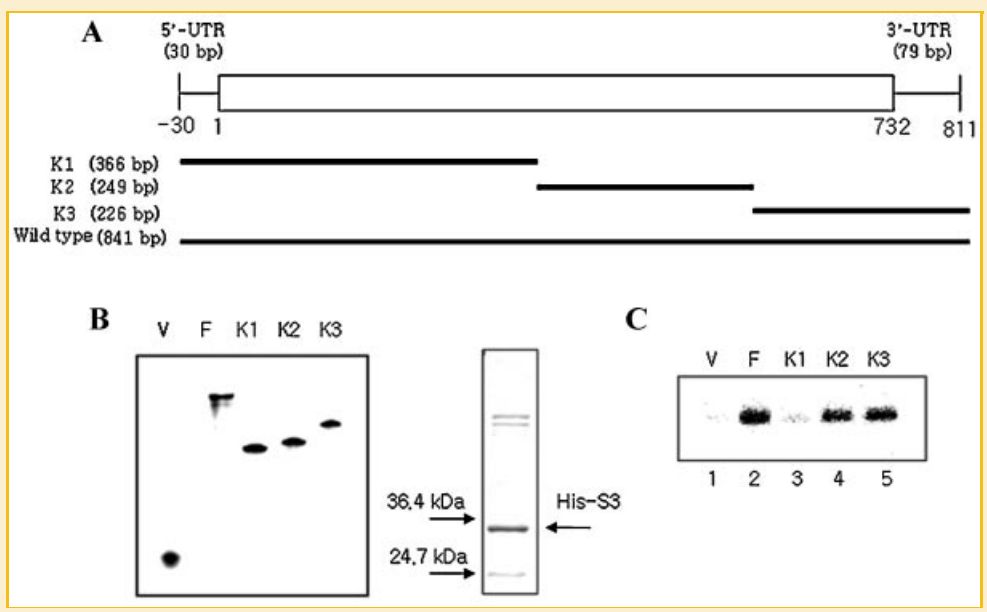

Fig. 3. RNA fragments and UV cross-linking analysis. The 841 nt rpS3 cDNA includes the coding region (732 nt) as well as 30 nt of the 5′-UTR and 79 nt of the 3′-UTR. (A): The cDNA fragment, -K1, -K2 or -K3 of each full-length cDNA was subcloned into the pGEM-T Easy vector downstream of the T7 promoter. (B): All in vitro RNA transcripts were resolved on a 5% polyacrylamide and 8 M urea gel (Left panel). Right panel is the Coomassie staining of purified His-tagged rpS3 protein (1 μg). (C): ^{32}P-radiolabeled rpS3 mRNA (160,000 cpm) was incubated with purified His-tagged rpS3 (2 μg) as described in Materials and Methods Section. After UV cross-linking, the reaction mixture was incubated with RNase A to digest the unprotected RNAs. The UV cross-linking complexes were then resolved on a 10% SDS–PAGE gel. Lane 1, Vector RNA; lane 2, Full-length wild type RNA; lane 3, K1 RNA; lane 4, K2 RNA; lane 5, K3 RNA.

