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QUARTERLY

Increased expression of ribosomal protein S2 in liver tumors, posthepactomized livers, and proliferating hepatocytes *in vitro*✪

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The ribosomal protein S2 (RPS2) is encoded by a gene from the highly conserved mammalian repetitive gene family LLRep3. It participates in aminoacyl-transfer RNA binding to ribosome, potentially affecting the fidelity of mRNA translation. These studies were designed to measure the expression of RPS2 during increased cell proliferation. Using Western and Northern blot analyses, we found that the levels of RPS2 protein and its corresponding mRNA were higher in mouse hepatocellular carcinoma, in mouse livers after one-third partial hepatectomy, and in serum-starved cultured hepatocytes following serum treatment. Our study shows that the increased expression of RPS2 correlates with increased cell proliferation. However, whether the altered expression of this protein reflects its involvement in cellular proliferation or represents an associated phenomena is still a key question that needs to be explored.

The eukaryotic ribosome consists of four ribosomal RNAs and approximately 80 highly conserved ribosomal proteins (Chen & Ioannou, 1999). Increased ribosomal protein synthesis is seen in malignancy (Stanners *et al.*, 1979; Kief & Warner, 1981; Tushinski & Warner, 1982; Ou *et al.*, 1987; Chester *et al.*, 1989; Pogue-Geile *et al.*, 1991; Aloni *et al.*, 1992; Shama *et al.*, 1995), but the higher expression level does not always correlate with increased cell proliferation. This is particularly true in colorectal cancers where the state of proliferation is often similar to that found in normal colonic mucosa (Pogue-Geile *et al.*, 1991). Although biosynthesis of all ribosomal components is coordinated with the changes

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Abbreviations: DLU, digital light unit; hnRNP, heterogeneous nuclear ribonucleoproteins; RPS2, ribosomal protein 2.

in cellular growth (Nygard & Nilsson, 1990), some ribosomal proteins may have a more specific role in regulating proliferation than simply influencing the rate of protein biogenesis. As such, their increased expression may be a separate phenomenon from the general increase in the synthesis of ribosomal proteins in dividing cells (Starkey & Levy, 1995).

The ribosomal protein S2 belongs to the highly conserve species of repetitive mammalian gene LLRep3, members of which hybridize to a single abundant poly A^+ RNA (Slynn *et al.*, 1990). It participates in aminoacyl-transfer RNA binding to the ribosome, potentially affecting the fidelity of mRNA translation. The S2 protein is localized on the external surface of the 40S ribosomal subunit (Lutsch *et al.*, 1990).

The hnRNP K protein is an evolutionarily conserved factor involved in a host of processes that comprise gene expression such as chromatin remodeling, transcription, pre-mRNA splicing, mRNA export and translation. It may act as a docking platform or a scaffold that bridges signal transduction pathways to sites of nucleic acid-directed processes (Ostrowski *et al.*, 2001; 2002). Using anti-K antibody #53 (Ostrowski *et al.*, 1994) we identified a 30-kDa protein that was highly expressed in tumors. This band was not K protein but the ribosomal protein S2 (RPS2). We show here that RPS2 expression is associated also with other states of increased proliferation.

MATERIAL AND METHODS

Screening of expression library. Immunoscreening of phage-expressed proteins was performed using replica nitrocellulose filters from plates containing 5×10^4 cDNA clones from a HeLa cDNA expression library (kindly provided by T. Hunter). Duplicate filters were screened for reactivity with #53 and #54 anti-K protein antibodies which were raised against synthetic peptides representing N-(2-12) and C-terminal (452–464) amino-acid residues of murine K protein, respectively (Ostrowski *et al.*, 1994; Van Seuingen *et al.*, 1995). The antigen–antibody complexes were visualized with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase, using BCIP/NBT phosphatase substrate. Three candidates that were immunostained by antibody #53 (but not by antibody #54) were further purified by rescreening until plaque purity was obtained. Since pGEX5, used for the construction of this expression library, contains plasmid sequence consisting of a ColE1 origin, an ampicillin resistance gene, and a GST gene between two *Not*I sites, cDNAs of interest were rescued as GST-fusion protein-expressing plasmids by *Not*I digestion and self ligation (Fukunaga & Hunter, 1997). Plasmids were purified from the positive clones and inserts were sequenced. Computer-based DNA analysis was done with Lasergene software package (DNAStar Inc., Madison, Wisconsin, U.S.A.). cDNA was expressed in *Escherichia coli* and recombinant proteins were tested in Western blots.

