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A novel nucleolar protein interacts with ribosomal protein S19 ☆

Noriko Maeda *, Seikichi Toku, Naoya Kenmochi [1], Tatsuo Tanaka

Department of Biochemistry, School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

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Abstract

The gene encoding ribosomal protein S19 (RPS19) is mutated in approximately 25% of patients with Diamond–Blackfan anemia (DBA), which is a rare congenital erythroblastopenia. DBA patients have a variety of clinical characteristics, and the role of the *RPS19* gene in the pathogenesis of the disease is presently unknown. To investigate a possible role for RPS19 in erythropoiesis, we looked for proteins associated with mouse RPS19 using a yeast two-hybrid system and identified a novel protein, which we named S19 binding protein (S19BP). The deduced amino acid sequence of S19BP derived from cDNA defines a calculated mass of 15,849 and an isoelectric point of 11.3. No known functional motifs were found in S19BP except a short polylysine tract embedded in a putative nucleolar localization signal. Immunolocalization experiments revealed that S19BP was highly concentrated in nucleoli after 6 h of transfection in Cos-7 cells. S19BP was expressed ubiquitously at a basal level but a significantly high level of expression was observed in some tissues.

Keywords: Ribosomal protein S19; Yeast two-hybrid system; Nucleolus; Ribosomal protein S19 binding protein

Diamond–Blackfan anemia (DBA) is a rare congenital hypoplastic anemia. Linkage analysis mapped the DBA gene to chromosome 19 at q13.2 [1] leading to the discovery of a gene that encodes ribosomal protein S19 (RPS19) [2,3]. However, mutations in the *RPS19* gene have been identified in only 25% of cases [2,4]. Subsequent linkage analysis indicated a second DBA gene at 8p23.3-p22 [5], where no ribosomal protein gene is located [6], and also suggested the existence of at least one more gene in addition to these two [5]. Candidate genes at these loci have not yet been specified. Other ribosomal protein genes of the patients were also tested but not mutated [7]. How a defect of *RPS19* leads to this serious anemia is a mystery. Although minor malformations occur in some cases, the major symptom is erythropoiesis. The pathogenesis, therefore, must be due not to a general failure of ribosomal function in protein synthesis but to a defect in the specific function of RPS19. Ribosomal proteins exhibit various extraribosomal functions including control of gene expression [8]. Such functions must be brought about through the interaction of the ribosomal protein with proteineous factors or probably with DNA whether directly or indirectly. The factors cooperating with RPS19 in the generation of erythrocytes may be involved in the pathogenesis of the anemia. It is interesting in this connection that RPS19 is expressed at much higher levels in some tissues than in others [9], although most ribosomal proteins are expressed equally [10]. The fact that patients with DBA do or do not have various physical anomalies [11] may reflect a difference in the causative factor.

We searched for proteins bound to RPS19 to reveal the mechanisms that involve RPS19 in erythropoiesis and found a novel protein that has a polylysine stretch and localizes to the nucleus especially the nucleolus in high concentrations.

☆ The sequence data described in this paper have been submitted to the DDBJ/EMBL/GenBank DNA databases under Accession Nos. AB233218 and AB233219.

* Corresponding author. Fax: +81 98 895 1404.
E-mail address: nmaeda@med.u-ryukyu.ac.jp (N. Maeda).
[1] Present address: Frontier Science Research Center, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan.

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Materials and methods

Construction of expression vector for yeast cells. The cDNA of full-length RPS19-coding sequences was obtained by PCR amplification from a rat liver cDNA library as a template. The primers were designed to introduce a *Bam*HI site at both ends: 5′-CCGGGATCCGTATGCCTGG AGTTACTGTA-3′ as the sense primer and 5′-TCGACGGATCCGTTC TAATGCTTCTTGTTG-3′ as the antisense primer. The PCR product was digested with *Bam*HI and purified on a 1% agarose gel to obtain a single band of expected size. The purified fragment was cloned in-frame into a pAS2-1 (Clontech) yeast expression vector. The resulting construct was designated pAS2-1-S19. The plasmids pAS2-1-S3a, pAS2-1-L7a, and pAS2-1-L5 were also prepared by the same method using the corresponding primer sets, respectively.

To make deletion mutants of S19BP, PCRs were performed using a cDNA having a 5′ end *Bam*HI site and a 3′ end *Xho*I site. After digestion with appropriate restriction enzymes, the products were ligated in frame into pACT2 using the *Bam*HI and *Xho*I sites.

Yeast two-hybrid library screening. A mouse 13-day embryonic cDNA library, constructed on pACT2 and contained in Y187 (Mat α) yeast cells (Clontech), was screened according to the manufacturer's instructions as follows. Yeast strain AH190 (Mat a) was transformed with pAS2-1-S19 using the yeast transformation system kit (Clontech). After the mating of Y187 and AH190 together, approximately 10^8 independent clones were screened. Diploid positive colonies were identified by growth on Ade^-, His^-, Leu^-, Trp^- synthetic dropout (QDO) agar plates containing 2 mM 3-amino-1,2,4-triazole. Transformants grown on QDO plates were screened further with a β-galactosidase colony-lift assay. Positive colonies were grown on QDO plates and subjected to the β-galactosidase assay again.