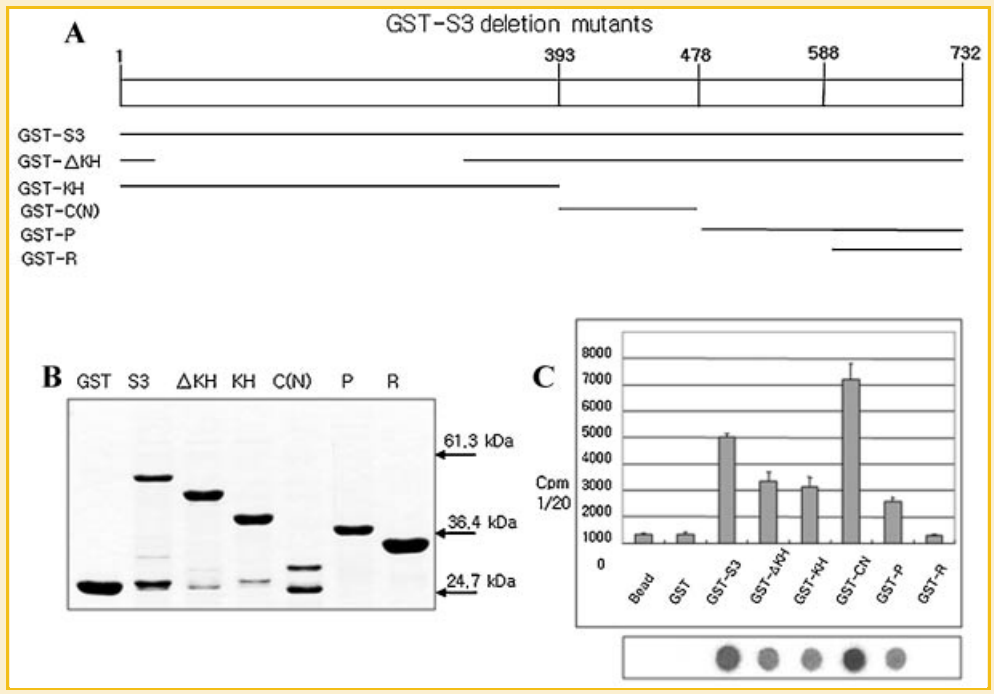

Fig. 4. Mapping analysis of the protein binding sites in rpS3 mRNA. (A): Schematic structure of the recombinant proteins GST-S3, GST-ΔKH, GST-KH, GST-C(N), GST-P, and GST-R. (B): GST fusion proteins were purified with glutathione-Sepharose 4B beads. One microgram of the protein was analyzed on 12% SDS–PAGE gel. (C) mRNA-binding activity of wild-type and mutant rpS3 proteins using in vitro binding assay. ^{32}P-radiolabeled rpS3 mRNA (160,000 cpm) was incubated with each of the recombinant proteins, which were previously immobilized on glutathione-Sepharose 4B beads as described in Materials and Methods Section. Each value represents the mean ± SD from six experiments and a twentieth of original values. Bead; GST-bead control.

indeed well-separated, RNAs were extracted from both fractions and resolved in an agarose gel containing 10% formaldehyde. 18S and 28S rRNA were found only in ribosomal fractions (Fig. 5A, upper panel) with no cross-contamination present. Both fractions were separated by SDS–PAGE and immunoblot analysis was performed (Fig. 5A, lower panel). As expected, the expression of Flag-tagged rpS3 is much higher in cytosolic fractions than in ribosomal fractions, and most endogenous rpS3 exists in ribosomal fractions. Additionally, immunobloting with anti-Erk antibody also confirmed ribosomal fractions were clearly separated from cytosolic fractions. Both fractions were continuously subjected to immunoprecipitation with anti-Flag antibody while RNA was extracted from the immunoprecipitate and RT-PCR was performed using oligo-dT_{15} and primers specific for rpS3 3′-UTR (Fig. 5B). Amplified rpS3 fragments were observed only in the cytosolic fractions of 293T cells expressing Flag-tagged rpS3. This result confirmed that rpS3 protein in the cytosol binds mainly to endogenous rpS3 mRNA.

TRANSLATION OF RpS3 IS REPRESSED BY INTERACTION OF rpS3 PROTEIN WITH ITS mRNA

From the previous data, it was established that rpS3 mRNA interacts with its own protein. What is the physiological role of this interaction in cells? It is general knowledge that over-produced ribosomal proteins self-regulate their amount via a feedback mechanism that involves interacting with their own mRNA. To confirm this interaction for rpS3, we performed in vitro translation experiments in which rpS3 trancripts were synthesized in capped form by guanylyltransferase, followed by ligation with poly-$(A)_{15}$ sequences at the 3′-UTR terminus. In vitro translation of the transcripts was performed, but upon addition of purified His-tagged rpS3 from *E.coli*, translation became inhibited in comparison with BSA (Fig. 6A, right panel). To exclude the possibility of global translational inhibition, total RNA extracted from 293T cells was incubated with rabbit reticulolysate in a similar manner. Results showed there was no change in the translational pattern of the total RNA (Fig. 6A, middle panel). And, the translational pattern of Firefly luciferase mRNA did not change either (Fig. 6A, left panel). In addition, we also confirmed that the inhibition occurred in a manner dependent on the amount His-tagged rpS3 protein (data not shown). Increasing amounts of His-tagged rpS3 further reduced the translation yield.