Cells. Rat hepatoma cells expressing human insulin receptors, HTC-IR, were grown in plastic cell culture flasks in DME media supplemented with 10% FBS, 2 mM glutamine, penicillin (100 units/ml), streptomycin (0.01%), in 7/93% CO_2/air gas mixture.

Animals. Mice were housed at the Cancer Center (Warszawa, Poland), breeding facilities were maintained under constant room temperature with a 12:12-h light-dark period and free access to water and standard food pellets. The animals received humane care in compliance with the regulations of the Cancer Center.

Experiments utilized CBA-T6/W mice, which had developed spontaneous hepatocellular neoplasms. Mice anaesthetized with ether were sacrificed, the livers were rapidly resected, and classified by gross examination into tumor and hepatic tissue. Two portions of each specimen were frozen in liquid nitrogen

and stored at –80°C until use. The remaining portions were fixed in formalin and embedded in paraffin for histological examination.

Partial hepatectomy was performed under light ether anesthesia on 2-month old BALB/c male mice. Under aseptic conditions, animals were subjected to mid-ventral laparatomy and resection of the left lateral lobe, which constitutes about one third of the total liver. The removed lobe was immediately frozen in liquid nitrogen and stored at –80°C until use. After the indicated times, animals were sacrificed and the remnant liver was rapidly collected and frozen. Sham-operated animals underwent mid-ventral laparatomy without resection of liver lobe.

Cytoplasmic and nuclear extracts were prepared by a modified method of Dignam *et al.* (1983) as described previously (Ostrowski *et al.*, 1991; 2000) using cells or frozen tissues which were pulverized under liquid nitrogen with a Mikro-Dismembrator II (B. Braun). In addition to dithiothreitol (DTT) (0.5 mM), phenylmethylsulfonyl fluoride (PMSF) (0.5 mM), and leupeptin (10 μg/ml), the lysis, extraction, and dilution buffers contained the following phosphatase inhibitors: 30 mM *p*-nitrophenyl phosphate (pNPP), 10 mM sodium fluoride (NaF), 0.1 mM sodium orthovanadate (Na_3VO_4), 0.1 mM sodium molybdate (Na_2MoO_4), and 10 mM β-glycerophosphate. Separation of the nuclear fraction from the cytosol was monitored with the cytosolic enzyme marker lactate dehydrogenase and the purity of nuclear extracts was greater than 92%. Protein concentration was measured using MicroBCA protein assay (Pierce Chemical).

Electrophoresis and immunoblotting. Equal amounts of sample protein (50 μg) were mixed with 2 × loading buffer (125 mM Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% β-mercaptoethanol) (1:1, v/v) and boiled for 5 min. Proteins were separated by 10% SDS/PAGE. After bathing the gel in transfer buffer (192 mM glycine, 25 mM Tris, 20% methanol, and 0.005% SDS) for 30 min, proteins were electroblotted to a PVDF membrane in transfer buffer. The membrane was blocked with 5% non-fat dry milk in TBST buffer (Tris-buffered saline containing 0.05% Tween-20) for 1 h at room temperature. Then, the membranes were probed for 1 h at room temperature with primary antibodies in 5% non-fat dry milk/TBST buffer. The membranes were washed three times in TBST buffer and incubated for 60 min at room temperature with secondary antibodies conjugated with alkaline phosphatase or horseradish peroxidase and immunoreactions were detected using BCIP/NBT phosphatase substrate or enhanced chemiluminescence (ECL) Western blotting detection system.

