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Human Ribosomal Protein L18a Interacts with hnRNP E1

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Abstract: Heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) is one of the primary pre-mRNA binding proteins in human cells. It consists of 356 amino acid residues and harbors three hnRNP K homology (KH) domains that mediate RNA-binding. The hnRNP E1 protein was shown to play important roles in mRNA stabilization and translational control. In order to enhance our understanding of the cellular functions of hnRNP E1, we searched for interacting proteins through a yeast two-hybrid screening while using HeLa cDNA library as target. One of the cDNA clones was found to be human ribosomal protein L18a cDNA (GenBank accession number BC071920). We demonstrated in this study that human ribosomal protein L18a, a constituent of ribosomal protein large subunit, interacts specifically with hnRNP E1 in the yeast two-hybrid system. Such an interaction was observed for the first time in this study, and was also verified by biochemical assay.

Key words: heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1, (poly(rC) binding protein 1), hnRNP K homology domain (KH domain), yeast two-hybrid system, ribosomal protein L18a

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are extremely abundant nuclear proteins and play important roles in various steps of pre-mRNA and mRNA metabolism, including transcriptional activation, mRNA processing, mRNA export, translation, and mRNA stability (Dreyfuss et al., 1993; Krecic and Swanson, 1999). The hnRNPs in human nuclei are composed of about 30 proteins and named as hnRNP A1 through U. (Choi and Dreyfuss, 1984; Krecic and Swanson, 1999). They are avid RNA-binding proteins and have their own independent RNA-binding activities and RNA sequence preferences (Dreyfuss et al., 1993).

hnRNP E1 is one of the major components of hnRNP particle. It binds to pyrimidine-rich sequence with high affinity and is one of the main poly(rC)-binding proteins in human cells. The hnRNP E1 protein is also known as αCP1 and poly(rC)-binding protein 1 (PCBP1) (Kiledjian et al., 1995; Leffers et al., 1995). It consists of 356 amino acid residues and bears a highly conserved triple repeats of the hnRNP K homology (KH) domains that mediate RNA-binding. hnRNP E1 is a ubiquitously expressed, nuclear protein. However, it is not restricted to the nucleus, and instead shuttles between the nucleus and the cytoplasm (Michael et al., 1995). hnRNP E1 was identified as a component of the RNP complexes that assemble at CU-rich region in the 3' UTR to stabilize mRNA for α-globin, collagen IA1 α-chain, tyrosine hydroxylase or erythropoietin (Weiss and Liebharber, 1995; Kiledjian et al., 1995; Holcik and Liebhaber, 1997; Paulding and Czyzyk-Krzeska, 1999; Czyzyk-Krzeska and Bendixen, 1999). hnRNP E1 was shown to be associated with translational silencing of 15-lipoxygenase mRNA in early erythroid precursor cells by binding to differentiation control element (DICE) in the 3' UTR (Ostareck et al., 1997; Ostareck et al., 2001). The protein has also been determined to be an essential factor of polioviral tralslation by binding to 5'UTR of viral mRNA (Gamarnik and Adino, 1997). Moreover, the hnRNP E1 protein was demonstrated to be phosphorylated by human p21-activated kinase 1 (Pak 1), and its phosphorylation was correlated to the increased translation of DICE-containing mRNA by releasing its binding and translational inhibition from target mRNA (Meng et al., 2007).

In order to understand the hnRNP E1 functions more precisely, we have attempted to detect hnRNP E1-interacting proteins in a HeLa cDNA library using a yeast two-hybrid screening system. We have identified several independent cDNA clones and analyzed their nucleotide sequences and the predicted amino acid sequences. One of these clones evidenced sequence identity with the human ribosomal protein L18a cDNA (GenBank accession number BC071920). Human ribosomal protein L18a (RPL18a) is a constituent of 60S subunit which is a ribosomal protein large subunit. This protein was demonstrated as one of the ribosomal

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proteins that interact with nucleolin (Bouvet et al., 1998). Our research is the first that showed, using both yeast two-hybrid and *in vitro* pull-down techniques, that human ribosomal protein L18a interacts specifically with hnRNP E1.

