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Preparation and Characterization of Antibodies against Human Ribosomal Proteins: Heterogeneous Expression of S11 and S30 in a Panel of Human Cancer Cell Lines

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Mutants of model eukaryotic organisms have revealed that most ribosomal proteins are essential for cell viability. However, the precise functional role of each ribosomal protein is largely unknown. Recent reports on the involvement of ribosomal proteins in various genetic diseases and studies on the extraribosomal functions of these proteins have cast some light on their localization and functions. Here we prepared rabbit polyclonal antibodies against 26 human ribosomal proteins; each of these reagents recognized a single band in immunoblots of the purified ribosome. We used these antibodies to evaluate a panel of human cancer cell lines. Although no deficiency of ribosomal proteins was observed, the abundance of S11 and S30 varied substantially among the cell lines, but the difference did not affect the biogenesis or composition of the ribosome. Therefore, the heterogeneity may be related to extraribosomal functions of S11 and S30. The antibodies described here are powerful tools for research into the molecular mechanisms of protein translation, cell-biological and medical studies on the ribosomal proteins, and ultimately a comprehensive understanding of all ribosomal proteins ("ribosomics").

Key words: Proteomics — Ribosomal proteins — Antibodies — Expression profiles — Human cancer

All living organisms have ribosomes, which are a protein complex that is essential for protein synthesis. Bacterial ribosomes are an excellent *in vivo* model system that has been studied for a long time, and much information on the roles of the ribosome in translation has accumulated.[1)] Therefore a conception prevalent among many scientists is that most of the important questions about the ribosome and its components have been resolved already. However, the following points relating to ribosomal components in higher organisms argue against this notion. Abnormality of ribosomal proteins causes diseases of specific tissues. For example, haploinsufficiency of S19 is associated with Diamond-Blackfan anemia.[2)] The absence of a ribosome-associated protein causes fragile X syndrome, which is characterized by mental retardation.[3)] In addition, mutation of the gene encoding a protein essential for the processing of pre-ribosomal RNA leads to dyskeratosis congenita.[4)] Ribosomal proteins appear to have roles in addition to those in the translation machinery (extraribosomal functions).[5)] In Jurkat T-lymphoma cells, overexpression of L7 suppresses the expression of nuclear proteins and arrests the cell cycle in G_1.[6, 7)] L32 may have bone-promoting potential.[8)] L29 is expressed on the cell surface, binds to heparin, and may be involved in embryo implantation.[9)] S19 is released as an oligomer from apoptotic cells into the extracellular space; this protein functions as a monocyte chemotactic factor, which is likely to be important *in vivo* for phagocytotic clearance of apoptotic cells.[10)] In addition, the regulation of ribosome-interacting proteins seems to be tightly associated with the stress response, apoptosis, and carcinogenesis.[11, 12)] Collectively, various ribosomal and ribosome-interacting proteins as well as changes in their expression and localization seem to participate in and modulate a wide variety of cellular activities.

To investigate the expression and roles of ribosomal proteins in the cell, specific probes are essential. Detection of the mRNA of ribosomal proteins by using their cDNA sequences has revealed that these proteins are associated with cell differentiation[13)] and malignant tumorigenesis.[14, 15)] Because the expression of ribosomal proteins is controlled translationally and post-translationally,[16, 17)] detection of translation products is very important. However, in contrast to those analyses of mRNA, few reports on analysis at the protein level have appeared. The paucity of specific probes for ribosomal proteins (that is, antibodies against them) has limited such biochemical analysis.

In the present study, we have prepared and characterized rabbit polyclonal antibodies against human ribosomal pro-

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teins. For the initial application of these reagents, we evaluated the expression of ribosomal proteins in a number of cancer cell lines. This analysis revealed an obvious variation in the expression of at least two ribosomal proteins.

MATERIALS AND METHODS

Antibody production and purification We obtained complete amino acid sequences of human ribosomal proteins from a BLAST homology search of the NCBI database and the review by Wool *et al.*[18] Using this information, we chemically synthesized oligopeptides (10–13 amino acids) to these human ribosomal proteins; we conjugated the oligopeptides with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl and keyhole limpet hemocyanin to prepare the immunogens.[19] Each immunogen was injected into two pathogen-free rabbits (six injections per rabbit). The fractions of immunoglobulin G were isolated from the antisera of the rabbits by using column chromatography with protein A-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden); the collected fractions were aliquoted and stored at −80°C until use.