Northern blot analysis. Total RNA was prepared from tissues and cell pellets by acid-guanidinium thiocyanate/phenol-chloroform extraction using TRIzol reagent. Ten μg of total RNA was denatured with formaldehyde/formamide and electrophoresed in a 1% agarose/formaldehyde gel. RNA was then transferred to Hybond N nylon membranes in 10 × SSC. The membranes were incubated at 65°C for 20 h with 2 × 10^7 d.p.m. of ^{32}P-labeled DNA probes in hybridization buffer (6 × SSC, 0.5% Ficoll, 0.5% PVP, 0.5% BSA, 0.5% SDS, 100 μg/ml herring sperm DNA). Excess probe was removed from the membrane by serial washes at 65°C in 1 × SSC/0.1% SDS. The hybridized probes were visualized by autoradiography.

RESULTS AND DISCUSSION

Anti-K antibody #53 recognizes ribosomal protein S2

The interaction of K protein with many of its protein and nucleic acid partners is regulated by extracellular signals. Acting as a docking platform, K protein may link signal transduction pathways to RNA and DNA targets. Immunoblot analyses of the same total amount of cytoplasmic and nuclear extracts

from normal mouse liver were carried out using three different anti-K antibodies; a monoclonal antibody (B4B6) (kindly provided by J.E. Celis) generated against unknown immunogenic epitopes of K protein (Dejgaard *et al.*, 1994) and rabbit polyclonal antibodies #54 and #53, raised against C-terminal and N-terminal peptides of murine K protein, respectively (Ostrowski *et al.*, 1994; Van Seuingen *et al.*, 1995). As shown in Fig. 1, all antibodies recognized the 65 kDa K protein in nuclear and cytoplasmic extracts. However, only antibody #53 immunostained an additional band of approx. 30 kDa.

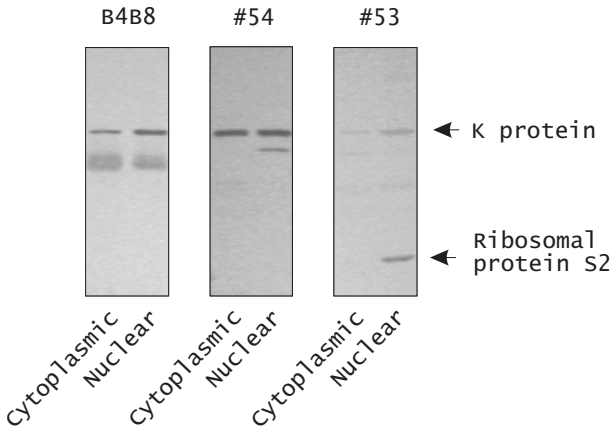

Figure 1. Immunoblotting of cytosolic and nuclear extracts from normal mouse liver.

Equal amounts of protein (50 μg) were separated by SDS/PAGE, proteins were transferred to PVDF membrane and probed with anti-hnRNP K protein monoclonal antibody B4B6 and polyclonal antibodies #53 and #54.

We screened an expression library to determine whether the 30-kDa protein, detectable by the anti-K polyclonal antibodies #53, represented a protein product of alternatively spliced K protein transcript or a band unrelated to K protein. Phage-expressed proteins were lifted onto nitrocellulose filters, and the filters were screened for reactivity with the anti-K protein antibodies #53 and #54. We purified three individual positive plaques that were immunostained with #53, but not with #54, antibodies. Plasmids from the positive clones were excised and then expressed in *E. coli* cells. GST-fusion proteins of the positive clones were analyzed by Western blotting using antibodies #53 and #54. cDNA-encoded proteins from all three positive clones revealed an easily detectable level of a protein of approx. 60 kDa with #53, but not with #54, antibody (not shown). Since the molecular mass of GST is approx. 30 kDa, the molecular mass of this cDNA-encoded fusion protein was in agreement with the 30 kDa protein detected in liver nuclear extracts.