MATERIALS AND METHODS

Plasmid constructions

The procedures for the construction of pGBT9/hnRNP E1 (2~356) and pACT2/hnRNP E1 (2~356) plasmids were reported by Choi (Choi, 2008). The plasmid pGAD424/hnRNP L (1~558) was kindly provided by Dr. S K Jang at Pohang University of Science and Technology (POSTECH) (Hahm et al., 1998; Kim et al., 2000). The procedures for the creation of plasmids pGBT9/hnRNP A1 (1~320) and pGAD424/hnRNP A1 (1~320) were described previously (Kim et al., 2000). The original clone #31, pGAD10/RPL18a (2~176+3'UTR), isolated from a HeLa cDNA library using yeast two-hybrid screening system includes the DNA fragment harboring the RPL18a coding sequences corresponding to amino acids 2~176. This plasmid was used for the *in vivo* analysis of the interaction occurring between hnRNP E1 and RPL18a.

In order to construct pGEX-KG/hnRNP E1 (2~356) plasmid, the pACT2/hnRNP E1 (2~356) plasmid was digested with *Eco*RI and *Xho*I. The DNA fragment including the hnRNP E1 coding sequence corresponding to amino acids 2~356 was inserted into the similarly treated pGEX-KG vector. The procedures for the construction of pTM1/hnRNP E1 (2~356) plasmid was previously described (Choi, 2008). For the creation of pCS3MT/RPL18a (2~176) plasmid, pGAD10/RPL18a (2~176+3'UTR) plasmid was digested with *Eco*RI and *Xho*I. The DNA fragment including the RPL18a coding sequence corresponding to amino acids 2~176 was inserted into the *Eco*RI and *Xho*I sites of pCS3MT vector (Turner and Weintraub, 1994).

Yeast strain and media

We followed yeast two-hybrid screening procedure as specified in the manufacturer's protocols (Clontech, Inc.). The pGBT9 plasmid was employed as a source of the GAL4 DNA-binding domain (DB) and encodes *TRP1* as a selectable marker gene. The pGAD424 and pGAD10 plasmids were employed as sources of the GAL4 activation domain (Ac) and also encode *LEU2* as a selectable marker gene. The *Saccharomyces cerevisiae* strain HF7c (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trpl-901, leu2-3, 112, gal4-542, gal80-538, cyh^{r}2, LYS2::GAL1$_{uas}$-GAL$_{tata}$-HIS3, URA3::GAL4$_{17mers}$(X3)-CyCl$_{tata}$-laZ*) was used for yeast transformation. HF7c encodes HIS3 and β-galactosidase as reporters and contains *trp*1, *leu*2, and *cyh^r*2 as marker genes (Feilotter et al., 1994). Transformed yeast cells were grown in minimal SD medium that allow maintenance of plasmids whereas untransformed yeast cells were grown in YEPD.

Yeast two-hybrid screen

HF7c was transformed with pGBT9/hnRNP E1 (2~356) cDNA and HeLa cDNA library, unidirectionally cloned into the *Eco*RI and *Xho*I sites of pGAD10. Transformants were plated onto synthetic medium (SD) lacking leucine, tryptophan, and histidine. The colonies grew on Trp$^-$ Leu$^-$ His$^-$ plates were transferred to filter paper and lysed in liquid nitrogen. Then the colonies on the filter were assayed for β-galactosidase activity by incubating in the Z buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$] containing 0.82 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). We rescued the library plasmid from positive clones which showed both β-galactosidase$^+$ and His$^+$. These library plasmids were transformed into and recovered from *E. coli* JBE181 and were then analyzed by restriction digestion with *Eco*RI and *Xho*I. In order to confirm that transcriptional activation was dependent on the presence of both DB-hnRNP E1 and Ac-library protein, the library plasmid was retransformed into HF7c strains with either pGBT9/hnRNP E1 (2~356) or pGBT9. Clones' being true positive was verified by the fact that they are able to transactivate *HIS3* and *LacZ* reporters when co-transforming HF7c only with pGBT9/hnRNP E1 (2~356). Plasmid inserts were sequenced (GenoTech, Korea; Bionex, Korea), and the sequences of them were identified through comparison with the GeneBank sequence data bank.

