Electrophoretic Characterization of Ribosomal Subunits and Proteins in Apoptosis: Specific Downregulation of S11 in Staurosporine-Treated Human Breast Carcinoma Cells†

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ABSTRACT: Stimulation of death receptors (Fas on human T-cell leukemia Jurkat cells and tumor necrosis factor receptor-1 on human monoblastic leukemia U937 cells) triggers the specific degradation of 28S ribosomal RNA, and this process may contribute to cell death through the inhibition of protein synthesis. We have developed an analytical method using a polyacrylamide−agarose composite gel to evaluate ribosomal subunits in apoptotic cells (human breast carcinoma MCF-7 cells treated with staurosporine and human 293T cells irradiated with ultraviolet light were used in addition to the two apoptosis systems described above). No alterations were detected by this method, suggesting that apoptosis, including the process of ribosomal RNA degradation, does not cause fragmentation or extensive conformational changes in the ribosome. We also examined the status of 21 different ribosomal proteins in apoptotic cells by immunoblotting with polyclonal antibodies. S11 was specifically downregulated in apoptotic MCF-7 cells and in other apoptotic breast carcinoma cells. Previous studies have shown that S11 is heterogeneously expressed in cancer cells. Taken together, it appears that particular intracellular environments regulate the expression of S11 protein. However, the mechanism by which this process is modulated is as yet unknown. Furthermore, we have demonstrated that our composite gel electrophoresis system can efficiently detect ubiquitination of ribosomal subunits.

Ribosomes are the protein synthesis machinery common to all organisms. Prokaryotic ribosomes are the primary model used to study the structure of ribosomal protein and RNA components and the molecular mechanisms for protein production, including synthesis initiation and protein elongation (*1*). Studies of bacteria (e.g., stringent control) have shown us that ribosomes, and their associated components, are directly involved in translational control (*2*). However, in the higher eukaryotes such as humans, the relationship between the ribosome and translational regulation is largely unknown at a molecular level. On the basis of recent publications, it has been proposed that eukaryotic translational control be grouped into two categories (*3*). The first includes the regulation of ribosome biosynthesis. Knockout mice lacking the ribosomal protein S6 gene (*4*) and *Drosophila* with mutations in the S6 kinase gene (*5*) have revealed the importance of ribosome biogenesis in vivo, especially with respect to cell cycle progression. The second category of eukaryotic translational control includes the regulation of protein synthesis initiation. Phosphorylation of initiation factors plays a role in this process (*6*). The capacity for direct control of protein expression at the translational level provides an obvious advantage with respect to speed. Thus, protein elongation, including that of the large ribosomal subunit, is likely to be regulated. These rapid regulatory systems would be effective during periods when a cell undergoes drastic changes in its metabolic state, for example, during transitions from cell growth to growth suppression or apoptosis.

Recently, 28S ribosomal RNA (rRNA), a component of the large subunit, has been reported to be degraded during apoptosis (*7*, *8*). Moreover, this degradation was implicated in protein synthesis inhibition (*7*) and in activation of c-Jun NH_2-terminal kinase (*8*) in promoting the implementation of cell death. In addition, initiation factors 4G (*9*, *10*) and 2α (*11*) are also degraded during apoptosis by caspase-3, a protease involved in death implementation.

The evidence clearly suggests that the translational system receives functionally important modulation from apoptosis signaling cascades. However, biochemical knowledge of the ribosome during apoptosis is still fragmentary. For example, ultrastructural analysis has shown that ribosomes dissociate from the rough endoplasmic reticulum during apoptosis, but the molecular basis underlying this phenomenon is not known (*12*). In the report presented here, we have analyzed ribosomal subunits and proteins during apoptosis using our anti-ribosomal protein antibodies. We prepared these antibodies for a comprehensive study of the human ribosome that we have termed ribosomics (*13*).

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EXPERIMENTAL PROCEDURES

Cells and Culture. Human breast carcinoma cell lines MRK-nu-1, YMB-1, and YMB-1-E, human colon adenocarcinoma LoVo cells, human cervical cancer cell line ME-180, human testicular germ cell tumor NEC8, human neuroblastoma cell line NH-12, and human ovarian teratocarcinoma cell line PA-1 were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). The human T-cell leukemia cell line, Jurkat, and human embryonic kidney cell line, 293T, were obtained from American Type Culture Collection (Rockville, MD). The human breast carcinoma cell line MCF-7 and human monoblastic leukemia cell line U937 (DE-4) were obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Miyagi, Japan), and RIKEN Cell Bank (Tsukuba, Ibaraki, Japan), respectively. These cell lines were maintained according to the suppliers' instructions, and grown in an atmosphere of 5% CO_2 at 37 °C.

Apoptosis Systems. U937 cells were washed once with serum-free RPMI1640 and resuspended in the same medium. To induce apoptosis, recombinant human tumor necrosis factor-α (TNF-α)[1] (R&D Systems, Minneapolis, MN) was added at a concentration of 30 ng/mL, and the cells were incubated for 8 h. Control cells were incubated under the same conditions but without TNF-α. Jurkat cells were washed once with serum-free RPMI1640 and resuspended in the same medium. To induce apoptosis, a mouse monoclonal anti-Fas antibody (clone CH-11; Molecular & Biological Laboratories, Nagoya, Aichi, Japan) was added at a concentration of 100 ng/mL, and the cells were incubated for 8 h. Control cells were incubated with 100 ng/mL unrelated mouse IgM (no. 02-6800; Zymed, San Francisco, CA). MCF-7 cells were incubated for 16 h with 1 μM staurosporine (Sigma, St. Louis, MO), which had been dissolved at 1 mM in dimethyl sulfoxide for storage. Control cells were treated with the same volume of dimethyl sulfoxide. 293T cells were irradiated with ultraviolet-C light (258 nm) using a 30 W germicidal lamp (GL-30, Matsushita Electronics, Osaka, Japan) for 30 min at room temperature 0.4 m away from the light source. Cells were then cultured for 1 h at 37 °C. Control cells were treated according to the same procedure except without ultraviolet irradiation.

Electrophoretic DNA fragmentation analysis (*7*) and detection of caspase-3 activation by immunoblotting were used to confirm execution of apoptosis. In the latter case, because the anti-caspase-3 antibody used here recognizes its 32 kDa pro form, caspase-3 activation was observed as the decrease in the intensity of the 32 kDa band. An MTT assay was performed to examine the viability of MCF-7 cells using a kit supplied from Boehringer-Mannheim (Mannheim, Germany).

Ribosome Preparation. For the isolation of rat ribosomes (*14*), the liver (15 g) from a Sprague-Dawley rat was homogenized in 30 mL of 50 mM Tris-HCl (pH 7.5) containing 0.1 M KCl, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.25 M sucrose and then centrifuged for 20 min at 12000*g* to collect the postmitochondrial fractions. The ribosomal fractions were obtained by ultracentrifugation through a 1 M sucrose cushion at 130000*g* overnight, treated with puromycin, and centrifuged for 15 min at 10000*g*. The resulting supernatants were used as the 80S ribosome fraction. The 80S ribosome fraction was dissociated into 40S and 60S subunits in the presence of 0.5 M KCl, and these subunits were separated in sucrose gradients (*14*) prepared by the Gradient Master 106 (Towa Kagaku, Tokyo, Japan). The sucrose gradient fractions were collected using the piston gradient fractionator (Towa Kagaku), and each fraction was concentrated using the Centricon YM-100 apparatus (Millipore, Bedford, MA). Ribosomes from human cultured cells were isolated by essentially the same ultracentrifugation method. Ribosomal RNA was isolated from ribosomes using the Trizol reagent (GibcoBRL, Rockville, MD).