Plasmid DNA from the positive clones was subjected to dideoxynucleotide chain termination sequencing followed by analysis on an ABI Prism 377 automated DNA sequencer (Perkin-Elmer). Comparison of the cDNA nucleotide sequences from each of the three positive clones with sequences in the database revealed the clones' identity as human RPS2 (EMBL accession No. X17206) (Slynn *et al.*, 1990). Amino acid analysis of the peptide represented by the N-terminal amino-acid residues 2–12 of murine K protein that was used to produce antibody #53 revealed a similarity to a fragment of the amino-acid sequence of RPS2 (Fig. 2). Thus, our polyclonal anti-K antibody #53 incidentally recognizes similar immunogenic epitopes from both K protein and RPS2.

Ribosomes are essential for cell growth and differentiation. The response to growth stim-

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E T E Q P E E T F P N - peptide from hnRNP K protein
  T .   P   : . F . : - similarity (. - medium; :  - high)
F T K S P Y Q E F T D - peptide from ribosomal protein S2
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Figure 2. Comparison of peptide sequences.

The sequences represent amino-acid residues 2–12 of murine hnRNP K protein (used for raising anti-K protein antibody #53) and amino-acid residues 189–199 of ribosomal protein S2.

uli controls ribosomal protein synthesis; growing cells more efficiently translate ribosomal protein mRNAs than resting cells (DePhilip *et al.*, 1980; Ignotz *et al.*, 1981; Hammond & Bowman, 1988; Warner & Nierras, 1998). During senescence, the level of ribosomal protein mRNA decreases (Seshadri *et al.*, 1993). Our studies were performed to measure RPS2 levels and expression in hepatic tumors, regenerating livers and cultured hepatocytes.

Expression of RPS2 in hepatic neoplasms

To examine the distribution of RPS2 in neoplastic tissues, we assayed murine spontaneous liver tumors histologically classified as hepatocellular carcinoma. Cytoplasmic and nuclear extracts were prepared from frozen tissues and RPS2 levels were measured by Western blot analysis using rabbit polyclonal antibody #53. The results are shown in Fig. 3A. Antibody #53 did not immunostain, or stained very weakly, RPS2 in the cytoplasmic extracts prepared from normal mouse livers and hepatic tumors (not shown). In contrast, easily detectable levels of RPS2 were detected by antibody #53 in nuclear extracts from these tissue samples. Nuclear levels of RPS2 were higher in tumors than in the normal part of the livers of animals with the tumors. The level of the corresponding mRNA in hepatic tumors and normal liver tissue(s) surrounding the tumors was studied by North-