Upon the gradual addition of exogenous rpS3 protein, its endogenous level needed to be examined. Flag-rpS3 was transiently transfected into 293T cells by increasing the amount of DNA and the lysates were analyzed by Western blot (Fig. 6B, upper panel, Fig. S4A). The result showed that increased levels of exogenous rpS3 reduced the level of endogenous rpS3. To exclude transcriptional regulation, the RNA from each sample was analyzed by semi-quantitative RT-PCR using primers specific for endogenous rpS3 and GAPDH. The reactions were performed for 25 cycles, which is within the linear range of amplification (Fig. S4B). All lanes were similarly proportioned to two PCR products (Fig. 6B, lower panel). Therefore, rpS3 protein experiences translational repression through the interaction with its own mRNA.

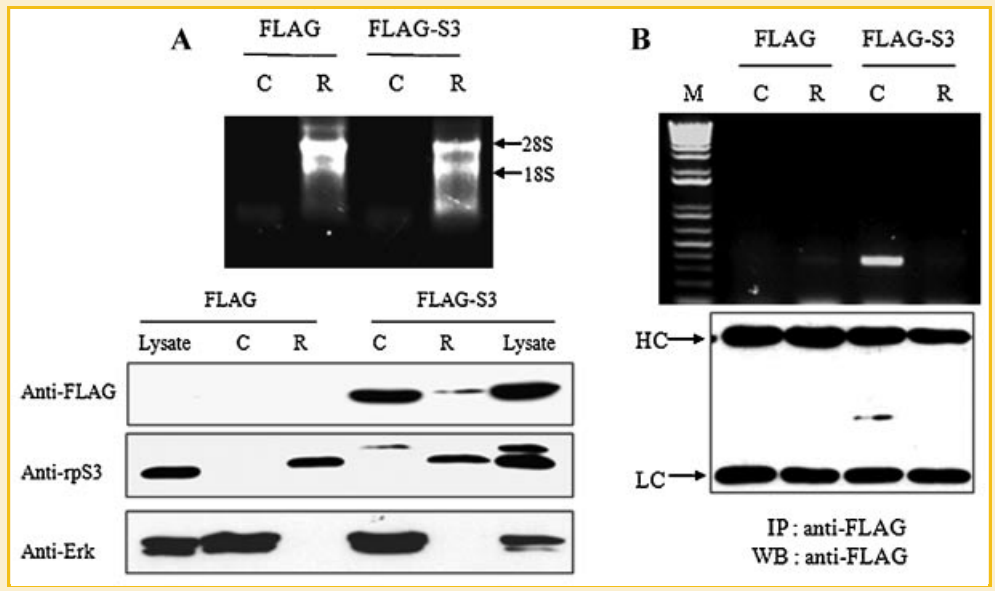

Fig. 5. The protein–RNA interaction occurs in cytoplasmic rather than ribosomal fractions. (A): Ectopically expressed rpS3 exists mainly in cytoplasmic fractions. Human 293T cells were transfected with pcDNA3-Flag or pcDNA3-Flag tagged rpS3 protein, followed by cell lysis. Ribosomes were precipitated by ultracentrifugation at 150,000*g* for 2 h through a sucrose cushion. To remove contaminating ribosomes, the supernatant was recentrifuged under the same conditions. Ribosomal and cytosolic fractions were subjected to immunoblot analysis with anti-rpS3, anti-Erk, and anti-Flag antibodies (lower panels). RNA was extracted from ribosomal and cytosolic fractions as described in Materials and Methods Section, then resolved on an agarose-formaldehyde gel under denaturing conditions. Ribosomal RNA was visualized by ethidium bromide staining (upper panel). (B): Ectopically expressed rpS3 only interacts with its own mRNA in the cytoplasmic fraction. Samples from each fraction were immunoprecipitated with anti-Flag antibody followed by immunoblot analysis with anti-Flag antibody (lower panel). RNAs were extracted from the immunoprecipitates, reverse-transcribed and then amplified by PCR with rpS3-specific primers (upper panel). HC; Ig heavy chain, LC; Ig light chain, M; molecular marker, C; cytosoplasmic fraction, R; ribosomal fraction.