Characterization of positive clones. The positive colonies were grown on Leu^- plates to separate Y187 cells containing the prey plasmids. The plasmid DNA was isolated using a YEASTMAKER plasmid isolation kit (Clontech) according to the manufacturer's directions and introduced into *Escherichia coli* HB101 cells using electroporation. Cells were grown on M9 agar plates containing 50 μg/ml ampicillin, 40 μg/ml proline, and 1 mM thiamine hydrochloride. The insert of each independent positive clone was sequenced using an ABI PRISM 310 Genetic Analyzer. A homology search was performed using the BLAST program on the web (NCBI).

In vitro transcription and translation. A linear DNA template was generated by PCR, transcribed from the T7 promoter, and translated in a TNT-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. Each 50 μl reaction mixture contained 25 μl rabbit reticulocyte lysate, 2 μl reaction buffer, 1 μg DNA template, 20 μM amino acid mixture, 10 U T7 polymerase, 40 U RNA inhibitor, and 20 μCi of L-[^{35}S]methionine (Amersham Bioscience). The reaction was carried out at 30 °C for 90 min.

Glutathione S-transferase-S19 fusion protein synthesis and in vitro binding assay. To synthesize glutathione *S*-transferase (GST)-S19 fusion protein, pAS2-1-S19 plasmid DNA was digested with *Bam*HI and the insert was subcloned in-frame at the *Bam*HI site of pGEX4T-1 (Amersham Bioscience). GST-fusion protein was expressed in *E. coli* strain BL21 by growing the culture at 37 °C to an optical density at 600 nm of about 0.8 and treating it with 0.1 mM isopropyl-β-thiogalactopyranoside at 30 °C for 2 h. Cells were collected by centrifugation, resuspended in sonication buffer (1 × PBS, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF), and disrupted by sonication. GST-tagged protein was purified from the supernatant using glutathione–Sepharose beads (Amersham Bioscience). The in vitro binding assay was performed as follows. Twenty microliters of the in vitro translated ^{35}S-labeled protein was incubated with beads carrying GST or GST-S19 fusion protein in the sonication buffer for 90 min at 4 °C. Complexes were washed extensively with the sonication buffer. The trapped proteins were eluted by boiling in loading buffer and separated on a 10% SDS–polyacrylamide gel. The gel was dried and exposed to an imaging plate for an appropriate period and scanned using the BAS1500 system (Fuji Film).

Dot blot hybridization. The expression pattern of S19BP was analyzed on a commercially available poly(A)$^+$ RNA dot blots panel (mouse RNA master blot, BD Bioscience). A cDNA probe was labeled with [α-^{32}P]dCTP using a random primer labeling kit (Takara). The filter was pre treated by ExpressHyb hybridization solution (BD Bioscience) for 30 min at 65 °C and then hybridized with the labeled probe for 18 h at 65 °C. After a wash according to the manufacturer's protocol, the blots were exposed to an imaging plate and analyzed. Hybridization levels were quantified with the Image Gauge program included in the BAS1500 system (Fuji Film).

Transient expression in cultured cells and immunofluorescence analysis. To examine the subcellular distributions of RPS19 and its binding proteins, mammalian expression vectors for both proteins were constructed in pCMV-HA and pCMV-Myc, respectively. Cos-7 cells were seeded on glass coverslips in 12-well plates and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% 10,000 IU/ml penicillin/streptomycin solutions, and 2 mM glutamine. Cells were grown until 50–60% confluent and co-transfected with 1 μg each of pCMV-HA-S19 and pCMV-Myc-S19BP using PolyFect transfection reagent (Qiagen).

Immunofluorescence staining was performed as below. At 6 or 12 h after transfection, cells were washed in 1× PBS and fixed with 3.7% formaldehyde in 1× PBS. The fixed cells were washed again and treated with a permeabilizing and blocking solution (10% FBS, 0.1 M phosphate buffer, pH 7.2, 1mM PMSF, and 0.1% Triton X-100) for 15 min. They were then incubated with the primary antibodies (1:100 anti-HA antibody and 1:100 anti-cMyc antibody, MBL) for 1 h at room temperature. After a wash with washing solution (1× PBS, 1 mM PMSF, and 0.1% Triton X-100), the cells were finally stained with a mixture of fluorescent dye-conjugated antibodies; FITC-labeled goat anti-rabbit IgG (Sigma Aldrich, 1:100) and TRITC-labeled goat anti-mouse IgG (Sigma Aldrich, 1:100). The labeled proteins were visualized under an Olympus BX51 fluorescence microscope.

Results

Identification of S19 binding protein by two-hybrid screening

We have searched for proteins that interact with RPS19, screening a mouse embryonic cDNA library with the yeast two-hybrid system using RPS19 cDNA as bait. Three clones, pS19bp-1, pS19bp-2, and pS19bp-3 were isolated from approximately 10^8 transformants. Preliminary sequence analyses indicated that these three clones originate from the same gene. Since most ribosomal proteins have similar chemical characteristics and share similar functions on the ribosome, the protein encoded by the new clone was tested for interaction with other ribosomal proteins. The plasmid pS19bp-1 was purified and introduced into Y187 cells for a β-galactosidase assay with clones having cDNAs of ribosomal proteins S3a, L5, and L7a, as well as S19. The products of pS19bp-1 showed interaction with RPS19 but not with the other ribosomal proteins tested (Table 1). The isolated clones were thus proved to have cDNAs that encode a specific S19 binding protein (S19BP).