Antibodies. Production, purification, and characterization of rabbit polyclonal antibodies recognizing 19 human ribosomal proteins (S4X, S11, S12, S14, S15, S18, S30, L5, L7, L8, L9, L13a, L14, L15, L17, L18, L34, L35a, and L36a) were described in our previous report (*13*). For the preparation of new antibodies against human ribosomal proteins S3a and L10a, the following oligopeptides were chemically synthesized (cysteine residues added for conjugation are underlined): MAVGKNKRLTKGGKKGAKKKVC (residues 1−21 of S3a) and CKKLNKNKKLVKKLAKKYDA (residues 91−109 of L10a). These peptides were conjugated with maleimide-activated keyhole limpet hemocyanin and injected into hens (four injections per hen). Immunoglobulin Y was purified from the yolk of the hens' eggs by the water-dilution method (*15*). The specificity of these antibodies was characterized by immunoblotting using the purified ribosome, the ribosomal protein fused with glutathione *S*-transferase, and human cell lysates (*13*). The specific reactivity of all these antibodies is shown in the Supporting Information (Figure 1S). Mouse monoclonal antibodies against caspase-3 (clone 19), α-tubulin (clone B-5-1-2), and ubiquitin (clone 1B3) were purchased from Transduction Laboratories (Lexington, KY), Sigma, and Molecular & Biological Laboratories, respectively.

Polyacrylamide−Agarose Composite Gel Electrophoresis for the Separation of Mammalian Ribosomal Subunits. A 2.0% polyacrylamide−0.7% agarose composite gel [1.0 mm (thickness) × 87 mm (width) × 77 mm (height)] was prepared as follows. Electrophoresis-grade agarose (105 mg; Iwai Chemicals, Tokyo, Japan) was added to 13 mL of sterilized distilled water. After the agarose was melted, the gel solution was cooled to ∼40 °C. The following solutions were then added to the gel solution and mixed well: 3.0 mL of 0.20 M Tris containing 0.125 M acetic acid, 35% sucrose, and 0.05% (v/v) Triton X-100, 1.0 mL of 29% acrylamide/1% *N*,*N*′-methylene-bis-acrylamide, 20 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, and 20 μL of 10% ammonium persulfate. The mixture was then poured into a glass gel mold (Nihon Eido, Tokyo, Japan) and, after the insertion of an eight-well comb (Nihon Eido) at the top of the mold, kept for 30 min at room temperature for polymerization of the polyacrylamide gel. To completely solidify the agarose and cool the composite gel, it was kept at 4 °C for 1 h. The same procedure was used for the preparation of a 1.5% polyacrylamide−0.7% agarose composite gel, except that agarose was dissolved in 13.25 mL of water and 0.75 mL of acrylamide-bis solution was added to the mixture. For the preparation of cell extracts, cells (1×10^7) were

[1] Abbreviations: PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate; TNF-α, tumor necrosis factor-α; TNFR, tumor necrosis factor receptor.

washed once with phosphate-buffered saline, lysed with 0.1 mL of 5 mM Tris-HCl (pH 7.5) containing 1% (v/v) Triton X-100, 50 mM KCl, 10 mM 2-mercaptoethanol, 1.5 mM $MgCl_2$, and 1 mM phenylmethanesulfonyl fluoride, for 30 min on ice, and centrifuged for 20 min at 12000*g*. The supernatant was mixed with an equal volume of 50 mM Tris-HCl (pH 7.5) containing 0.5% (v/v) Triton X-100, 1 M sucrose, 50 mM KCl, 10 mM 2-mercaptoethanol, 1.5 mM $MgCl_2$, 0.04% bromophenol blue, and 0.04% xylele cyanole, and then loaded onto the gel. Gels were run for 45 min at 200 V in precooled buffer (40 mM Tris containing 25 mM acetic acid). The intact ribosomal subunits were electrophoresed from the negative electrode to the positive electrode. Red cytochrome *c* (Sigma) was used as a marker protein to detect electrophoresis from the positive electrode to the negative electrode. After electrophoresis, the gels were incubated with 1 μg/mL ethidium bromide in water to observe RNA bands. Pure ribosomes were also visualized by staining with 0.2% Coomassie Brilliant Blue R-250 (Sigma) in a methanol/acetic acid/water mixture (4:1:5) followed by destaining with the same solvent.

Immunoblotting after Composite Gel Electrophoresis of Ribosomes. After being stained with ethidium bromide, gels were incubated for 30 min with gentle agitation in 25 mM Tris containing 0.19 M glycine, 8 M urea, 0.03% sodium dodecyl sulfate (SDS), and 10 mM 2-mercaptoethanol. This incubation was repeated once using fresh buffer with the same composition. Ribosomal proteins were electroblotted onto a PVDF membrane (Immobilon, Millipore) in 25 mM Tris containing 0.19 M glycine, 0.03% SDS, and 10 mM 2-mercaptoethanol, using the Mini Transblot Cell (Bio-Rad, Hercules, CA) at 0.3 A for 60 min. The blots were probed with primary antibodies and then incubated with horseradish peroxidase-conjugated goat antibody against rabbit or mouse immunoglobulin G (Bio-Rad) or rabbit anti-chicken immunoglobulin Y antibody (Promega, Madison, WI). The signal was visualized using the ECL detection system (Amersham, Little Chalfont, England) and X-OMAT films (Kodak, New Haven, CT).

SDS−Polyacrylamide Gel Electrophoresis (PAGE) Followed by Immunodetection of Ribosomal Proteins. This procedure was described in detail previously (*13*). Briefly, cells (1×10^7) lysed in 0.5 mL of 1× Laemmli's sample buffer (100 °C, 10 min) were subjected to SDS−PAGE with 12% gels. The proteins were electroblotted onto Immobilon membranes using the Mini Transblot Cell. The blots were incubated with a primary antibody, and then with a horseradish peroxidase-conjugated secondary antibody, and finally, the signal was visualized using the ECL detection system as described above. Band intensity was evaluated using the Epson (Tokyo, Japan) ES-8000 image analyzer and NIH Image software (National Institutes of Health, Bethesda, MD). The details of densitometric analysis using NIH Image have been described previously.

Northern Blotting Analysis of S11 mRNA. The open reading frame of human ribosomal protein S11 cDNA (GenBank accession no. NM001015) was amplified from a human fetal brain cDNA library (Clontech, Palo Alto, CA) by polymerase chain reaction and subcloned into pCMV-Tag 2B (Stratagene, La Jolla, CA). After sequence confirmation, the cDNA insert was isolated, random-primed using the Prime-It labeling kit (Stratagene) and [α-^{32}P]dCTP (Amersham), and used as the probe for Northern blotting. Total RNA was isolated from apoptotic and control MCF-7 cells (8×10^6 each) using the Trizol reagent and dissolved in 50 μL of ribonuclease-free water. Duplicated total RNA samples (9 μL/lane) were electrophoresed in a 1% agarose/formaldehyde gel (*16*). One set of the samples in the gel was then subjected to ethidium bromide staining, and the other set was subjected to upward capillary transfer using a Hybond-N+ membrane (Amersham). The blot was hybridized for 1 h at 68 °C with the ^{32}P-labeled S11 probe in the QuickHyb solution (Stratagene), washed at room temperature with 2× SSC (1× SSC consists of 15 mM sodium citrate containing 0.15 M NaCl) and 0.1% SDS, washed at 50 °C with 0.2× SSC and 0.1% SDS, and then subjected to autoradiography.