DISCUSSION

Many ribosomal proteins possess extra-ribosomal functions despite also being components of the ribosome complex [Wool, 1996; Chen and Ioannou, 1999; Naora, 1999; Oh et al., 2002; Zimmermann, 2003]. As RNPs, ribosomal proteins that interact with rRNA and mRNA have been identified in prokaryotes and eukaryotes where they play important roles in mRNA stabilization, splicing, and control of translation. The RNA-binding domains of ribosomal proteins are diverse as other RNA-binding proteins [Bycroft et al., 1997; Draper and Reynaldo, 1999; Jones et al., 2001; Liu et al., 2003]. The K homolog (KH) domain is a well-characterized RNA-binding domain consisting of 70–100 amino acids that was originally identified as a repeated sequence in heterogeneous nuclear ribonucleoprotein K (hnRNP K) [Siomi et al., 1993; Klimek-Tomczak et al., 2004]. Sequence similarity analysis detected the KH domain in many proteins and classified it as types I and II, according to the protein folding involved [Grishin, 2001]. In addition, RpS3 has a type II KH domain in its N-terminus.

In this report, we revealed through RNP immunoprecipitation and RT-PCR that rpS3 protein interacts with its own mRNA. To determine which domain of the mRNA is responsible for protein binding, we performed an in vitro binding assay with rpS3 protein deletion mutants. Our data in Figure 4 surprisingly demonstrated that even in the presence of the KH domain, the N-terminus of the S3-C terminal domain [S3-C (N)] is a major binding site for its own mRNA. Since the deletion mutants in our experiment may not have been in their native conformation, it is entirely possible the S3-C (N) binding epitope is partly buried inside the rpS3 protein. In addition, we tried to determine which region of the rpS3 mRNA binds to the protein. A UV cross-linking assay and EMSA (data not shown) found that the binding region is located in the 3′-untranslated region (UTR) of the rpS3 mRNA, between base pairs 337 to 841. Translational control mediated by mRNA binding proteins usually involves mRNA binding regions located in the 5′- or 3′-UTR [Mazumder et al., 2003b]. Also, our results indicate that the S3-C terminal domain and C-terminus of rpS3 mRNA were important for this interaction. According to RNA structure analysis by the MFOLD prediction [Zuker, 2003], two common structural motives (a and b) having an internal loop and a hairpin loop, respectively, were found in K3 RNA. And two similar motives (a′ and b′) were also found in K2 RNA as well. They have significant sequence similarities with 37.5% homology between a and a′, 47.4% homology between b and b′ (Fig. S5). In spite of reports detailing the role of the S3-C terminal domain in eukaryotes, its function has not been fully established. In this study, we propose that the conservative S3-C terminal domain might play a role in translational auto-regulation. Therefore, further studies such as RNase foot-printing experiments are needed to identify the accurate RNA-binding regions.

It is now clear that most ribosomal proteins exist in the ribosome complex with only a small portion present in non-ribosomal fractions [Spence et al., 2000; Mazumder et al., 2003a]. Therefore, there is only a small quantity of protein in the cytosol, making it relatively hard to study the extra-ribosomal function of rpS3 in cells. Sucrose gradient fractionation found that ectopically expressed rpS3 exists in free form in cytosolic fractions as opposed