Cells and culture The cell lines used in this study were obtained from the Japanese Collection of Research Bioresources (JCRB; Tokyo), maintained according to the supplier's instructions, and grown in an atmosphere of 5% CO_2 at 37°C. These lines are A549 (lung cancer, catalog no. JCRB0076), Caki-1 (renal cancer, JCRB0801), C32TG (amelanotic melanoma, JCRB0227), HuO-3N1 (osteosarcoma, JCRB0413), HuO9 (osteosarcoma, JCRB0427), LoVo (colon adenocarcinoma, JCRB9083), LU99 (lung carcinoma, JCRB0080), ME-180 (cervical cancer, JCRB0810), MRK-nu-1 (mammary carcinoma, JCRB0628), NEC8 (testicular germ cell tumor, JCRB0250), NH-6 (neuroblastoma, JCRB0832), NH-12 (neuroblastoma, JCRB0833), NY (osteosarcoma, JCRB0614), PA-1 (ovarian teratocarcinoma, JCRB9061), RPMI1788 (peripheral blood, JCRB0035), SBC-3 (small cell carcinoma, JCRB0818), YMB-1 (breast cancer, JCRB0823), and YMB-1-E (breast cancer, JCRB0825). These cell lines have no mutation in the *p53* tumor suppressor gene.

Ribosomes and polysomes Rat liver ribosomes were isolated by ultracentrifugation as described previously.[20] Briefly, the liver from a Sprague-Dawley rat was homogenized in 50 m*M* Tris-HCl, pH 7.5, containing 0.1 *M* KCl, 5 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, and 0.25 *M* sucrose. We collected the post-mitochondrial fractions by using a brief centrifugation. The ribosomal fractions were obtained by ultracentrifugation through a 1 *M* sucrose cushion at 130 000*g* overnight, treated with puromycin and 0.5 *M* KCl, and centrifuged. The resulting supernatants were used in subsequent analyses.

To collect polysomes from cell lines, cells (2×10^8) were cultured for 10 min in the presence of cycloheximide (5 μg/ml) then harvested.[21] Cells were washed with phosphate-buffered saline (PBS) and lysed by incubating them for 15 min on ice with 2.5 ml of buffer A (50 m*M* Tris-HCl, pH 7.5, containing 25 m*M* KCl, 5 m*M* $MgCl_2$, and 10 m*M* 2-mercaptoethanol), to which was added 1% Triton X-100, 1% sodium deoxycholate, 0.25 *M* sucrose, 10 μg/ml cycloheximide, and 1 m*M* phenylmethylsulfonyl fluoride. The lysates were then centrifuged for 10 min at 15 000*g* to collect the post-mitochondrial supernatants. Ultracentrifuge tubes (model no. 349622; Beckman, Palo Alto, CA) were filled with 0.75 ml of 2.0 *M* sucrose and 0.63 ml of 0.5 *M* sucrose solution (solvent, buffer A). The post-mitochondrial supernatants (0.9 ml per tube) were added as the top zone to the tubes containing the layers of sucrose solution; the tubes were centrifuged for 3 h at 2°C and 105 000*g*.[22] The polysome-containing pellets were resuspended in buffer A. Using the conversion factor of 13 absorbance units/ml at 260 nm equals a ribosome concentration of 1 mg/ml,[20] we calculated the concentration of the isolated ribosomes and polysomes.

Immunoblotting Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli.[23] Cells (1×10^7) were washed with PBS, lysed in 0.5 ml of 1× SDS-PAGE sample buffer, and immediately boiled for 10 min. After electrophoresis of the lysates, proteins were transferred to Immobilon membrane (Millipore, Bedford, MA) in 25 m*M* Tris-HCl, pH 7.5, containing 0.19 *M* glycine and 20% methanol by using the Mini Transblot Cell (Bio-Rad, Hercules, CA) at 0.3 A for 90 min. The blots were then probed with antibodies against ribosomal proteins by using one of the two following methods. For the Tween-plus method, the blots were blocked in PBS containing 5% skim milk (Difco, Sparks, MD) and 0.1% Tween 20 for 1 h at room temperature then incubated for 1 h at room temperature with primary antibodies diluted in 0.5% skim milk in PBS. After five 5-min washes with 0.5% Tween 20 in PBS, the blots were incubated for 30 min at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) diluted in 0.5% skim milk in PBS and washed five times with 0.5% Tween 20 in PBS. The Tween-plus method was suitable for all antibodies listed in Table I except the anti-L32 antibody. In addition, the blots were stained with monoclonal anti-α-tubulin antibody (clone B-5-1-2; Sigma, Saint Louis, MO) by using the Tween-plus method.