Production of proteins and glutathione S-transferase pull-down assay

The GST (glutathione S-transferase) and GST-hnRNP E1 (2~356) were expressed in *E. coli* BL21(DE3)pLysS from the pGEX-KG and pGEX-KG/hnRNP E1 (2~356) plasmids, respectively. The induction of proteins and preparation of bacterial cell lysates were performed according to the published procedures reported by Park et al (Park et al., 2007).

The [^{35}S]-labeled proteins were synthesized in a rabbit reticulocyte lysate (Promega) via an *in vitro* transcription-translation reaction in the presence of [^{35}S] methionine and cysteine (Perkin Elmer Life Sciences, Inc.). The plasmids pTM1/hnRNP E1 (2~356) and pCS3MT/RPL18a (2~176) were used in order to obtain [^{35}S]-labeled hnRNP E1 and RPL18a, respectively. The *in vitro* pull-down assay was carried out as explained previously (Choi, 2008; Park et al., 2007).

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                                                    (nt#1)  CGAACGCGGAGAGCACGCC  (nt#19)
┌─
ATG AAG GCC TCG GGC ACG CTA CGA GAG TAC AAG GTA GTG GGT CGC TGC CTG CCC ACC CCC  (nt#79)
Met Lys Ala Ser Gly Thr Leu Arg Glu Tyr Lys Val Val Gly Arg Cys Leu Pro Thr Pro   20

AAA TGC CAC ACG CCG CCC CTC TAC CGC ATG CGA ATC TTT GCG CCT AAT CAT GTC GTC GCC
Lys Cys His Thr Pro Pro Leu Tyr Arg Met Arg Ile Phe Ala Pro Asn His Val Val Ala   40

AAG TCC CGC TTC TGG TAC TTT GTA TCT CAG TTA AAG AAG ATG AAG AAG TCT TCA GGG GAG
Lys Ser Arg Phe Trp Tyr Phe Val Ser Gln Leu Lys Lys Met Lys Lys Ser Ser Gly Glu   60

ATT GTC TAC TGT GGG CAG GTG TTT GAG AAG TCC CCC CTG CGG GTG AAG AAC TTC GGG ATC
Ile Val Tyr Cys Gly Gln Val Phe Glu Lys Ser Pro Leu Arg Val Lys Asn Phe Gly Ile   80

TGG CTG CGC TAT GAC TCC CGG AGC GGC ACC CAC AAC ATG TAC CGG GAA TAC CGG GAC CTG
Trp Leu Arg Tyr Asp Ser Arg Ser Gly Thr His Asn Met Tyr Arg Glu Tyr Arg Asp Leu  100

ACC ACC GCA GGC GCT GTC ACC CAG TGC TAC CGA GAC ATG GGT GCC CGG CAC CGC GCC CGA
Thr Thr Ala Gly Ala Val Thr Gln Cys Tyr Arg Asp Met Gly Ala Arg His Arg Ala Arg  120

GCC CAC TCC ATT CAG ATC ATG AAG GTG GAG GAG ATC GCG GCC AGC AAG TGC CGC CGG CCG
Ala His Ser Ile Gln Ile Met Lys Val Glu Glu Ile Ala Ala Ser Lys Cys Arg Arg Pro  140

GCT GTC AAG CAG TTC CAC GAC TCC AAG ATC AAG TTC CCG CTG CCC CAC CGG GTC CTG CGC
Ala Val Lys Gln Phe His Asp Ser Lys Ile Lys Phe Pro Leu Pro His Arg Val Leu Arg  160

CGT CAG CAC AAG CCA CGC TTC ACC ACC AAG AGG CCC AAC ACC TTC TTC TAG GTGCAGGGCCC  (nt#561)
Arg Gln His Lys Pro Arg Phe Thr Thr Lys Arg Pro Asn Thr Phe Phe  *                 176
                                                                ┐
CTCGTCCGGGTGTGCCCCAAATAAACTCAGGAACGCCCCGGTGAAAAAAAAAAAAAAAAAAA┘                (nt#622)
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Fig. 1. The nucleotide sequences and the deduced amino acid sequences of human ribosomal protein L18a. The nucleotide sequence is numbered from nucleotide #1 of RPL18a cDNA. A putative initiation codon ATG at nt 20-22 and the stop codon of the open reading frame TAG at nt 548-550 are shown. An open reading frame comprised of 176 amino acid residues is shown. The arrows show the starting point (nt#21) and end point (nt#622) of the original library plasmid, clone #31.