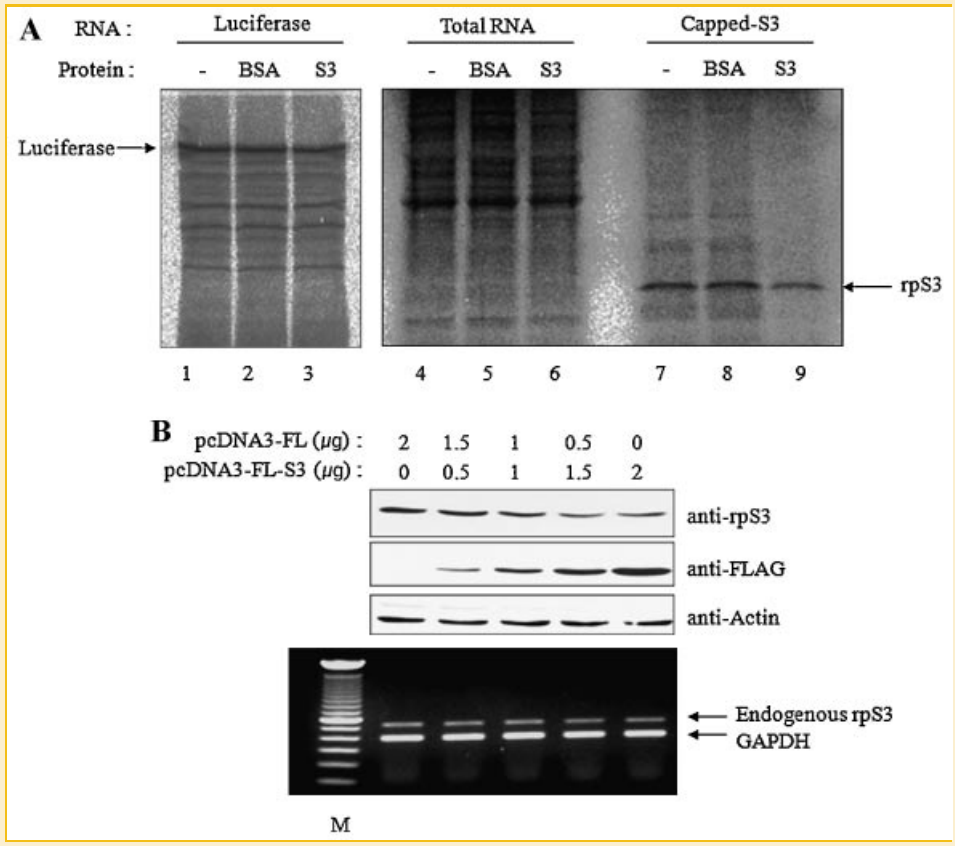

Fig. 6. In vitro and in vivo inhibition of rpS3 translation caused by interaction with its own mRNA. (A): Translational inhibition of capped rpS3 mRNA. Right panel; 0.25 μg of capped rpS3 mRNA were incubated with rabbit reticulolysate in the absence (lane 7) or presence of 4 μg of BSA (lane 8), or purified His-tagged rpS3 (lane 9), as described in Materials and Methods Section. Left panel; The luciferase mRNA (0.5 μg) was incubated with rabbit reticulolysate as described above. Middle panel; Total RNA extracted from 293T cells was incubated with rabbit reticulolysate in the same manner. (B): In vivo inhibition of translation is not dependent on transcription. Human 293T cells grown in 25 mm culture dishes were co-transfected with pcDNA3-Flag and pcDNA3-Flag-rpS3. The amount of plasmid used in the transfections is indicated at the top of the panel. Immunoblot analysis was performed on the cell lysate (20 μg) with anti-Flag, anti-rpS3 and anti-actin antibodies (upper panel). Total RNA was extracted from each sample and semi-quantitative RT-PCR was performed with two primer pairs; rpS3-specific (rpS3-F2 and rpS3-R) and GAPDH-specific (lower panel), as an internal control. M; molecular marker.

to ribosomal fractions. Since the ribosome itself interacts with mRNA during translation, any result produced by RNP immunoprecipitation may be a false positive result. To exclude that possibility, we performed RNP immunoprecipitation in both fractions and found that the interaction occurs only in the cytosolic fraction. Therefore, we conclude that free rpS3 proteins interact with their own mRNA.

Many RNA binding proteins including ribosomal proteins interact with their own mRNAs resulting in translational repression [Standart and Jackson, 1994; Zengel and Lindahl, 1994; Tai et al., 2002; Serganov et al., 2003]. For example, when yeast rpS14 accumulates in excess of its assembly partners, only then is it available to interact with *RPS14B* pre-mRNA and prevent gene expression [Fewell and Woolford, 1999; Antunez de Mayolo and Woolford, 2003]. In vitro translation experiments have shown that recombinant His-tagged rpS3 inhibits the specific translation of capped rpS3 mRNA, possibly due to global as opposed to specific translational inhibition. To examine whether this inhibition is global or specific to rpS3, we performed an in vitro translation with total RNA from 293T cells. If global translation inhibition occurs, the addition of His-tagged rpS3 will result in the disappearance of almost all translated product. However, there was no change in translated products, indicating that translational inhibition is specific for rpS3. Although the ribosome filter hypothesis proposes that specific interactions between mRNA and sites on the ribosomal subunit are important for translational control [Brandt and Gualerzi, 1992; Dontsova et al., 1992; Mauro and Edelman, 2002], our results demonstrated that in addition this interaction specifically inhibits its own translation.