Effect of the Inhibition of the Proteasome and Phosphatases on S11 in Apoptotic MCF-7 Cells. To examine the effect of proteasome inhibition, 13 h after the addition of staurosporine to MCF-7 cells, (*Z*)-Ile-Glu(OtBu)-Ala-Leucinal (proteasome inhibitor I; Peptide Institute, Osaka, Japan) was added at a concentration of 50 μM, and cells were further cultured for 3 h at 37 °C. Control cells incubated with dimethyl sulfoxide were also treated with this proteasome inhibitor according to the same procedure. Cells were then harvested, lysed in 1× Laemmli's sample buffer, and then subjected to SDS−PAGE and immunodetection. To examine the effect of phosphatase inhibition, apoptotic cells and control cells were treated for 30 min with 0.1 mM pervanadate before cell harvesting according to the method described in the previous report (*17*). The following analytical procedues were the same as those described above.

Ribosome Biosynthesis Analysis. Staurosporine-treated and control cells were prepared from MCF-7 cells (3×10^7 each) as described above. The cells were placed in methionine-free DMEM (Sigma) containing 1% dialyzed fetal calf serum and pulse-labeled with 110 μCi/mL L-[^{35}S]methionine (EASYTAG; New England Nuclear, Boston, MA) for 1 h. The 80S ribosome was purified from these cells by ultracentrifugation, and 1/30 of the ribosome fraction from every (apoptotic and control) cell sample was subjected to 2.0% polyacrylamide−0.7% agarose composite gel electrophoresis according to the procedures described above. After electrophoresis, the composite gel was stained with Coomassie Brilliant Blue, placed on Whatman 3MM Chr paper, dried up at 60 °C using the model 583 gel dryer (Bio-Rad), and then subjected to autoradiography. Ribosome biogenesis in staurosporine-treated Jurkat cells was examined by the same method that was used for staurosporine-treated MCF-7 cells except that RPMI1640 medium was used instead of DMEM medium.

RESULTS

Separation of Mammalian Ribosomal Subunits and Detection of Their Ubiquitination Were Achieved by Polyacrylamide−Agarose Composite Gel Electrophoresis. The mammalian ribosome (sedimentation coefficient, 80 S) is formed by the noncovalent association of the 60S large subunit (including 47 proteins and 28S, 5.8S, and 5S rRNAs) and the 40S small subunit (including 32 proteins and 18S rRNA) (*18*, *19*). Since these complexes are too large to separate using standard polyacrylamide gels, polyacrylamide−agarose composite gels (*20*) were used to overcome

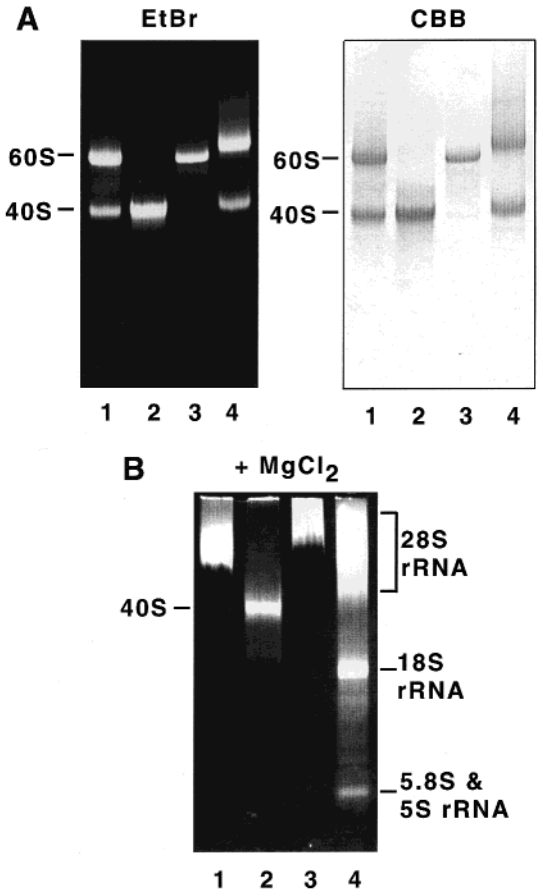

FIGURE 1: Polyacrylamide−agarose composite gel electrophoresis of mammalian ribosomes and subunits. (A) Electrophoric patterns under our standard conditions. Approximately 10 μg of the ribosomal complex was applied to each sample well, electrophoresed on a 2.0% polyacrylamide−0.7% agarose composite gel, stained with ethidium bromide (left panel), and then stained with Coomassie Brilliant Blue (right panel): lane 1, rat 80S ribosome; lane 2, rat 40S small subunit; lane 3, rat 60S large subunit; and lane 4, human 80S ribosome isolated from Jurkat cells. (B) Patterns obtained after electrophoresis in the presence of 5 mM $MgCl_2$. After electrophoresis on a 2.0% polyacrylamide−0.7% agarose composite gel, the gel was stained with ethidium bromide: lane 1, rat 80S ribosome; lane 2, rat 40S small subunit; lane 3, rat 60S large subunit; and lane 4, rRNAs isolated from the rat 80S ribosome. The negative electrode is at the top of all the panels.

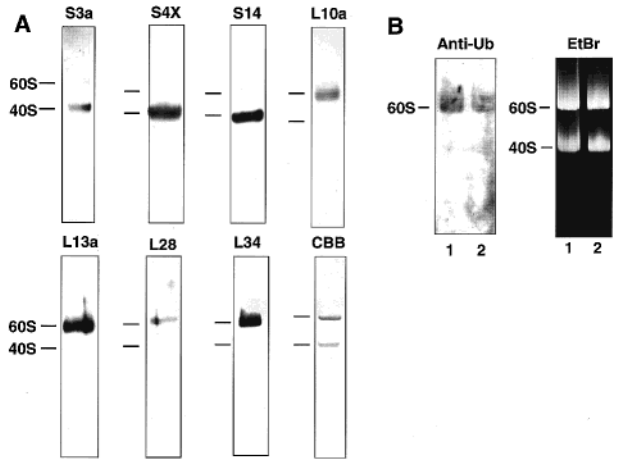

FIGURE 2: Immunoblotting of the mammalian ribosome after polyacrylamide−agarose composite gel electrophoresis. (A) Approximately 5 μg of the rat 80S ribosome was applied to each sample well, electrophoresed on a 2.0% polyacrylamide−0.7% agarose composite gel, and electroblotted on the PVDF membranes. The blots were stained with antiribosomal protein antibodies. Antibody target proteins are indicated at the tops of each panel. The CBB panel shows a blot stained with Coomassie Brilliant Blue. (B) Ubiquitination of the human ribosome. 80S ribosomes (5 μg/lane) from Lovo cells (lane 1) and NEC8 cells (lane 2) were electrophoresed on a 2.0% polyacrylamide−0.7% agarose composite gel, stained with ethidium bromide (right panel), and then immunostained using the anti-ubiquitin antibody following electroblotting (left panel). The negative electrode is at the top of all the panels.

this obstacle. The gel composition and electrophoresis conditions were significantly modified to maximize the separation of ribosomal subunits (for details, see Experimental Procedures). Because more than half of the mass of mammalian ribosomal subunits is composed of RNA, these complexes were electrophoresed from the negative electrode to the positive electrode in our protocol. Figure 1A shows the clear separation of the purified 40S and 60S subunits detected by ethidium bromide staining of RNA (lanes 2 and 3 of the left panel) and subsequent protein staining with Coomassie Brilliant Blue (lanes 2 and 3 of the right panel).

Under the electrophoresis conditions described above, the purified 80S ribosomes from rats and humans were spontaneously dissociated into 60S and 40S subunits (lanes 1 and 4 of Figure 1A, respectively). Because the association of the two subunits is known to be dependent on Mg^{2+} ions (*21*), ribosomal complexes and RNA were electrophoresed in the presence of 5 mM $MgCl_2$ (Figure 1B). Under these conditions, the 80S ribosome barely penetrated into the gels, resulting in a smeared band around the top of the gel (lane 1). Such smeared bands were also observed for the 60S subunit (lane 3) and 28S rRNA (upper band of lane 4), but not for the 40S subunit (lane 2) or 18S rRNA (middle band of lane 4), suggesting that these electrophoretic patterns of 60S and 80S complexes are mainly due to the unique properties of 28S rRNA in the presence of $MgCl_2$. The data presented here indicate that, although we are unable to analyze polysomes (80S ribosomes associated with mRNA), our method is suitable for the separation of mammalian ribosomal subunits.