RESULTS AND DISCUSSION

Human ribosomal protein L18a is an hnRNP E1-interacting protein

In order to find proteins interact with hnRNP E1, a yeast two-hybrid screening system was carried out. The hnRNP E1 cDNA fused to the DNA-binding domain of the GAL4 yeast transcription factor (DB-hnRNP E1) was employed as the 'bait'. HeLa cDNA library fused to the GAL4 activation domain (Ac-library protein) was utilized as the 'target'. Approximately 1.7×10^6 transformants were screened by conducting the selection in the HF7c yeast strain harboring both *HIS3* and *LacZ* reporter genes. 35 of the *HIS3* gene-positive clones exhibited expression of *LacZ* gene. The library plasmids isolated from positive clones were amplified and analyzed by agarose gel electrophoresis after restriction digestion. The library plasmids that enabled β-galactosidase activity and histidine prototrophy to reporter strains only in the presence of DB-hnRNP E1 were considered to be true positive. Plasmid inserts from true positives were sequenced and the sequences were identified by a BLAST search. Our results showed that one of the positive clones, clone #31, harbored cDNA encoding human ribosomal protein L18a (GenBank accession number BC071920). The cDNA of the human ribosomal protein L18a is comprised of 622 nucleotides and bears a single open reading frame of 531 nucleotides. This coding sequence, located in between

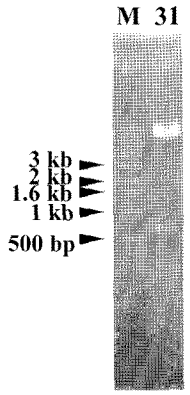
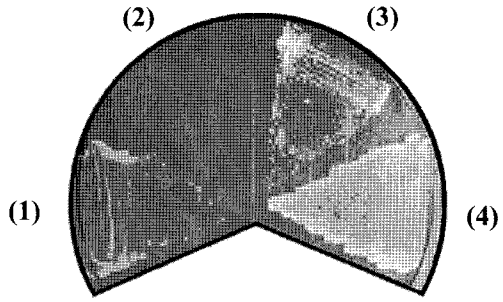


Fig. 2. Analysis of insert DNA of library plasmid. Library plasmid, #31 was rescued from the β-Gal$^+$ and His$^+$ yeast colony and transformed into *E. coli* JBE181. Bacterial colonies were grown on M63-Leu plate. The plasmid DNA was purified and the size of insert DNA was analyzed on the 0.8% agarose gel after digestion with *Eco*RI and *Xho*I (indicated as 31). Size marker was indicated as M.

nt#20 and nt#550, encodes for 176 amino acid residues (Fig. 1). The size of the insert DNA of the clone #31 was determined to be approximately 600 bp (Fig. 2). We found that the clone #31, pGAD10/RPL18a (2~176), carried a partial cDNA of human ribosomal protein L18a (RPL18a) from nt#21 to nt#622. It corresponds to amino acid residues 2~176 among 176 amino acid residues. The clone #31 has the same nucleotide sequences as those in GenBank for



(1) pGBT9/hnRNP A1 + pGAD10/hnRNP A1

(2) pGBT9 + pGAD10/RPL18a

(3) pGBT9/hnRNP E1 + pGAD10/RPL18a

(4) pGBT9/hnRNP E1 + pGAD424/hnRNP L

Fig. 3. RPL18a binds to hnRNP E1 in a yeast two-hybrid assay. The pairs of indicated plasmids were cotransfected into HF7c yeast cells. The protein-protein interactions between the two proteins were monitored via a viability test of the yeast cells on Trp^- Leu^- His^--SD plates. The growth of the yeast cells reveals interactions between the hybrid proteins. pGBT9/hnRNP E1 (2~356) and pGAD424/hnRNP L (1~558) plasmids were employed as positive controls. pGBT9/hnRNP A1 (1~320) and pGAD10/hnRNP A1 (1~320) plasmids were also used as positive controls.