From these data, we propose that rpS3 possesses a feedback mechanism in which its own expression is repressed. When there exists an excess amount of free rpS3, interaction between rpS3 and its mRNA results in the repression of translation. The precise mechanism by which the interaction blocks translation has not yet been identified, but two possibilities can be considered; First, rpS3 interacts with eukaryotic initiation factors [Westermann et al., 1979; Tolan et al., 1983; Westermann and Nygard, 1984] and aminoacyl-tRNA [Ohsawa and Gualerzi, 1983]. Second, rpS3 interacts with other ribosomal proteins in the ribosome [Ramakrishnan et al., 1986]. Therefore, we can imagine that free rpS3 binds either its own

mRNA or initiation factors such as eIF2 and eIF3, along with other ribosomal proteins such as rpS5 and rpS10. Thus, these interactions will inhibit the initiation and elongation of translation. Ultimately, if the feedback mechanism of rpS3 remains unregulated in cells, it may influence ribosome biogenesis, which could affect cell fate operations such as proliferation and apoptosis.

REFERENCES

Antunez de Mayolo P, Woolford JL, Jr. 2003. Interactions of yeast ribosomal protein rpS14 with RNA. J Mol Biol 333:697–709.

Brandt R, Gualerzi CO. 1992. Ribosomal localization of the mRNA in the 30S initiation complex as revealed by UV crosslinking. FEBS Lett 311:199–202.

Bycroft M, Hubbard TJ, Proctor M, Freund SM, Murzin AG. 1997. The solution structure of the S1 RNA binding domain: A member of an ancient nucleic acid-binding fold. Cell 88:235–242.

Chen FW, Ioannou YA. 1999. Ribosomal proteins in cell proliferation and apoptosis. Int Rev Immunol 18:429–448.

Dontsova OA, Rosen KV, Bogdanova SL, Skripkin EA, Kopylov AM, Bogdanov AA. 1992. Identification of the *Escherichia coli* 30S ribosomal subunit protein neighboring mRNA during initiation of translation. Biochimie 74:363–371.

Draper DE, Reynaldo LP. 1999. RNA binding strategies of ribosomal proteins. Nucleic Acids Res 27:381–388.

Fewell SW, Woolford JL, Jr. 1999. Ribosomal protein S14 of *Saccharomyces cerevisiae* regulates its expression by binding to RPS14B pre-mRNA and to 18S rRNA. Mol Cell Biol 19:826–834.

Grabowski DT, Deutsch WA, Derda D, Kelley MR. 1991. Drosophila AP3, a presumptive DNA repair protein, is homologous to human ribosomal associated protein P0. Nucleic Acids Res 19:4297.

Grishin NV. 2001. KH domain: One motif, two folds. Nucleic Acids Res 29:638–6343.

Hegde V, Kelley MR, Xu Y, Mian IS, Deutsch WA. 2001. Conversion of the bifunctional 8-oxoguanine/beta-delta apurinic/apyrimidinic DNA repair activities of Drosophila ribosomal protein S3 into the human S3 monofunctional beta-elimination catalyst through a single amino acid change. J Biol Chem 276:27591–27596.

Ivanov AV, Malygin AA, Karpova GG. 2005. Human ribosomal protein S26 suppresses the splicing of its pre-mRNA. Biochim Biophys Acta 1727:134–140.

Jones S, Daley DT, Luscombe NM, Berman HM, Thornton JM. 2001. Protein-RNA interactions: A structural analysis. Nucleic Acids Res 29:943–954.

Jung SO, Lee JY, Kim J. 2001. Yeast ribosomal protein S3 has an endonuclease activity on AP DNA. Mol Cells 12:84–90.

Kanno S, Iwai S, Takao M, Yasui A. 1999. Repair of apurinic/apyrimidinic sites by UV damage endonuclease; a repair protein for UV and oxidative damage. Nucleic Acids Res 27:3096–3103.

Kim J, Chubatsu LS, Admon A, Stahl J, Fellous R, Linn S. 1995. Implication of mammalian ribosomal protein S3 in the processing of DNA damage. J Biol Chem 270:13620–13629.