The use of 1 mm thick slab gels for this analysis enabled the transfer of separated ribosomal subunits to membranes, which could then be subjected to immunoblotting with various antibodies against ribosomal components. Separated ribosomal subunits were electroblotted onto PVDF membranes to determine the feasibility of this procedure. The following modifications were added to achieve effective transfer of ribosomal proteins. (a) Ribosomal proteins are tightly packed with RNA in the large complex. To facilitate the exposure of antigenic sequences recognized by antibodies, the ribosomal subunits in the gels were incubated with 8 M urea prior to blotting. (b) Apart from three acidic proteins (P0−P2), ribosomal proteins are highly basic (isoelectric point > 10) (*18*). To transfer all ribosomal proteins in the same direction (from the negative electrode to the positive electrode), 0.03% SDS was included throughout the blotting procedure. We found that higher concentrations of SDS inhibited the absorption of the proteins onto PVDF membranes (data not shown). The successful transfer of ribosomal proteins was confirmed by staining the blots with Coomassie Briliant Blue (panel CBB of Figure 2A). The transfer efficiency of ribosomal proteins was examined using serially diluted rat liver ribosomes as electrophoresis samples as

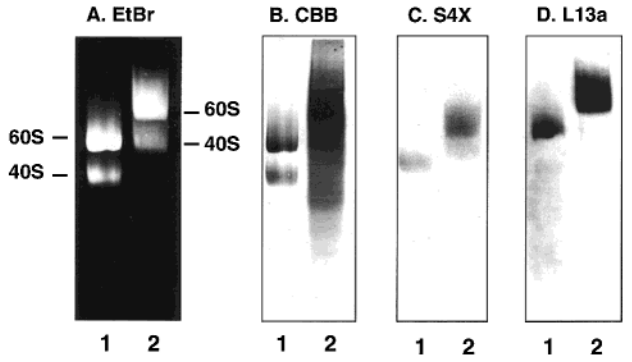

FIGURE 3: Specific detection of human ribosomal subunits from crude cell extracts. Purified ribosomes (10 μg, lane 1) and crude extracts (from 5×10^5 cells, lane 2) of MCF-7 cells were electrophoresed in a 1.5% polyacrylamide−0.7% agarose composite gel, stained with ethidium bromide (A), and electroblotted onto PVDF membranes. The blots were stained with Coomassie Brilliant Blue (B) and antibodies against S4X (C) and L13a (D). The negative electrode is at the top of all the panels.

follows (data not shown). (a) When the composite gels after transfer were stained with Coomassie Briliant Blue, no remaining ribosomal protein was detected. (b) Two sheets of PVDF membranes were placed on the positive electrode side of the gel in transfer. Coomassie Briliant Blue staining of the two blots after transfer revealed that more than 95% of proteins were detected on the membrane directly abutted on the positive electrode side of the gel. (c) When a PVDF membrane was placed on the negative electrode side of the gel, less than 3% of the proteins were transferred to the membrane. Therefore, under our blotting conditions, ribosomal proteins were likely to be retained effectively on a single PVDF membrane on the positive electrode side of the gel. The lower limit of detection of the ribosome on the blot was 0.6 μg with Coomassie Briliant Blue staining. The large and small ribosomal subunits were detected using antibodies specific for proteins within the large and small subunits (other panels of Figure 2A). The lower limit of immunodetection of a ribosomal subunit was, for example, 0.06 μg when our anti-S14 antibody was used.

It has recently been reported that both yeast and human ribosomal proteins are modified by ubiquitination (*22*). We asked whether this modification could be detected by our immunoblotting protocol using an anti-ubiquitin antibody. As shown in Figure 2B (left panel), the 60S large subunit of the purified human ribosome was selectively immunoreactive with the antibody. This is consistent with the previous finding that ubiquitin is ligated to ribosomal protein L27a of the human large subunit (*22*). Our results indicate that this electrophoretic method is suitable for the in situ detection of ribosomal protein ubiquitination.

Specific Detection of Ribosomal Subunits from Crude Biological Samples Was Applied to Characterization of Ribosomal Subunits in Apoptosis. The successful detection of ribosomal subunits led us to examine whether our technique could be used to detect subunits in cruder samples. Ribosomes were extracted using Triton X-100 from cultured human cells and analyzed by the combination of polyacrylamide−agarose composite gels and immunoblotting with antibodies against ribosomal proteins. Typical results, which were obtained using human breast carcinoma MCF-7 cells, are shown in Figure 3. Ribosomal subunits were identified by ethidium bromide staining of the gel before electroblotting (Figure 3A). Alter blotting had been carried out, however, ribosomal subunits could not be visualized by Coomassie Brilliant Blue staining due to the presence of other proteins and protein complexes which obscured the characteristic pattern of purified ribosomes (Figure 3B). However, small subunits and large subunits were specifically detected by immunoblotting using antibodies against ribosomal proteins S4X (Figure 3C) and L13a (Figure 3D), respectively. The method of ribosome preparation affected the mobility of ribosomal subunits. Comigration of many other proteins present in the crude lysates (Figure 3B) might interfere with the mobility of ribosomal subunits. We therefore examined various extraction conditions, including the use of deoxycholate and higher concentrations of potassium or sodium salt, but no improvement in yield or subunit separation was obtained. For example, extraction with 0.5 M KCl resulted in disturbed electrophoretic patterns (data not shown). In conclusion, our method was shown to be applicable to the separation of ribosomal subunits in crude biological samples and the detection of their structural alterations (conformational change and modification) which was visualized in the analysis of purified ribosomes in the preceding section.

Once our method was optimized, we examined whether it could be used to detect the alteration of ribosomal subunits in apoptosis. The following four apoptosis systems were used in this study: (a) TNF-α stimulation of the TNF receptor 1 (TNFR1, CD120a) on human monoblastic leukemia U937 cells, (b) anti-Fas antibody stimulation of Fas (CD95) on human T-cell leukemia Jurkat cells, (c) staurosporine treatment of human breast carcinoma MCF-7 cells, and (d) ultraviolet irradiation of human embryonic kidney 293T cells. Apoptosis in U937, Jurkat, and 293T cells was confirmed by DNA fragmentation analysis (Figure 4A). MCF-7 cells are deficient in caspase-3 protein expression (*23*, *24*), which was also confirmed by immunoblotting with an anti-caspase-3 antibody (data not shown). As expected, no caspase-3-activated DNA ladder formation (*25*) was detected in apoptotic MCF-7 cells (Figure 4A). Loss of cell viability by staurosporine treatment was confirmed by an MTT assay (data not shown). Crude cell extracts, including the ribosome from apoptotic and control cells, were subjected to electrophoresis in 1.5% polyacrylamide−0.7% agarose gels, followed by staining with ethidium bromide, and immunoblotting with anti-S4X, S14, and L13a antibodies (Figure 4B and data not shown). No alterations (i.e., mobility difference, generation of degraded particles, etc.) in ribosomal subunits were observed in apoptotic cells in any of the four systems. Since most ribosomal proteins have a high isoelectric point, it was possible that basic protein particles liberated from the ribosome migrated in a direction that is the opposite of that of intact ribosomal subunits and failed to be detected in our electrophoresis system. To exclude this possibility, cell lysate samples were electrophoresed toward the negative electrode and then subjected to immunoblotting. Under these conditions, no protein bands were detected. rRNA has been reported to be fragmented in the Fas−Jurkat and TNFR−U937 systems (*7*), and more complex damage of rRNA by exposing cells to ultraviolet light has been reported (*26*). Our data imply that apoptosis, including fragmentation of rRNA, does not cause extensive degradation or conformational changes in ribosomal subunits.