RPL18a (GenBank accession number BC071920). The cDNA of clone #31 was inserted in-frame with the Gal4 activation domain to pGAD10 vector.

The hnRNP E1-RPL18a interaction was verified after re-transformation of pGBT9/hnRNP E1 (2~356) and pGAD10/RPL18a (2~176) plasmids into a host strain. Individual colonies of HF7c yeast cells harboring both plasmids were examined to detect the presence of histidine prototrophy as well as β-galactosidase activity. Visual inspection of *HIS3* and *LacZ* reporter activation ascertained that hnRNP E1 interacted with RPL18a. RPL18a did not induce β-galactosidase activity and histidine prototrophy in cells harboring only the GAL4 DNA-binding domain (Fig. 3). These results demonstrate that hnRNP E1 (2~356) interacts specifically with human RPL18a (2~176) in yeast cells. Since as hnRNP E1 and hnRNP L can utilize heteromeric interactions, yeast cells expressing both hnRNP E1 and hnRNP L fusion proteins evidenced both β-galactosidase$^+$ and His^+ (Fig. 3) (Choi, 2008). In addition, hnRNP A1 can lead to homomeric interactions, yeast cells expressing two hnRNP A1 fusion proteins show both β-galactosidase activity and histidine prototrophy are present (Fig. 3) (Kim et al., 2000; Choi, 2008).

Human ribosomal protein L18a interacts with hnRNP E1 in *in vitro* pull-down assay

In order to verify yeast-two-hybrid data, *in vitro* pull-down experiments using GST-hnRNP E1 fusion protein (Fig. 4A) were carried out. The GST protein expressed in bacteria was employed as a negative control (Park et al., 2007). The