For the Tween-minus method, the blots were blocked overnight at 4°C in 10% skim milk in H_2O and incubated for 1 h at room temperature with primary antibodies diluted with 50 m*M* Tris-HCl, pH 7.5, containing 0.5 *M* NaCl and 0.5% skim milk. After five 15-min washes with PBS, the blots were incubated for 30 min at room temperature with the secondary antibody diluted in 0.5% skim milk in PBS then washed five times with PBS. The signal

Table I. Peptide Sequences of Human Ribosomal Proteins for Polyclonal Antibodies

Antibody name	Target protein			Antigen peptide	
	Name	Accession no.	Mol. weight	Sequence	Homology to rat (%)
HSa1	Sa	J03799	32 852	RMRGTISREHP	100
HS4X1	S4X	M58458	29 596	DKRLAAKQSSG	100
HS82	S8	X67247	24 204	GGKRKPYHKKR	100
HS111	S11	X06617	18 430	DYLHYIRKYNR	100
HS121	S12	X53505	14 525	KESQAKDVIEE	100
HS141	S14	M13934	18 489	RRKGGRRGRRL	100
HS152	S15	J02984	17 039	QRRRLNRGLRR	100
HS182	S18	P25232	17 718	RGRTVGVSKKK	100
HS242	S24	U12202	15 068	KQRKERKNRMK	100
HS302	S30	X65921	14 389	FGKKKGPNANS	100
HL52	L5	U14966	34 446	HSTKRFPGYDSES	100
HL71	L7	X52967	29 224	DAGNREDQINR	100
HL7a2	L7a	M36072	29 994	TNYNDRYDEIR	100
HL81	L8	Z28407	28 023	RGTKTVQEKEN	100
HL92	L9	D14531	21 862	RLRVDKWWGNR	100
HL13a2	L13a	X56932	23 576	KIHYRKKKQLM	91
HL142	L14	D87735	23 802	QKYVRQAWQKA	82
HL151	L15	L25899	23 917	DTQWITKPDHK	91
HL171	L17	X53777	21 396	AKQWGWTQGRW	100
HL182	L18	L11566	21 633	RSKGRKFERAR	100
HL281	L28	U14969	15 761	RKRTRPTKSS	100
HL322	L32	X03342	15 859	KRNWRKPRGID	100
HL341	L34	P49207	13 304	KTRLSRTPGNR	100
HL35a2	L35a	X52966	12 494	KRGLRNQREHT	100
HL36a2	L36a	M15661	12 468	KHQPHKVTQYK	100
HL392	L39	L05096	6 292	KQKQNRPIPQW	100

was visualized by using the ECL detection system (Amersham, Little Chalfont, England) and X-OMAT films (Kodak, New Haven, CT).

RESULTS

Preparation of polyclonal antibodies against human ribosomal proteins According to a recent review,[18] the human ribosome (80 S) comprises a large subunit (60 S) of 46 proteins and a small subunit (40 S) of 33 proteins. To accomplish our final goal of surveying all the ribosomal proteins in the cell, we started by preparing antisera against all 79 ribosomal proteins.[24] For each ribosomal protein, we conjugated an appropriate oligopeptide with keyhole limpet hemocyanin then used the conjugated protein to immunize two rabbits. According to a BLAST search, the selected oligopeptide sequences showed no significant homology to other proteins, including other ribosomal proteins. The reactivity of the 158 antiserum samples thus obtained was evaluated by immunoblotting using the purified ribosome. We used rat liver ribosomes for this primary screening because we can obtain a large amount of very pure ribosomes by using an established method. The antigen peptide structures of human ribosomal proteins are identical or very similar to the corresponding structures of their rat homologs (70 of 79 structures were identical; Table I and data not shown). In light of this evaluation, we selected antibodies against 26 ribosomal proteins (Table I). Immunoblotting with any of these antibodies gave a single band (Fig. 1), whose mobility was close to that expected from the calculated molecular weight of the rat protein.[18] Therefore, these antibodies can be used to specifically identify the target proteins in the ribosome fraction isolated by using a simple, conventional method.