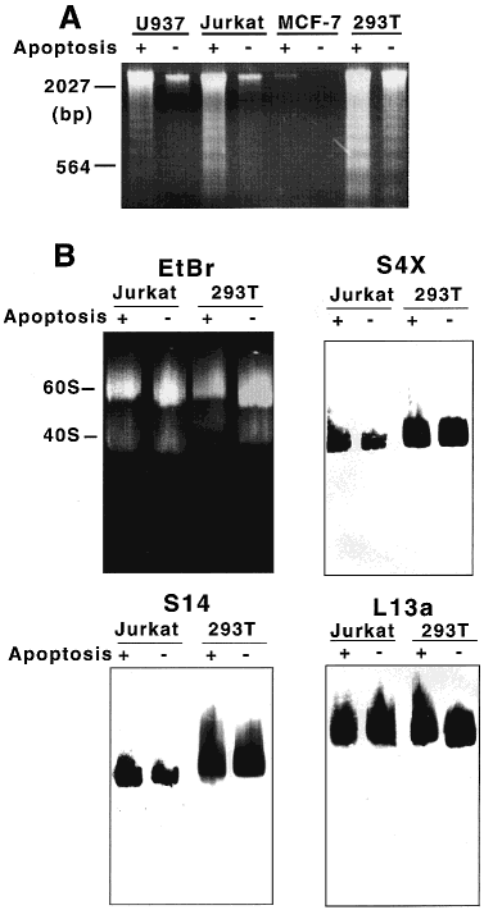

FIGURE 4: Ribosomal complexes in apoptosis. (A) DNA fragmentation analysis. The four apoptosis systems (TNF-α-stimulated U937 cells, Jurkat cells treated with anti-Fas antibody, MCF-7 cells treated with staurosporine, and UV-irradiated 293T cells) are described in Experimental Procedures. Triton-extracted DNA samples from all sets of apoptotic and control cells were electrophoresed in a 1.4% agarose gel and stained with ethidium bromide. (B) Electrophoretic patterns of ribosomal complexes. Apoptotic and control cells of the Fas−Jurkat system and the UV−293T system were lysed with the Triton-containing buffer, electrophoresed in a 1.5% polyacrylamide−0.7% agarose composite gel, stained with ethidium bromide (top left panel), and electroblotted onto PVDF membranes. The blots were stained with antibodies against S4X (top right panel), S14 (bottom left panel), and L13a (bottom right panel). The negative electrode is at the top of all the panels.

S11 Was Specifically Downregulated in Staurosporine-Mediated Apoptosis of Human Breast Carcinoma Cell Lines. A mobility shift caused by structural modification (probably fragmentation) of the acidic ribosomal protein, P0, has been observed on two-dimensional electrophoresis of apoptotic cell lysates from a human lymphoma cell line (*27*). When combined with mass spectrometry, two-dimensional gel electrophoresis is an excellent proteomics tool. However, the separation range of isoelectric focusing is limited under the conditions that are used, particularly for highly basic regions (isoelectric point > 10) (*28*). Therefore, it is possible that basic ribosomal proteins were overlooked in that study. In the study presented here, we examined 21 ribosomal proteins (S3a, S4X, S11, S12, S14, S15, S18, S30, L5, L7, L8, L9, L10a, L13a, L14, L15, L17, L18, L34, L35a, and L36a) in four apoptosis systems using our antibodies against them. Apoptotic and control cells were lysed in 1× Laemmli's SDS−PAGE sample buffer, boiled for 10 min to achieve complete cell lysis, and then subjected to SDS−PAGE followed by immunodetection. No fragmentations or changes in molecular form (molecular weight) were detected in this vast screening. However, in the case of apoptotic MCF-7 cells, expression levels of the S11 protein were reproducibly decreased by approximately 80%. We can conclude that this downregulation was not a passive result of overall protein synthesis inhibition or nonspecific protein degradation during cell death since there were no differences in the levels of numerous other ribosomal proteins. Furthermore, a cytoskeletal protein, α-tubulin, did not exhibit any differences between apoptotic cells and control MCF-7 cells (Figure 5A,B).

We next tried to clarify the mechanisms of specific S11 downregulation. To examine the possibility of transcriptional control, Northern blotting was performed. The same amount of S11 mRNA in apoptotic and control cells (Figure 5C) excluded downregulation at a transcriptional level. Recently, post-translational modification has been reported as an important mechanism for protein downregulation. For example, polyubiquitinated proteins are subjected to rapid degradation in the proteasome (*29*). No additional bands generated by polyubiquitination or phosphorylation of S11 were observed from apoptotic MCF-7 cells, as judged from immunoblotting with the anti-S11 antibody (Figure 5A). Furthermore, treatment of apoptoic MCF-7 cells with a proteasome inhibitor or a phophatase inhibitor before cell harvest (methods in Experimental Procedures) did not visualize any change in S11 band patterns during immunoblotting (data not shown). Although S11 in apoptotic MCF-7 was found to be downregulated at a post-transcriptional level, the precise mechanism remains to be elucidated.

S11 was significantly downregulated in the staurosporine−MCF-7 system, but not in the other three apoptosis systems (data not shown). We screened additional tumor cell lines that are killed by the same staurosporine treatment to determine whether this S11 downregulation was unique to MCF-7. The execution of apoptosis was confirmed by activation of caspase-3 visualized by immunoblotting using the anti-caspase-3 antibody (data not shown). The cell lysates were then subjected to immunoblotting using the anti-S11 antibody. In three different human breast carcinoma cell lines (MRK-nu-1, YMB-1, and YMB-1-E), S11 was downregulated after apoptosis execution, while such drastic downregulation was not observed during apoptosis in other types of tumors, including leukemia Jurkat, cervical ME-180, testicular NEC8, neuroblastoma NH-12, and ovarian PA-1 tumors (Figure 5D). Downregulation of the other 20 ribosomal proteins or the control protein, α-tubulin, was not detected in any of these cells (data not shown).

Although the essential molecules for apoptosis execution are the same in most cell types, some tissue specific variations in the execution pathway have been revealed (*30*). Staurosporine is a broad spectrum inhibitor of protein kinases, including protein kinase C. Its potency and inhibition mechanisms appear to be different from kinase to kinase (*31*). The cell-type specific downregulation of S11 shown here may reflect a difference in the kinds of kinases affected in staurosporine-mediated apoptosis in tissues. Despite the common use of staurosporine as an inducer of apoptosis, the direct target of this drug in the cell is still unknown. Therefore, an initial approach to clarifying the mechanism