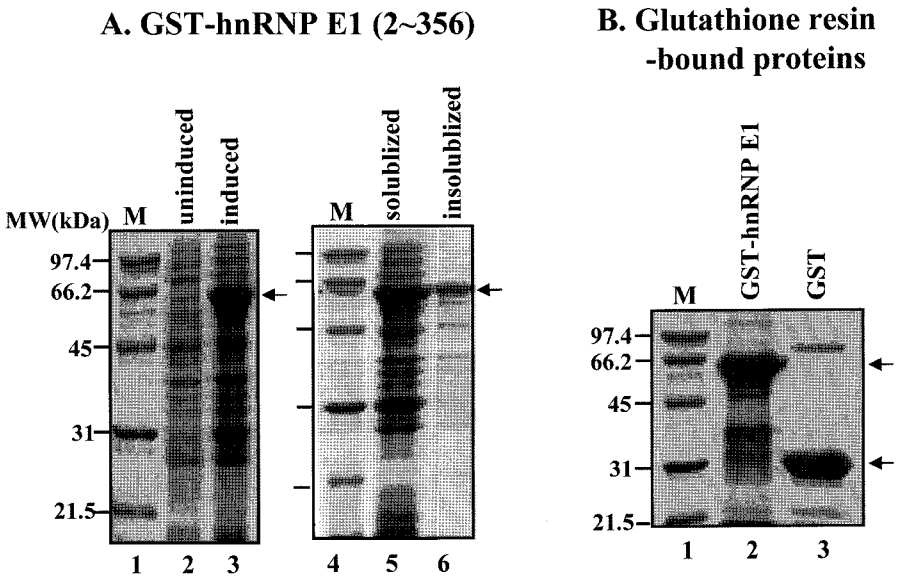


Fig. 4. (A) Bacterially expressed GST-hnRNP E1 (2~356) protein in Coomassie blue-stained 12% SDS-polyacrylamide gel. The GST-hnRNP E1 (2~356) was expressed in *E. coli* BL21(DE3)pLysS from the pGEX-KG/hnRNP E1 (2~356) plasmid. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cells up to 0.5mM. After 6 hours of further incubation at 27°C, the cells were harvested and resuspended in lysis buffer [20 mM Na-phosphate (pH 7.6), 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluroride (PMSF), 1 mM β-mercaptoethanol, and 10% glycerol]. The cell extracts from uninduced and induced cells were shown in lanes 2 and 3, respectively. The cells were then ruptured by sonication. After centrifugation, the supernatant and pellet were resolved in lanes 5 and 6, respectively. The arrow indicates GST-hnRNP E1 (2~356). (B). The amounts of glutathione sepharose 4B resin-bound GST-hnRNP E1 (2~356) and GST proteins are shown on Coomassie blue-stained 12% SDS-polyacrylamide gel. The arrows indicate GST-hnRNP E1 (2~356) and GST.

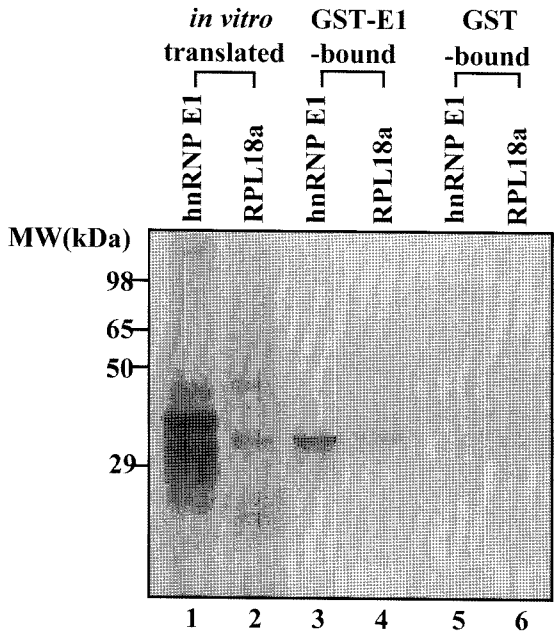


Fig. 5. RPL18a binds to hnRNP E1 *in vitro*. The proteins were produced and labeled with methionine and cysteine via *in vitro* transcription-translation in the reticulocyte lysates. The *in vitro* translated hnRNP E1 and RPL18a proteins are shown in lanes 1 and 2, respectively. These [^{35}S]-labeled proteins were incubated with resin-bound GST-hnRNP E1 (2~356) (lanes 3 and 4) or GST (lanes 5 and 6) in the presence of RNase A and RNase T1. After washing the samples with binding buffer, the resin-bound proteins were dissociated by boiling in SDS-containing buffer, and then analyzed on 12% SDS-polyacrylamide gel.

pCS3MT/RPL18a (2~176) plasmid, which harbors six c-myc epitope tag sequences upstream of the RPL18a coding sequence, was utilized in order to obtain [^{35}S]-RPL18a. The GST-hnRNP E1 (2~356) fusion protein immobilized on glutathione-Sepharose resin (Fig. 4B) was incubated either with [^{35}S]-labeled RPL18a (2~176) or, as a positive control, with the [^{35}S]-labeled hnRNP E1 (2~356) protein. The amount of [^{35}S]-RPL18a input we loaded on lane 2 was 50% of the one used for the pull-down assay with GST-hnRNP E1 or GST (Fig. 5, lanes 4 and 6). In a similar manner, the amount of [^{35}S]-hnRNP E1 input loaded on lane 1 was 50% of the one for the pull-down assay with GST-hnRNP E1 or GST (Fig. 5, lanes 3 and 5). In order to exclude the possibility that the protein interactions were mediated by protein-RNA-protein interactions, RNase A and RNase T1 were included in the reaction mixture. We found that bacterially expressed GST-hnRNP E1 fusion protein was able to pull-down [^{35}S]-labeled RPL18a (Fig. 5, lane 4). However, GST alone could not precipitate *in vitro* translated proteins (Fig. 5, lane 5 and 6). Therefore, we conclude that human ribosomal protein L18a interacts specifically with hnRNP E1 *in vitro*.