Evaluation of ribosomal proteins in human cancer cell lines by using the polyclonal antibodies We next examined whether our antibodies were useful for detecting ribosomal proteins in crude samples, lysates of human cancer cells. To confirm the detection of ribosomal proteins in various tissues, we used cell lines derived from distinct organs and cells. Lysates of 17 human cancer cell lines were prepared by using 1× Laemmli sample buffer, electrophoresed in 12% gels, blotted onto membrane, and sub-

jected to staining with the antibodies. Because samples of normal human tissues are difficult to obtain, we included a lysate of RPMI1788 cells, which are derived from normal peripheral blood leukocytes,[25] as a normal control. Of the 26 antibodies, the 19 antibodies against S4X, S11, S12, S14 (Fig. 2A), S15, S18, S30, L5, L7, L8, L9, L13a (Fig. 2B), L14, L15, L17, L18, L34, L35a, and L36a showed a single strong band that co-migrated with the rat homolog. The remaining seven antibodies (anti-Sa, S8, S24, L7a, L28, L32, and L39) each yielded a putative positive band in the midst of a high background, which probably resulted from nonspecific binding of the primary antibodies. These results suggest specific binding of the 19 antibodies to ribosomal proteins in crude cell extracts.

In addition, we evaluated the amounts of the 19 ribosomal proteins in the 18 cell lines. We used α-tubulin, which is a main component of microtubules that is expressed in all cells, as an internal standard (Fig. 3D). None of the 19 ribosomal proteins was missing, a finding consistent with the essential roles of ribosomal proteins as demonstrated by mutant analysis (see "Discussion"). Most of the ribosomal proteins showed high, uniform band intensities among the cell lines (for instance, Figs. 2 and 3C)—a finding that agrees with the commonly held opinion of ubiquitous, abundant ribosomal proteins. However, the expression levels of ribosomal proteins S11 (Fig. 3A) and S30 (Fig. 3B) obviously differed among these cell lines. More specifically, the expression of S11 in HuO9 osteo-