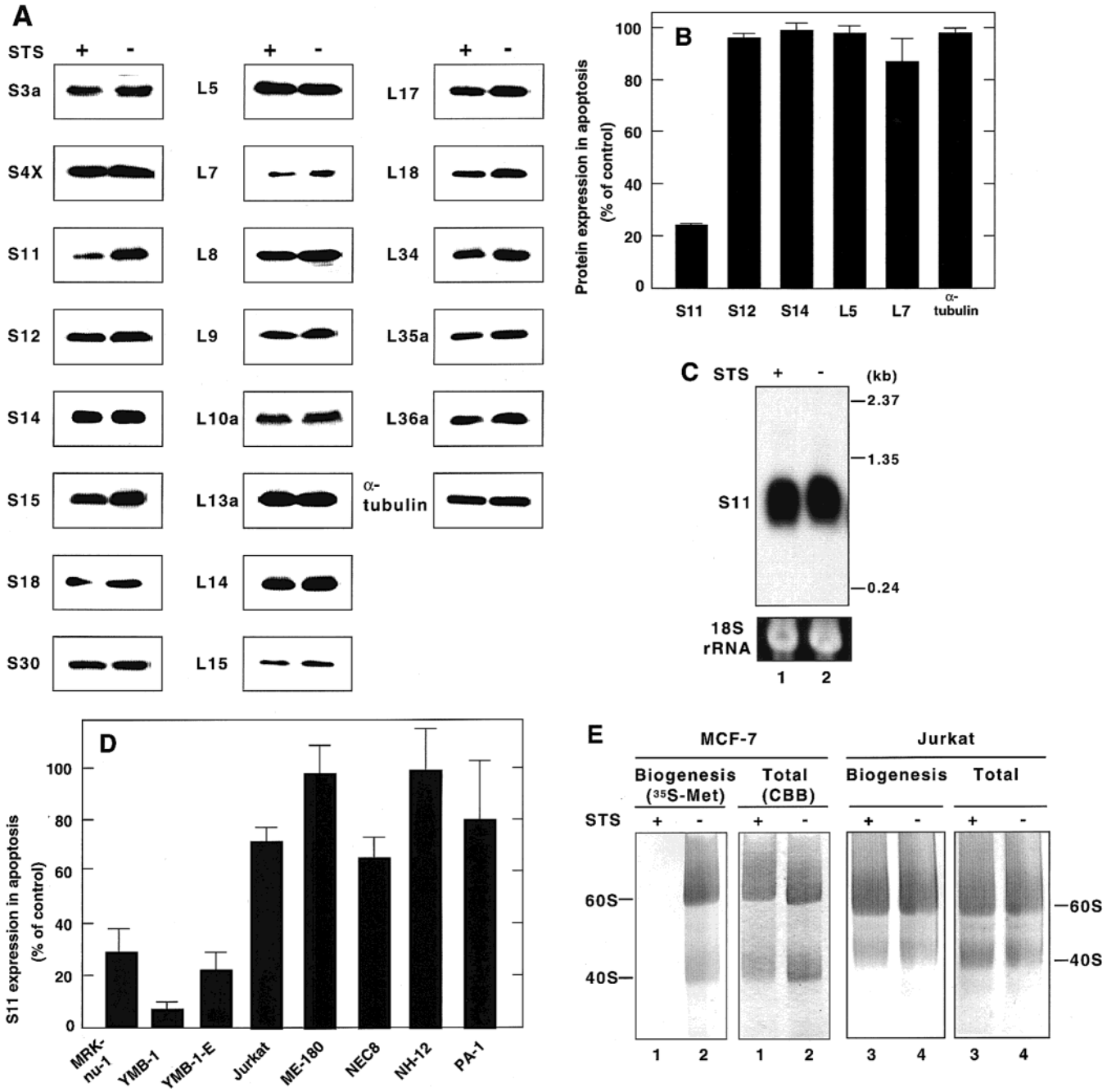

FIGURE 5: Downregulation of S11 in apoptotic breast carcinoma cells. (A) Expression of 21 ribosomal proteins in MCF-7 with and without staurosporine (STS) treatment. Lysates (from 1×10^5 cells/lane) of STS-treated and control cells were subjected to SDS−PAGE on 12% gels and electroblotted onto PVDF membranes. The blots were stained with antibodies, whose target proteins are indicated at the left sides of all the panels. (B) Specific downregulation of S11 in apoptotic MCF-7 cells was reproducible. Immunoblots were prepared and immunostained as described for panel A. The protein band intensity of staurosporine-treated samples relative to controls is shown. The data represent the means ± the standard deviation for three independent experiments. (C) S11 mRNA in apoptotic MCF-7 cells. Total RNA from apoptotic and control cells was subjected to Northern blotting analysis as described in Experimental Procedures. S11 mRNA detected by autoradiography and 18S rRNA stained with ethidium bromide are shown in the top and bottom panels, respectively. The migration positions of RNA molecular weight markers (GibcoBRL) are indicated on the right of the top panel: lane 1, apoptotic MCF-7 cells; and lane 2, control MCF-7 cells. (D) Downregulation of S11 was also observed in other breast carcinoma cell lines. The procedures for staurosporine treatment, immunoblotting, and measurement of band intensity were as described for panels A and B. The data represent the means ± the standard deviation for three independent experiments. (E) Suppression of ribosome biogenesis in apoptotic MCF-7 cells. After pulse-labeling of apoptotic and control cells with [^{35}S]methionine had been carried out, the ribosome was isolated from these cells and subjected to 2.0% polyacrylamide−0.7% agarose composite gel electrophoresis. Separated ribosomal subunits were stained with Coomassie Brilliant Blue (Total) and then analyzed by antoradiography (Biogenesis): lane 1, apoptotic MCF-7 cells; lane 2, control MCF-7 cells; lane 3, apoptotic Jurkat cells; and lane 4, control Jurkat cells. The negative electrode is at the top of all the panels.

of S11 downregulation specific in breast carcinoma is to search for a target kinase in breast carcinoma. Very recently, protein kinase B (Akt) has been reported to be inhibited by staurosporine, and the inhibition of protein kinase B signaling appeared to be involved in the pro-apoptotic action of staurosporine (*32*). Examining whether protein kinase B is the target of this drug under our conditions may give us a good clue about the S11 downregulation mechanism.

Because S11 is an essential component of the ribosome (see the Discussion for details), it is possible that downregulation of S11 leads to the inhibition of ribosome biosynthesis in staurosporine-treated MCF-7 cells. To pursue this possibility, apoptotic and control MCF-7 cells were pulse-labeled with [^{35}S]methionine and the amounts of newly synthesized ribosomes in the cytoplasm were examined using our polyacrylamide−agarose composite gel electrophoresis. As shown in Figure 5E (lanes 1 and 2), ribosome biosynthesis was suppressed by staurosporine treatment. The mechanism of ribosome biogenesis appears to be well conserved throughout eukaryotes. According to reports about yeast (*33*), many ribosomal proteins are synthesized in the cytoplasm and imported into the nucleus, where they associate with pre-rRNA to generate a preribosomal particle (early 90S particle). The 90S particle is subsequently cleaved into 66S and 43S particles, the precursors to the mature cytoplasmic 60S and 40S subunits, respectively. We can speculate that the downregulation of essential S11 inhibits the assembly of these precursors (such as an early 90S particle) inside the nucleus, resulting in the loss of newly synthesized ribosomal subunits in the cytoplasm. Ribosome biogenesis was not suppressed in staurosporine-treated Jurkat cells, where S11 was not drastically downregulated (Figure 5D), excluding the possibility that apoptosis shuts down ribosome biogenesis (lanes 3 and 4 of Figure 5E). Our finding implicates a link between S11 downregulation and the inhibition of ribosome biosynthesis.

In our previous report (*13*), we demonstrated that S11 was heterogeneously expressed in human cancer cell lines, whereas most other ribosomal proteins were consistently expressed across all cell lines. In other words, although no deficiency of the S11 protein was observed, the expression level of this protein was obviously different among the cell lines. Expression of S11 appears to be modulated in particular intracellular environments by unknown mechanisms.

DISCUSSION

Studies of yeast and fly mutants have revealed that many eukaryotic ribosomal proteins are essential for cell viability (see references in ref *13*). However, the precise structure, functional role, and regulation of individual ribosomal proteins are largely unknown. There is evidence concerning ribosomal components in higher organisms, including humans, that argues against the prevailing notion that most of the important questions about the ribosome have been resolved. For example, abnormalities in specific ribosomal proteins cause genetic diseases and carcinogenesis (*34*, *35*). In addition, ribosomal proteins appear to have roles unrelated to the translation machinery (extraribosomal functions) (*18*, *35*).