Human ribosomal protein L18a (RPL18a), a constituent of ribosomal protein large subunit, belongs to the L18AE family of ribosomal protein. We scanned the GenBank data bank for amino acids sequence similarities. The amino acid sequences, reported in the database showing high homology with *Homo sapience* RPL18a was compared and aligned (Fig. 6). Human ribosomal protein L18a showed 76% homology with *Drosoplila melanogaster* RPL18a (GenBank accession number P41093), *Podocoryne carnea* RPL18a (GenBank accession number O76968), and *Schizosaccharomyces pombe* RPL20 (GenBank accession number P05732). This fact may indicate that RPL18a is crucial for the function or structure of ribosome.

Human ribosomal protein L18a was determined as one of the ribosomal proteins that interact with nucleolin (Bouvet et al., 1998). The protein was shown to interact with hepatitis C virus (HCV) internal ribosome entry site (IRES). It suggested that RPL18a might influence the HCV IRES mediated translation (Dhar et al., 2006). In this study,

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L18a (H. sapiens)       6 TLREY KVVGRCLPTP KCHTPPLYRM RIFAPNHVVA KSRFWYFVSQ LKKMKKSSGE 60
L18a (D. melanogaster)  6 LLKEY EVVGRKLPSE KEPQTPLYKM RIFAPDNIVA KSRFWYFLRQ LKKFKKTTGE 60
L18a (P. carnea)        4 ELKEY KIIGRRLPSE KTPNTPLYRM RIFAPDVVSA KSRFWYYMKK LKKLKKTVGE 58
L20 (S. pombe)          2 ALKEYQVVGRKVPTE HEPVPKLFRM RLFAPNESVA KSRYWYFLKM INKVKKATGE 56
                            ****  ***** **  * *  * **** ******  ** **** *** *  ***  ** ****

L18a (H. sapiens)       61 IVYCGQVFEK SPLRVKNFGI WLRYDSRSGT HNMYREYRDL TTAGAVTQCY RDMGARHRAR 120
L18a (D. melanogaster)  61 IVSIKQVYET SPVKIKNFGI WLRYDSRSGT HNMYREYRDL TVGGAVTQCY RDMGARHRAR 120
L18a (P. carnea)        59 VVMCSEVFDK SPTTVKNFGVWIRYNSRSGT HNMYREYRDV TVTGAVTQCY TDMAARHRAR 118
L20 (S. pombe)          57 IVAINQVFEK KPLKAKVFGI WIRYDSRSGT HNMYKEFRDT TRAGAVEAMY ADMAARHRAR 116
                            **  ****** **** *  *** **********  *** ******  *  *** ** **  ** **** ***

L18a (H. sapiens)       121 AHSIQIMKVE EIAASKCRRP AVKQFHDSKI KFPLPHR_LR RQ_KPRFTTK RPNTFF 176
L18a (D. melanogaster)  121 AHSIQ IIKVD SIPAAKTRRV HVKQF HDSKI KFPLVQR_HH KG_RKLFSFR KPRTYF 176
L18a (P. carnea)        119 ASSVQILKVE EVPSSKCRRP HVKQMHNSKM RFPMTHR_MR SQ_RSTFQAK RPHTFL 174
L20 (S. pombe)          117 FRSIR ILKVV EVEKKDVRRN YVKQLLNPHL KFPLPHR_VG LA_KKVFAPH RPSTFY 176
                            **** * **  **    *  **   ***  ***** **** **   **   ** *   * ******
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Fig. 6. Amino acid sequence alignment of human ribosomal protein L18a. Amino acid sequences of human ribosomal protein L18a are aligned with the sequences of *Drosoplila melanogaster* RPL18a, *Podocoryne carnea* RPL18a, and *Schizosaccharomyces pombe* RPL20. Numbers indicate the position of amino acid residue of the protein. Highly conserved positions are marked as star (*). They showed 76% homology.

we have shown for the first time that human ribosomal protein L18a binds specifically to hnRNP E1 both in yeast two-hybrid system and *in vitro*. Our results suggest that hnRNP E1 plays an important role in translational regulation, not only by binding to specific site on mRNA but also by interacting directly with ribosome complexes.

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