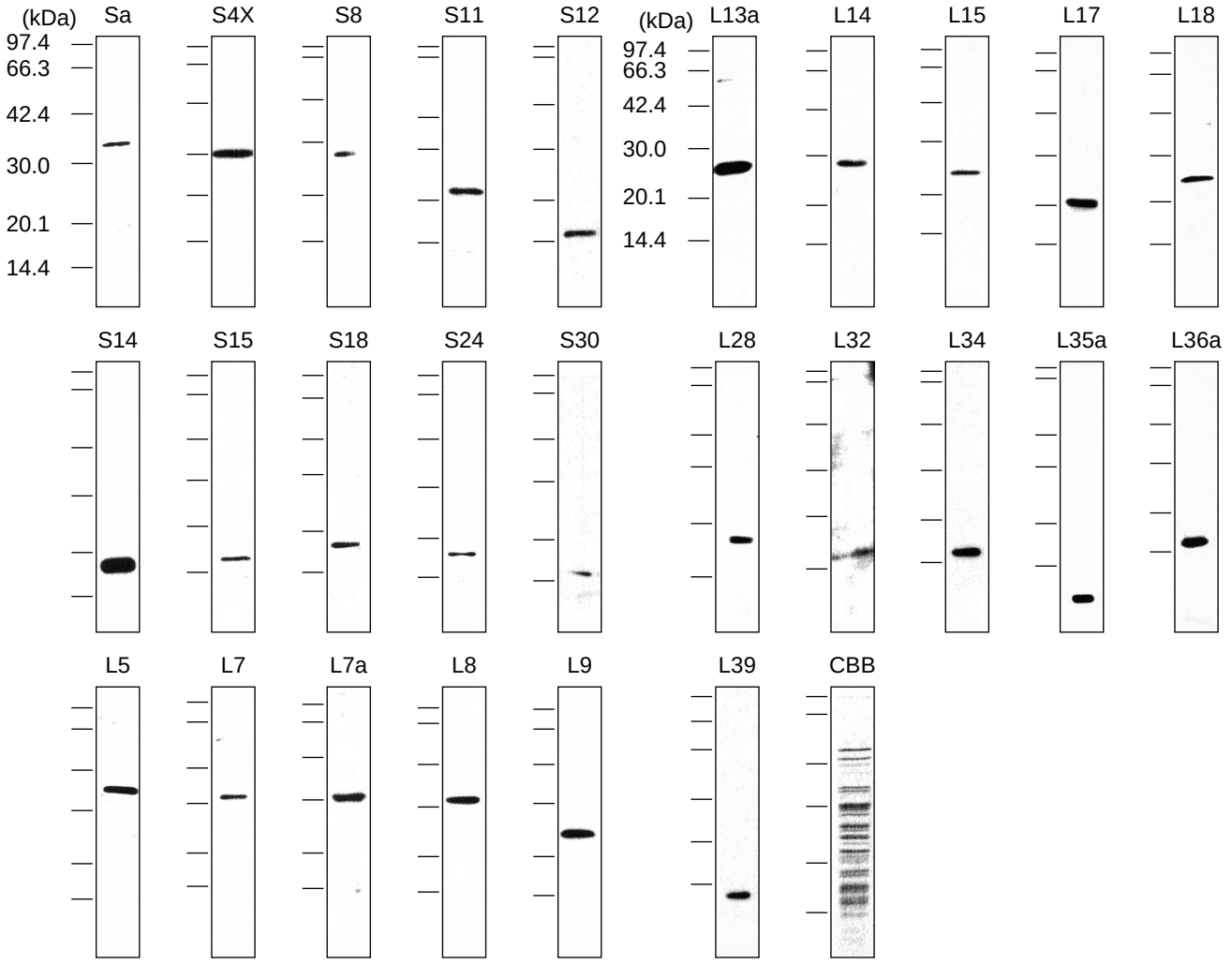

Fig. 1. Reactivity of antibodies to proteins in the ribosome. Immunoblotting of rat liver ribosomes (10 μg/lane) by using antibodies against 26 ribosomal proteins was performed as described in "Materials and Methods." At the top of each panel, the target protein of the antibody is indicated. CBB, blot stained with Coomassie Brilliant Blue. The migration positions of the protein markers (Daiichi Pure Chemicals, Tokyo) are shown in the far left panel.