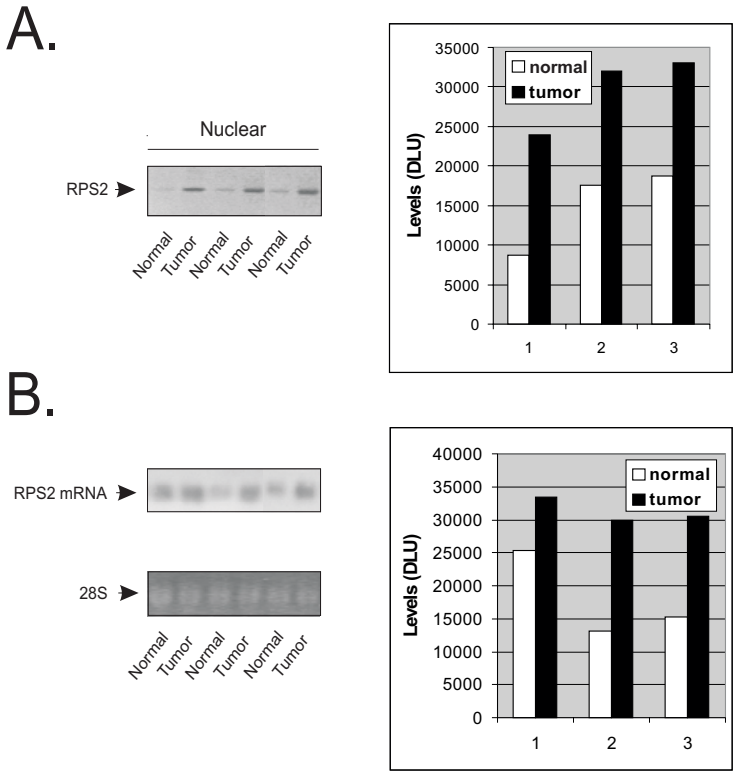

Figure 3. Expression of ribosomal protein S2 in spontaneous hepatic adenocarcinoma in mice.

A. Nuclear protein extracts prepared from hepatic adenocarcinomas (tumor) and normal liver tissue (normal) surrounding these tumors were resolved by SDS/PAGE followed by Western blotting with #53 antibody. Blots were scanned (Expression 638, Epson). Densitometric analysis of RPS2 bands, shown in the graph, was done using OptiQuant™ Image Analysis Software. **B.** Total RNA extracted from adenocarcinoma and livers harboring these tumors was electrophoresed in 1% agarose/formaldehyde gel, RNA was visualized with ethidium bromide and photographed with a digital camera (DC40, Kodak). After RNA transfer, the nylon membranes were probed with ^{32}P-labeled RPS2 cDNA, autoradiographed and densitometric analysis was done. The mRNA levels (upper panel), shown in the graph, were normalized to the levels of 28S RNA (lower panel). Results represent three out of seven independent experiments.

ern blot analysis using RPS2 cDNA as a probe. As shown in Fig. 3B, a single RPS2 mRNA species was detected in mouse liver. The level of RPS2 transcript was higher in hepatic tumors than in the adjacent hepatic tissues.

Increased expression of several ribosomal protein genes was commonly found in colorectal cancer tissues and in established cell lines that were derived from colorectal tumors (Chester *et al.*, 1989; Cartwright *et al.*, 1990; Pogue-Geile *et al.*, 1991). However, unlike other ribosomal genes, overexpression of RPS2 was observed only in a subset of human squamous cell carcinoma samples and cell lines, and in colon and breast tumor samples (Chiao *et al.*, 1992). RPS2 was expressed at much higher levels in tumorigenic cell lines transformed by *ras*, SV-40, polyoma virus, Rous sarcoma virus, Abelson murine leukemia virus, and chemical carcinogen than in nontumorigenic cells (Scott *et al.*, 1983; Chiao *et al.*, 1992; Shin *et al.*, 1993). Ribosomal protein genes L37, RPP-1 and RPS2 were shown to be upregulated in the presence of an oncogenic form of the mutated p53 tumor suppressor (Loging & Reisman, 1999). The RPS2 gene can be also downregulated in normal liver sinusoidal endothelial cells (Liliensiek *et al.*, 1998). Thus, although the RPS2 gene can be differentially regulated, it is not clear whether increased expression of RPS2 may contribute to the transformed phenotype.

Expression of RPS2 in injured livers

To test whether the increased level of nuclear RPS2 found in murine hepatic tumors may reflect its involvement in the neoplastic process or rather be due to the increased rate of cellular proliferation we determined RPS2 protein and mRNA levels in murine livers after partial hepatectomy. Liver exhibits a remarkable potential to regenerate (Fausto & Mead, 1989; Michalopoulos, 1990; Taub, 1996); the majority of hepatocytes in the adult liver are quiescent, but within hours after liver injury or following partial hepatectomy they undergo one or two rounds of replication. Thus, the use of animals after partial hepatectomy has become an excellent model for studying cellular proliferation in intact organ. As shown in Fig. 4A, increased levels of nuclear RPS2 were detected in the extracts from the livers following partial hepatectomy as compared to normal livers taken during surgery at all time points. For better detection of these differences, immunoreactions were done for a short period of time using BCIP/NBT phosphatase substrate, and for this reason, RPS2 in the presented blot is not immunostained in normal livers. Longer development showed immunostained RPS2 also in nuclear extracts from normal livers (not shown). In contrast, no RPS2 was found in the cytoplasmic extracts from both posthepactomized livers and the surgically removed lobe (not shown). Northern analysis revealed that the RPS2 transcript levels increased in the remnant liver at each time point as compared to transcript levels in the lobes removed during hepatectomy (Fig. 4B). The levels of RPS2 and its mRNA were similar in the livers obtained during hepatectomy and those from sham-operated controls (not shown).