Kim TS, Jang CY, Kim HD, Lee JY, Ahn BY, Kim J. 2006. Interaction of Hsp90 with ribosomal proteins protects from ubiquitination and proteasome-dependent degradation. Mol Biol Cell 17:824–833.

Kim TS, Kim HD, Kim J. 2009a. PKCdelta-dependent functional switch of rpS3 between translation and DNA repair. Biochim Biophys Acta 1793:395–405.

Kim TS, Kim HD, Shin HS, Kim J. 2009b. Phosphorylation status of nuclear ribosomal protein S3 is reciprocally regulated by protein kinase C{delta} and protein phosphatase 2A. J Biol Chem 284:21201–21208.

Klimek-Tomczak K, Wyrwicz LS, Jain S, Bomsztyk K, Ostrowski J. 2004. Characterization of hnRNP K protein-RNA interactions. J Mol Biol 342:1131–1141.

Lee CH, Kim SH, Choi JI, Choi JY, Lee CE, Kim J. 2002. Electron paramagnetic resonance study reveals a putative iron-sulfur cluster in human rpS3 protein. Mol Cells 13:154–156.

Liu W, Seto J, Sibille E, Toth M. 2003. The RNA binding domain of Jerky consists of tandemly arranged helix-turn-helix/homeodomain-like motifs and binds specific sets of mRNAs. Mol Cell Biol 23:4083–4093.

Loflin P, Chen CY, Shyu AB. 1999. Unraveling a cytoplasmic role for hnRNP D in the in vivo mRNA destabilization directed by the AU-rich element. Genes Dev 13:1884–1897.

Mager WH. 1988. Control of ribosomal protein gene expression. Biochim Biophys Acta 949:1–15.

Malygin AA, Parakhnevitch NM, Ivanov AV, Eperon IC, Karpova GG. 2007. Human ribosomal protein S13 regulates expression of its own gene at the splicing step by a feedback mechanism. Nucleic Acids Res 35:6414–6423.

Matter N, Herrlich P, Konig H. 2002. Signal-dependent regulation of splicing via phosphorylation of Sam68. Nature 420:691–695.

Mauro VP, Edelman GM. 2002. The ribosome filter hypothesis. Proc Natl Acad Sci USA 99:12031–12036.

Mazumder B, Sampath P, Seshadri V, Maitra RK, DiCorleto PE, Fox PL. 2003a. Regulated release of L13a from the 60S ribosomal subunit as a mechanism of transcript-specific translational control. Cell 115:187–198.

Mazumder B, Seshadri V, Fox PL. 2003b. Translational control by the 3'-UTR: The ends specify the means. Trends Biochem Sci 28:91–98.

Mili S, Shu HJ, Zhao Y, Pinol-Roma S. 2001. Distinct RNP complexes of shuttling hnRNP proteins with pre-mRNA and mRNA: candidate intermediates in formation and export of mRNA. Mol Cell Biol 21:7307–7319.

Naora H. 1999. Involvement of ribosomal proteins in regulating cell growth and apoptosis: Translational modulation or recruitment for extraribosomal activity? Immunol Cell Biol 77:197–205.

Naora H, Takai I, Adachi M. 1998. Altered cellular responses by varying expression of a ribosomal protein gene: sequential coordination of enhancement and suppression of ribosomal protein S3a gene expression induces apoptosis. J Cell Biol 141:741–753.

Neumann F, Hemmerich P, von Mikecz A, Peter HH, Krawinkel U. 1995. Human ribosomal protein L7 inhibits cell-free translation in reticulocyte lysates and affects the expression of nuclear proteins upon stable transfection into Jurkat T-lymphoma cells. Nucleic Acids Res 23:195–202.

Oh HS, Kwon H, Sun SK, Yang CH. 2002. QM, a putative tumor suppressor, regulates proto-oncogene c-yes. J Biol Chem 277:36489–36498.

Ohsawa H, Gualerzi C. 1983. Chemical modification in situ of Escherichia coli 30 S ribosomal proteins by the site-specific reagent pyridoxal phosphate. Inactivation of the aminoacyl-tRNA and mRNA binding sites. J Biol Chem 258:150–156.