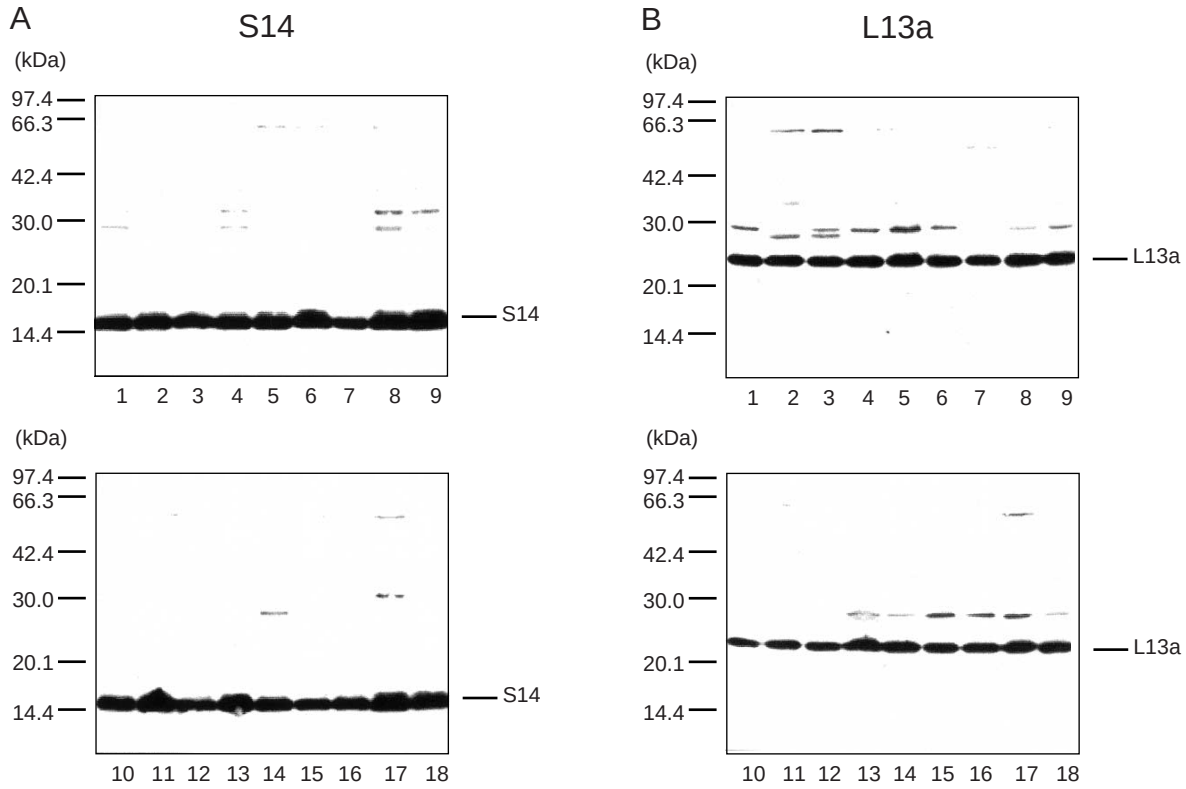

Fig. 2. Reactivity of antibodies with ribosomal proteins in various cancer cell lines. The lysates of 18 human cell lines were subjected to immunoblotting using antibodies against S14 (panel A) and L13a (panel B). Proteins from about 1×10^5 cells were loaded into each well. The migration positions of the protein markers are shown in the far left panel. The migration positions of the rat homologs of these markers are indicated in the rightmost panel. Lane 1, YMB-1; 2, YMB-1-E; 3, LU99; 4, RPMI1788; 5, NH-12; 6, HuO-3N1; 7, HuO9; 8, NEC8; 9, MRK-nu-1; 10, ME-180; 11, A549; 12, SBC-3; 13, C32TG; 14, NY; 15, PA-1; 16, Caki-1; 17, LoVo; 18, NH-6.

sarcoma cells (Fig. 3A, lane 7) was lower than that in RPMI1788 (Fig. 3A, lane 4), whereas the amounts of S14, L13a, and S4X were equal in these two cell lines (lanes 7 and 4, Figs. 2 and 3C). Leukemic blast cells have been reported to have independent uncoordinated mRNA levels of S11 and S14.[26] These findings suggest that the expression of ribosomal proteins is distinctly regulated in various cancers.

Because the samples here were cells lysed with Laemmli sample buffer, ribosomal proteins in all cellular compartments were solubilized. To examine whether the observed differences in the expression of ribosomal proteins affect the amount and composition of the ribosome involved in protein synthesis, the polysome fractions from ten cell lines were isolated and analyzed by immunoblotting. Although HuO9 weakly expressed S11 (as described in the preceding paragraph), the concentration of polysomes in this cell line was not lower than that of other cell lines (Table II). This result suggests that the low expression of S11 did not compromise the biogenesis of ribosomes involved in protein synthesis. Further, the amounts of S11 and S30 in the polysomes were equal among the ten cell lines (Fig. 4) like S4X, which is uniformly expressed among the cell lines (Fig. 3C). This result suggests that the stoichiometry of the ribosomal proteins in these ribosomes is identical. Therefore, the differential expression of S11 and S30 does not seem to affect ribosome complexes; rather, this difference appears to be related to the role of these proteins in an extraribosomal localization.

DISCUSSION

A deficiency in the gene encoding a ribosomal protein undermines cell viability. This view is supported by a growing body of evidence obtained by using eukaryotic model organisms. For example, analysis of yeast mutants has shown that 30 of 32 ribosomal proteins play essential roles in cell growth (Briones *et al.*[27] and references cited therein). Even a reduced dose of these proteins impairs the