Expression of RPS2 in serum-treated hepatocyte culture

Fetal calf serum is an essential agent for cell growth in culture, supplying the cells with the required proliferation signals and growth factors. Under serum-deprived conditions, cultured cells become growth-arrested, and after addition of serum they re-enter the cell cycle (Squinto *et al.*, 1989). RPS2 protein and mRNA levels were studied in growth-arrested and proliferating hepatocytes *in vitro*. HTC-IR cells were arrested in G_0 by 48-h serum starvation, and then were treated with 15% fetal calf serum. At given time points following the treatment, cells were harvested and cytoplasmic and nuclear extracts were prepared. In contrast to hepatic tissue extracts, RPS2 from

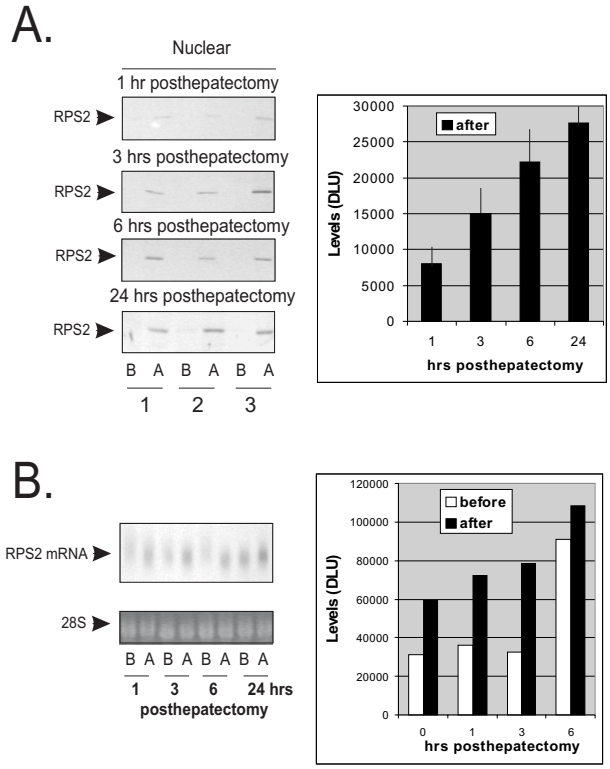

Figure 4. Expression of ribosomal protein S2 after partial hepatectomy in mice.

A. Nuclear protein extracts prepared from resected lobes (B) and from the remnant livers (A), obtained in three independent experiments, were analyzed by anti-RPS2 Western blots. Blots were scanned and RPS2 bands from posthepactomized livers were quantified. Results shown in the graph are expressed as DLU and represent means ± S.D. of 3 experiments. **B.** Total RNA was extracted from livers before and after partial hepatectomy. Northern blots were probed with ^{32}P-labeled K protein cDNA. Autoradiograms of the RPS2 transcripts were analyzed by densitometry. The intensity of the RPS2 mRNA bands (upper panel) was normalized to the levels of 28S RNA (lower panel). The results, shown in the graph, are expressed as normalized mRNA levels after (A) and before (B) partial hepatectomy and are average of three independent experiments.

cultured hepatocytes was easily detected by antibody #53 in both nuclear and cytoplasmic extracts (Fig. 5A). Serum treatment of growth-arrested cells increased the levels of RPS2 in both the cytoplasm and nuclei, peaking at 6 h.