Passmore LA, Schmeing TM, Maag D, Applefield DJ, Acker MG, Algire MA, Lorsch JR, Ramakrishnan V. 2007. The eukaryotic translation initiation factors eIF1 and eIF1A induce an open conformation of the 40S ribosome. Mol Cell 26:41–50.

Polakiewicz RD, Munroe DJ, Sait SN, Tycowski KT, Nowak NJ, Shows TB, Housman DE, Page DC. 1995. Mapping of ribosomal protein S3 and internally nested snoRNA U15A gene to human chromosome 11q13.3-q13.5. Genomics 25:577–580.

Ramakrishnan V, Graziano V, Capel MS. 1986. A role for proteins S3 and S14 in the 30 S ribosomal subunit. J Biol Chem 261:15049–15052.

Serganov A, Polonskaia A, Ehresmann B, Ehresmann C, Patel DJ. 2003. Ribosomal protein S15 represses its own translation via adaptation of an rRNA-like fold within its mRNA. EMBO J 22:1898–1908.

Shin HS, Jang CY, Kim HD, Kim TS, Kim S, Kim J. 2009. Arginine methylation of ribosomal protein S3 affects ribosome assembly. Biochem Biophys Res Commun 385:273–278.

Siomi H, Matunis MJ, Michael WM, Dreyfuss G. 1993. The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. Nucleic Acids Res 21:1193–1198.

Spence J, Gali RR, Dittmar G, Sherman F, Karin M, Finley D. 2000. Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain. Cell 102:67–76.

Standart N, Jackson RJ. 1994. Regulation of translation by specific protein/mRNA interactions. Biochimie 76:867–879.

Tai N, Ding Y, Schmitz JC, Chu E. 2002. Identification of critical amino acid residues on human dihydrofolate reductase protein that mediate RNA recognition. Nucleic Acids Res 30:4481–4488.

Tasheva ES, Roufa DJ. 1995. Regulation of human RPS14 transcription by intronic antisense RNAs and ribosomal protein S14. Genes Dev 9:304–316.

Tolan DR, Hershey JW, Traut RT. 1983. Crosslinking of eukaryotic initiation factor eIF3 to the 40S ribosomal subunit from rabbit reticulocytes. Biochimie 65:427–436.

Wan F, Anderson DE, Barnitz RA, Snow A, Bidere N, Zheng L, Hegde V, Lam LT, Staudt LM, Levens D, Deutsch WA, Lenardo MJ. 2007. Ribosomal protein S3: A KH domain subunit in NF-kappaB complexes that mediates selective gene regulation. Cell 131:927–939.

Westermann P, Nygard O. 1984. Cross-linking of mRNA to initiation factor eIF-3, 24 kDa cap binding protein and ribosomal proteins S1, S3/3a, S6 and S11 within the 48S pre-initiation complex. Nucleic Acids Res 12:8887–8897.

Westermann P, Heumann W, Bommer UA, Bielka H, Nygard O, Hultin T. 1979. Crosslinking of initiation factor eIF-2 to proteins of the small subunit of rat liver ribosomes. FEBS Lett 97:101–104.

Wool IG. 1996. Extraribosomal functions of ribosomal proteins. Trends Biochem Sci 21:164–165.

Wool IG, Chan YL, Gluck A. 1995. Structure and evolution of mammalian ribosomal proteins. Biochem Cell Biol 73:933–947.

Wormington M. 1991. Preparation of synthetic mRNAs and analyses of translational efficiency in microinjected Xenopus oocytes. Methods Cell Biol 36:167–183.

Yacoub A, Augeri L, Kelley MR, Doetsch PW, Deutsch WA. 1996. A Drosophila ribosomal protein contains 8-oxoguanine and abasic site DNA repair activities. EMBO J 15:2306–2312.

Zengel JM, Lindahl L. 1994. Diverse mechanisms for regulating ribosomal protein synthesis in *Escherichia coli*. Prog Nucleic Acid Res Mol Biol 47:331–370.

Zhang Y, Wolf GW, Bhat K, Jin A, Allio T, Burkhart WA, Xiong Y. 2003. Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. Mol Cell Biol 23:8902–8912.

Zimmermann RA. 2003. The double life of ribosomal proteins. Cell 115:130–132.

Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31:3406–3415.