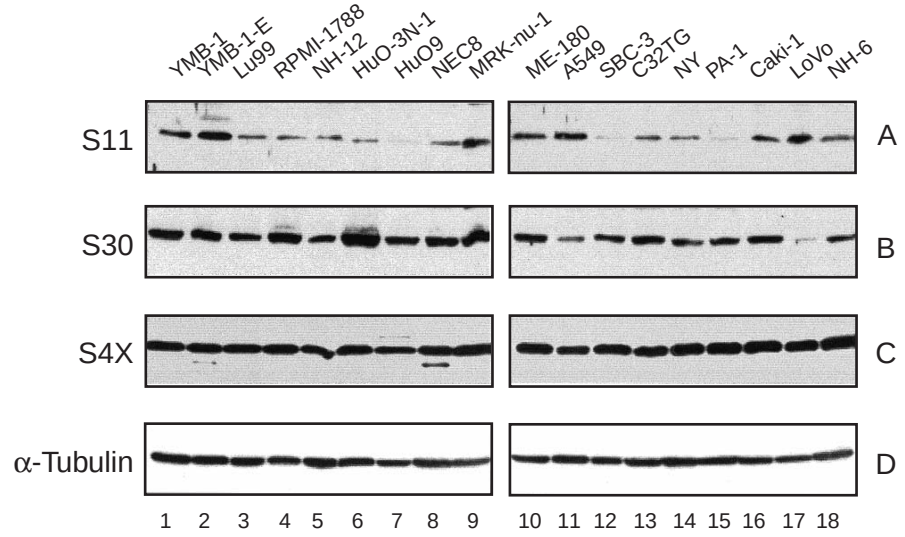

Fig. 3. Heterogeneous expression of ribosomal proteins S11 and S30 in cancer cell lines. The lysates of 18 human cell lines were subjected to immunoblotting using antibodies against S11 (panel A), S30 (panel B), S4X (panel C), and α-tubulin (panel D). Proteins from about 1×10^5 cells were loaded into each well of the gels. Lane 1, YMB-1; 2, YMB-1-E; 3, LU99; 4, RPMI1788; 5, NH-12; 6, HuO-3N1; 7, HuO9; 8, NEC8; 9, MRK-nu-1; 10, ME-180; 11, A549; 12, SBC-3; 13, C32TG; 14, NY; 15, PA-1; 16, Caki-1; 17, LoVo; 18, NH-6.

Table II. Amounts of Polysome in Human Cell Lines

Cell line	Polysome (μg/10^6 cells)
YMB-1	5.43
RPMI1788	1.08
HuO-3N1	4.17
HuO9	4.63
NEC8	19.3
MRK-nu-1	21.4
A549	6.45
Caki-1	7.62
LoVo	9.39
NH-6	7.11

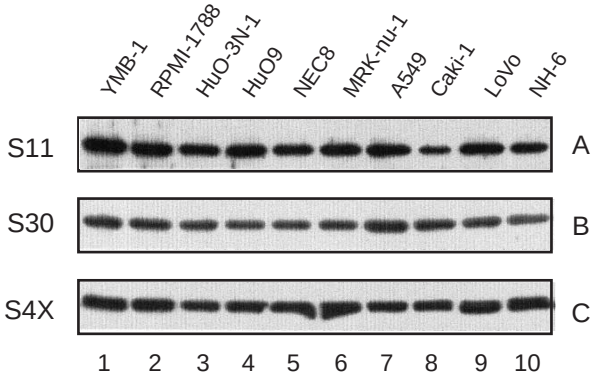

Fig. 4. The proteins in the ribosomes of cancer cell lines. Polysome fractions were isolated from the various cell lines and subjected to immunoblotting with antibodies against S11 (panel A), S30 (panel B), and S4X (panel C). We loaded 9 μg of polysomes into each well. Lane 1, YMB-1; 2, RPMI1788; 3, HuO-3N1; 4, HuO9; 5, NEC8; 6, MRK-nu-1; 7, A549; 8, Caki-1; 9, LoVo, 10, NH-6.

viability of a living organism. Haploinsufficiency of more than ten *Drosophila* ribosomal proteins (the *Minute* mutants) leads to malformation of various tissues.[28] In contrast, hemizygotes of ribosomal protein S6 showed overgrowth of the lymph glands (the hematopoietic organs) in the fly,[29, 30] demonstrating that reduced expression of ribosomal proteins can promote cell growth *in vivo*. To our knowledge, *in vivo* manipulation of the expression of mammal ribosomal proteins has not been reported. Here, we have prepared anti-human ribosomal protein antibodies to detect ribosomal proteins with high sensitivity and specificity. Specificity of these antibodies was also confirmed by direct binding of every antibody to its antigenic protein fused with glutathione-*S*-transferase, but not to glutathione-*S*-transferase itself (our unpublished data). Because the primary structures of ribosomal proteins are well conserved in eukaryotes,[18] the antibodies likely will cross-react with ribosomal proteins from other, non-human eukaryotic species. Therefore, our antibodies may be widely applicable to studying the biology and genetics of ribosomal proteins in model animals and organisms.