RPS2 subcellular localization is different in tissue and cellular extracts. In sharp contrast to protein extracts from hepatic tissue and tumors, in cultured hepatocytes this protein was easily detected in both the cytoplasm and nuclei. In another cell line, AGS derived from human gastric carcinoma, RPS2 was also detected in cytoplasmic and nuclear extracts, but in cultured fibroblasts (3T3 cells) it was predominantly localized in the nuclei (not shown). Our observations suggest that while in all cells and tissues that have thus far been studied RPS2 may play a role in ribosome assembly, mature cytosolic ribosomes contain RPS2 only in some types of cultured cells.

In contrast to tissues, the level of RPS2 mRNA expression in resting hepatocytes was very similar to that observed in serum treated cells (Fig. 5B), suggesting that the upregulation of RPS2 biosynthesis during cellular proliferation *in vitro* occurs primarily at the level of translation.

This study shows that the expression of RPS2 is increased in proliferating hepatocytes *in vitro* and *in vivo*, as well as in hepatic carcinomas. Since increased ribosomal biogenesis is an early event whenever cell growth rate increases, in both normal and malignant tissues, the presence of an increased level of RPS2 gene transcript and of

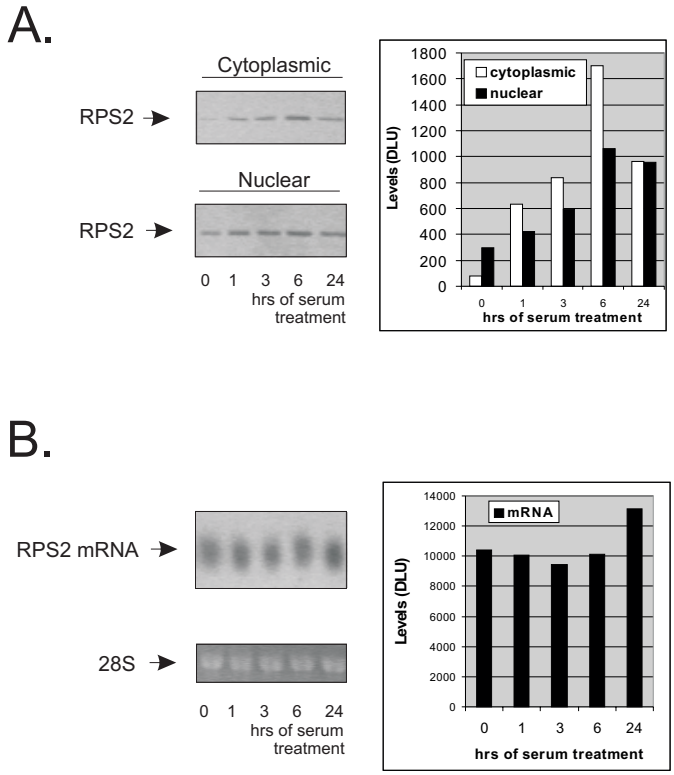

Figure 5. Expression of ribosomal protein S2 in serum treated hepatocytes.

A. After 48 h of serum deprivation, HTC-IR cells were supplemented with media containing 15% FCS. At given time-points, cells were harvested, nuclear and cytoplasmic proteins were extracted, and proteins were analyzed by anti-RPS2 Western blots. Blots were scanned and RPS2 bands were quantified as shown in the graph. Results are average of three independent experiments. **B.** Northern blots were probed with ^{32}P-labeled RPS2 cDNA. The RPS2 transcripts were analyzed by densitometry. The graph shows the intensity of the RPS2 mRNA bands (upper panel) normalized to the levels of 28S RNA (lower panel). Results are average of three independent experiments.

its protein product may reflect active proliferation rather than direct contribution to tumorigenesis. However, whether the altered expression of this protein reflects its involvement in cellular proliferation or represents associated phenomena is still a key question that needs to be further explored. Moreover, it is possible that not only the protein level but also post-translational modifications, such as phosphorylation, may regulate the functioning of RPS2 in proliferating cells.

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