In the present era of genomics, copious genetic information and revolutionary technologies make it possible for researchers to systematically characterize complicated macromolecular machines and organelles such as the nuclear pore complex (*36*, *37*). Studies carried out by an international consortium have provided a great deal of information about the mammalian mitochondorial ribosome (*38*). Our group has proposed to analyze the human cytoplasmic ribosome and its components at the levels of the gene, the RNA, the protein, and the molecular complex. We have coined the term "ribosomics" to describe this comprehensive field of study (*13*).

In a previously published study, we determined the status of four rRNAs during apoptosis triggered by death receptor activation and found that 28S rRNA was specifically degraded (*7*). In this report, we examined ribosomal proteins and subunits in apoptosis.

Specific probes are essential for the systematic study of all ribosomal proteins. Unfortunately, very few antibodies against human ribosomal proteins are commercially available. For this reason, we have undertaken to prepare antibodies against all ribosomal proteins. In our initial screening, we obtained rabbit antibodies against 26 proteins, among which 19 exhibited specific binding to their antigenic protein in crude biological samples (*13*). To obtain antibodies against the remaining proteins, we have used the chicken as a host for antibody production and new oligopeptides for antigens. Very recently, two new chicken antibodies have exhibited specific reactivity to S3a and L10a. These new antibodies resulted in a total of 21 antibodies for use in the present characterization of ribosomal proteins in apoptosis.

Ribosomal protein S11 was found to be specifically downregulated in four different human breast carcinoma cell lines treated with staurosporine. Staurosporine and its derivatives cause cell cycle arrest, inhibition of growth, and apoptosis in breast carcinoma cells (*31*, *39*). Because staurosporine is a candidate anticancer drug (*31*), the analysis of molecular targets of staurosporine could help in the development of new cancer therapies. Although rat S11 was first identified as an 18 kDa polypeptide in 1985 (*40*), the function of mammalian S11 remains unknown. S11 is well conserved during evolution, and homologues have been identified in *Saccharomyces cerevisiae* (S18) and *Escherichia coli* (S17). The yeast and bacterial homologues are essential for the assembly of small and large ribosomal subunits (*41*, *42*). Furthermore, merely reducing the dosage of the yeast gene product causes biologically important phenotypes, including changes in the accuracy of ribosomal translation (*41*). In this study, biogenesis of the ribosome was found to be suppressed in MCF-7 cells treated with staurosporine (Figure 5E). These data raise the possibility that downregulation of the S11 protein in mammalian cells leads to inhibition of the biosynthesis of the ribosome and/or modulation of translation products and subsequently promotes cell death in staurosporine-mediated apoptosis. If so, overexpression of S11 in apoptotic breast carcinoma cells would suppress or delay the execution of apoptosis. To pursue this possibility, we screened MCF-7 cells transfected with an expression vector of FLAG-tagged human S11 and obtained six stable transfectants expressing the exogenous FLAG−S11 protein, which was confirmed by immunoblotting with the anti-FLAG antibody. However, overexpression of the total S11 protein was not achieved as judged from immunoblotting with our anti-S11 antibody that can bind to exogenous and endogenous S11 (our unpublished data).

The expression of ribosomal protein genes is strictly regulated in yeast (*43*). To prohibit uncontrolled overexpression, the exogenous S11 protein in human MCF-7 cells might suppress the expression of endogenous S11. Alternatively, excess protein might be rapidly degraded. Although a precise control mechanism for S11 expression has not been described, translational regulation of expression by binding to their own pre-mRNA has been reported in yeast for a few ribosomal proteins (*33*). Such autoregulation by

feedback mechanisms in a ribosomal protein gene cluster (operon) has been widely observed in bacteria (*2*). However, since human ribosomal protein genes are dispersed within a much larger genome (*44*), the regulation mechanism is not likely to be so simple.

Ribosomal protein genes are generally considered to be housekeeping genes, although, at a message level, previous reports (refs *45* and *46*, for example) have revealed changes in the expression of mammalian ribosomal protein genes under some conditions. Indeed, most of the genes were found to be constitutively expressed, as determined by immunoblotting analysis of human cells using antiribosomal protein antibodies (*13*) and in this study (Figure 5). However, the previous systematic survey revealed that S11 was heterogeneously expressed across human cancer cell lines. In the study presented here, we found cell-type specific downregulation of S11 in response to staurosporine. These data are contrary to the widely held belief that all ribosomal proteins are expressed constantly under every condition and point out the potential for ribosomics in revealing unexpected properties of ribosomal proteins.

In addition to the study of ribosomal components, functional ribosomal complexes must be investigated for a full understanding of the ribosome. Distinct methods have been developed to observe the ribosome in the cell. Intracellular localization of ribosomes has been visualized by conventional electron microscopy (*12*). Polysomal profiles can be analyzed by sucrose gradient centrifugation (*14*). X-ray crystallography is currently the best available approach for the structural analysis of ribosomal complexes (*47*). However, crystallography requires a large amount of ultrapure ribosomes and special techniques. We provide here a simple electrophoretic approach that is suitable for the rapid, specific detection of ribosomal subunits in biological samples. Since we have optimized our technique using mammalian ribosomes, the largest and thus most challenging to study, we are confident that ribosomal subunits from any species can be analyzed by this method.

While our analytical method is unsuitable for the isolation of ribosomal subunits for carrying out chemistry on the subunits and their components, our results indicate the potential usefulness of this method. The working ribosome changes its conformation dynamically in the cytoplasm (*48*, *49*). Although nuclear magnetic resonance is one of the best methods for observing high-order structure in water, its application to large biopolymers (for instance, a >30 kDa protein) is still difficult (*50*). The difference we observed in the electrophoresis patterns of 80S and 60S complexes in the absence and presence of magnesium ions (Figure 1) suggests that this method is sensitive to some kinds of structural alteration in a ribosomal complex in solution. Ribosomes receive post-translational modifications in the cell, which alter their biological functions. For example, phosphorylation of S6 in the 40S ribosomal subunit selectively promotes the translation of mRNAs related to cell proliferation (*51*), and defects in ubiquitination of the large subunit reduce ribosomal function and cell viability (*22*). We have shown that our method can detect modifications of the ribosome, as indicated in Figure 2B.

Finally, another potential application should be pointed out, which could not have been anticipated when the original composite gel electrophoresis was reported more than 30 years ago (*20*). It is now known that cellular events, such as growth promotion and suppression, differentiation, and death, are controlled by a complex array of molecules in response to environmental cues. Interacting molecules often associate and form a large complex to promote the efficient execution of these phenomena and to protect themselves from proteolysis. There are many known examples of these multimolecule complexes. For example, a multiprotein complex remodels chromatin DNA structure (*52*), and mRNA is synthesized by RNA polymerase II which is composed of 12 subunits (*53*). Spliceosomes manage the processing of the precursor of mRNA (*54*). Proteasomes for protein catabolism are recognized as key players in signal transduction (*29*). Because the pore size and components of composite gels and antibodies for detection are easily modified, this flexible technique could be applied to the analysis of other macromolecules and large protein complexes (and subcomplexes) and their post-translational modifications, including acetylation and glycosylation, by combination with immunoblotting.