The papers described in the introduction and our data suggest abnormal expression of ribosomal proteins in mammalian tumor cells. How can a change in the expression of ribosomal proteins be connected with tumor progression and development? Only a few plausible links at a molecular level have been described. The ribosomal protein L18 is an inhibitor of double-stranded RNA-activated protein kinase (PKR).[31] Because an active form of PKR autophosphorylates, phosphorylates eukaryotic translation initiation factor-2, and eventually inhibits protein synthesis, up-regulation of PKR activity seems to suppress uncontrolled cell overgrowth.[12, 32] Therefore, overexpression of L18[14] has been implicated in the unregulated growth of colon carcinoma cells through the protein's inhibition of PKR. Another indicator of the connection between ribosomal proteins and tumorigenesis is the association of ribosomal L5 with the tumor suppressor p53, mdm-2, and protein kinase CK2 in the cell.[33, 34]

In the present study, we identified heterogeneous expression of ribosomal S11 and S30 by using our antibodies (Fig. 3). Because neither the biogenesis of the polysome (Table II) nor the protein makeup of the ribosomes (Fig. 4) differed as a result of the heterogeneity, the heterogeneity may be related to extraribosomal functions of S11 and S30. No extraribosomal function of S11 has been established as yet, and HuO9 cells with their low endogenous S11 will be useful for pursuing this question. One approach to elucidating the function of proteins is the production of null mutants. However, because ribosomal proteins are essential for cell growth, S11-null mutants likely are lethal. Manipulation of S11 expression by using the HuO9 cell line, such as overexpression of S11 in stable transfectants, may reveal the role of S11 in the cell. Dramatic but controlled change in the expression level of ribosomal protein S3a in culture cells has revealed the protein's pro-apoptotic nature.[35] Moreover, the unbalanced levels of S11 and L13a gene products in HuO9 cells are obvious (Fig. 3A and Fig. 2B, respectively). Coordinated regulation of the transcription of the genes for S11 and L13a has been proposed because they are located close to each other on chromosome 19 and have similar promoter regions.[36] Analysis of the regulation of these two genes in the HuO9 cell line may reveal a new mechanism underlying the aberrant expression of ribosomal proteins.

The heterogeneous expression of S30 is interesting because the gene encoding this protein is a putative tumor suppressor gene, the *fau* gene[37]; the initial gene product is a ubiquitin-like protein that is fused with S30. The *fau* gene is the cellular homolog of the fox sequence in the Finkel-Biskis-Reilly murine sarcoma virus; this virus contains the inverse of the mouse *fau* cDNA sequence. Expression of fox as the antisense of the *fau* gene increases the transforming capacity of the virus, probably by inactivating expression of the *fau* gene product. In addition, low expression of the Fau protein may sensitize cells to arsenite-associated genotoxicity and carcinogenesis.[38] Further, the Fau protein is an immune suppressor.[39] The signaling pathways in these various phenomena remain unclear. Further analysis of cancer cells with our anti-S30 antibody may uncover a connection between S30 and carcinogenesis. Although the exact biological significance of the heterogeneous expression of S11 and S30 is unclear at this stage, it is considered that the expression level of these ribosomal proteins is intimately linked to a specific intracellular environment. Actually, S11 has been found to be specifically down-regulated in apoptosis of human breast carcinoma cell line, MCF7, induced by staurosporine (our unpublished data). The relationship between ribosomal proteins and apoptosis is under investigation in our laboratory.

Although our antibodies will be used for basic research on translational machinery, they also are potentially powerful tools for medical and clinical applications, for example, for "ribosomapathy"[4] and "ribosomics" (genomics, transcriptomics, and proteomics of the ribosome). The evaluation of cancer cell lines in the present report suggests that our antibodies are applicable to analyses (e.g., immunohistochemistry) of tissue samples from patients, and the resulting information will complement message-level data. Further, our antibodies will make it possible to screen ribosomal proteins as a new marker, promoter, or suppressor of malignant neoplasms and other diseases. This process will improve our understanding of extraribosomal functions.

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