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SUPPORTING INFORMATION AVAILABLE

Figure 1S, which shows the specific reactivity of antibodies with ribosomal proteins in crude cell lysates. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Green, R., and Noller, H. F. (1997) *Annu. Rev. Biochem. 66*, 679−716.
2. Nomura, M., Gourse, R., and Baughman, G. (1984) *Annu. Rev. Biochem. 53*, 75−117.
3. Schmidt, E. V. (1999) *Oncogene 18*, 2988−2996.
4. Volarevic, S., Stewart, M. J., Ledermann, B., Zilberman, F., Terracciano, L., Montini, E., Grompe, M., Kozma, S. C., and Thomas, G. (2000) *Science 288*, 2045−2047.
5. Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C., and Thomas, G. (1999) *Science 285*, 2126−2129.
6. Clemens, M. J., and Bommer, U.-A. (1999) *Int. J. Biochem. Cell Biol. 31*, 1−23.
7. Nadano, D., and Sato, T. (2000) *J. Biol. Chem. 275*, 13967−13973.
8. Iordanov, M. S., Paranjape, J. M., Zhou, A., Wong, J., Williams, B. R. G., Meurs, E. F., Silverman, R. H., and Magun, B. E. (2000) *Mol. Cell. Biol. 20*, 617−627.
9. Marissen, W. E., and Lloyd, R. E. (1998) *Mol. Cell. Biol. 18*, 7565−7574.
10. Clemens, M. J., Bushell, M., Jeffrey, I. W., Pain, V. M., and Morley, S. J. (2000) *Cell Death Differ. 7*, 603−615.
11. Marissen, W. E., Guo, Y., Thomas, A. A. M., Matts, R. L., and Lloyd, R. E. (2000) *J. Biol. Chem. 275*, 9314−9323.
12. Clarke, P. G. H. (1990) *Anat. Embryol. 181*, 195−213.
13. Nadano, D., Ishihara, G., Aoki, C., Yoshinaka, T., Irie, S., and Sato, T. (2000) *Jpn. J. Cancer Res. 91*, 802−810.
14. Bommer, U. A., Burkhardt, N., Junemann, R., Spahn, C. M. T., Triana-Alonso, F. J., and Nierhaus, K. H. (1997) in *Subcellular Fractionation. A Practical Approach* (Graham, J. M., and Rickwood, D., Eds.) pp 271−301, Oxford University Press, New York.

15. Akita, E. M., and Nakai, S. (1993) *J. Immunol. Methods 160*, 207−214.
16. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
17. Garton, A. J., Flint, A. J., and Tonks, N. K. (1996) *Mol. Cell. Biol. 16*, 6408−6418.
18. Wool, I. G., Chan, Y.-L., and Gluck, A. (1996) in *Translational Control* (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., Eds.) pp 685−732, Cold Spring Harbor Laboratory Press, Plainview, NY.
19. Olvera, J., and Wool, I. G. (1996) *Biochem. Biophys. Res. Commun. 220*, 954−957.
20. Peacock, A. C., and Dingman, C. W. (1968) *Biochemistry 7*, 668−674.
21. Ts'o, P. O. P., and Vinograd, J. (1961) *Biochim. Biophys. Acta 49*, 113−129.
22. Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M., and Finley, D. (2000) *Cell 102*, 67−76.
23. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) *J. Biol. Chem. 273*, 9357−9360.
24. Tang, D., and Kidd, V. J. (1998) *J. Biol. Chem. 273*, 28549−28552.
25. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) *Nature 391*, 43−50.
26. Iordanov, M. S., Pribnow, D., Magum, J. L., Dinh, T.-H., Pearson, J. A., and Magun, B. E. (1998) *J. Biol. Chem. 273*, 15794−15803.
27. Brockstedt, E., Rickers, A., Kostka, S., Laubersheimer, A., Dorken, B., Wittmann-Liebold, B., Bommert, K., and Otto, A. (1998) *J. Biol. Chem. 273*, 28057−28064.
28. Nadano, D., Yasuda, T., Sawazaki, K., Takeshita, H., and Kishi, K. (1996) *Electrophoresis 17*, 104−109.
29. DeMartino, G. N., and Slaughter, C. A. (1999) *J. Biol. Chem. 274*, 22123−22126.
30. Los, M., Wesselborg, S., and Schulze-Osthoff, K. (1999) *Immunity 10*, 629−639.
31. Gescher, A. (2000) *Crit. Rev. Oncol. Hematol. 34*, 127−135.
32. Hill, M. M., Andjelkovic, M., Brazil, D. P., Ferrari, S., Fabbro, D., and Hemmings, B. A. (2001) *J. Biol. Chem. 276*, 25643−25646.
33. Woolford, J. L., Jr., and Warner, J. R. (1991) in *The Molecular and Cellular Biology of the Yeast Saccharomyces* (Broach, J. R., Pringle, J. R., and Jones, E. W., Eds.) pp 587−626, Cold Spring Harbor Laboratory Press, Plainview, NY.
34. Draptchinskaia, N., Gustavsson, P., Andersson, B., Pettersson, M., Willig, T.-N., Dianzani, I., Ball, S., Tchernia, G., Klar, J., Matsson, H., Tentler, D., Mohandas, N., Carlsson, B., and Dahl, N. (1999) *Nat. Genet. 21*, 169−175.
35. Naora, H., and Naora, H. (1999) *Immunol. Cell Biol. 77*, 197−205.
36. Blobel, G., and Wozniak, R. W. (2000) *Nature 403*, 835−836.
37. Pandey, A., and Mann, M. (2000) *Nature 405*, 837−846.
38. O'Brien, T. W., Liu, J., Sylvester, J. E., Mougey, E. B., Fischel-Ghodsian, N., Thiede, B., Wittmann-Liebold, B., and Graack, H.-R. (2000) *J. Biol. Chem. 275*, 18153−18159.
39. Seynaeve, C. M., Stetler-Stevenson, M., Sebers, S., Kaur, G., Sausville, E. A., and Worland, P. J. (1993) *Cancer Res. 53*, 2081−2086.
40. Tanaka, T., Kuwano, Y., Ishikawa, K., and Ogata, K. (1985) *J. Biol. Chem. 260*, 6329−6333.
41. Folley, L. S., and Fox, T. D. (1994) *Genetics 137*, 369−379.
42. Herzog, A., Yaguchi, M., Cabezon, T., Corchuelo, M.-C., Petre, J., and Bollen, A. (1979) *Mol. Gen. Genet. 171*, 15−22.
43. Warner, J. R. (1999) *Trends Biochem. Sci. 24*, 437−440.
44. Kenmochi, N., Kawaguchi, T., Rozen, S., Davis, E., Goodman, N., Hudson, T. J., Tanaka, T., and Page, D. C. (1998) *Genome Res. 8*, 509−523.
45. Shimbara, N., Sato, C., Takashina, M., Tanaka, T., Tanaka, K., and Ichihara, A. (1993) *FEBS Lett. 322*, 235−239.
46. Scheurle, D., DeYoung, M. P., Binninger, D. M., Page, H., Jahanzeb, M., and Narayanan, R. (2000) *Cancer Res. 60*, 4037−4043.
47. Cech, T. R. (2000) *Science 289*, 878−879.
48. Stark, H., Rodnina, M. V., Wieden, H.-J., van Heel, M., and Wintermeyer, W. (2000) *Cell 100*, 301−309.
49. Jerinic, O., and Joseph, S. (2000) *J. Mol. Biol. 304*, 707−713.
50. Arata, Y., Kato, K., Takahashi, H., and Shimada, I. (1994) *Methods Enzymol. 239*, 440−464.
51. Edgar, B. A. (1999) *Nat. Cell Biol. 1*, E191−E193.
52. Ito, T., Ikehara, T., Nakagawa, T., Kraus, W. L., and Muramatsu, M. (2000) *Genes Dev. 14*, 1899−1907.
53. Kornberg, R. D. (2000) *Trends Cell Biol. 9*, M46−M49.
54. Kaufer, N. F., and Potashkin, J. (2000) *Nucleic Acids Res. 28*, 3003−3010.

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