Confirmation of the binding of RPS19 and S19BP with a pull-down assay

Full-length RPS19 fused to GST (GST-S19) was expressed in *E. coli* cells, extracted, trapped on glutathione–

Table 1
Specific interaction of S19BP

Binding domain construct	Activation domain construct	HIS3 and ADE2[a]	β-gal[b]
pVA3-1	pTD1-1	+	+
pAS2-1-S19	pACT2-S19bp-1	+	+
pAS2-1-S19	pACT2	−	−
pAS2-1-S3a	pACT2-S19bp-1	−	−
pAS2-1-L7a	pACT2-S19bp-1	−	−
pAS2-1-L5	pACT2-S19bp-1	−	−

[a] Co-transformants were examined for HIS3 and ADE2 expression: +, expression; −, no expression.
[b] Co-transformants were also examined for β-galactosidase expression using the colony-lift filter assay.

Sepharose beads, and incubated with ^{35}S-labeled S19BP that was transcribed and translated in vitro with the rabbit reticulocyte TNT system. An electrophoretic analysis of the bound material showed clearly the binding of ^{35}S-labeled S19BP to GST-S19 in vitro (Fig. 1).

Nucleotide sequences of S19BP

The nucleotide sequences of the three isolated clones were determined. They each had the same sequence except that the 5′ ends started from different points. Nucleotide sequences of the clones and amino acid sequences predicted from the cDNAs are shown in Fig. 2A. The protein consists of 142 amino acids with a molecular weight of 15,849 and isoelectric point of 11.3 assuming no posttranslational modification. No similar protein has been characterized or described before, although many ESTs with the same nucleotide sequence have been compiled in public databases. It is a small basic protein having a polylysine tract that corresponds to triplet (AAG)-repeats in the gene. The results of our sequencing and the comparison of EST sequences revealed variation in the length of polylysine. The number of lysine residues varies from six to seven. A search for the S19BP gene was made using the MGI Blast server on the web with the cDNA sequence as a query. The gene matched a single sequence on chromosome 15. It consists of four exons and three introns as shown in Fig. 2B. We also proved the gene by sequencing a PCR from product mouse genome template. Since groups of genes represented by ribosomal protein genes have many processed pseudogenes, we checked for the existence of a pseudogene on the web. No other similar sequences, however, were detected in the mouse genome.

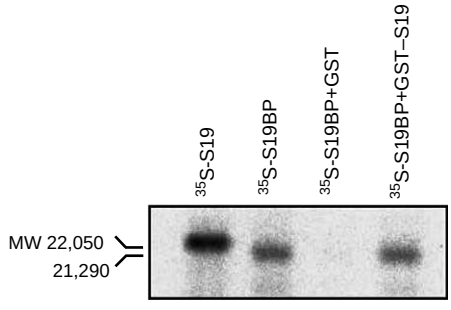


Fig. 1. In vitro binding assay for S19BP. RPS19 and S19BP were synthesized in vitro with [^{35}S]methionine (^{35}S-S19 and ^{35}S-S19BP). ^{35}S-labeled S19BP was incubated with GST or GST-tagged S19 at 4 °C and trapped by glutathione beads. The labeled protein bands were visualized as described under Materials and methods.

Binding of S19BP deletion mutants to RPS19

For confirmation of the specificity of S19BP and for locating a binding motif, we made deletion mutants of S19BP and tested their ability to bind RPS19. As shown in Fig. 3, S19BP/dN80-pACT which includes amino acid residues 81–142 interacted with pAS2-1-S19 and activated the lacZ reporter gene. On the other hand, S19BP/dC81-pACT2, containing amino acid residues 1–80, did not interact with pAS2-1-S19, nor did it activate the lacZ reporter gene.

Tissue specificity of S19BP expression

Information about the specificity of the expression of S19BP might help to reveal its function. We investigated its expression using a dot blot hybridization of mRNAs from various mouse tissues (Fig. 4). The expression was detected in all tissues, but significantly stronger in some specific tissues, e.g., the submaxillary gland and epididymis. The expression of RPS19 in these tissues was not significantly different from the average.

Subcellular localization of S19BP

The subcellular distribution of a protein is also an important clue to its function. To examine the subcellular localization of S19BP and RPS19, Cos-7 cells were co-transfected with myc-tagged S19BP and HA-tagged RPS19 expression vectors, and signals were analyzed by indirect immunofluorescence microscopy using anti-myc or anti-HA antibodies. In these experiments, S19BP concentrated in the nucleolus after 6 h of transfection, colocalizing with RPS19 (Fig. 5, upper panel). After 12 h, S19BP was still in the nucleolus but part had diffused into the nucleoplasm, whereas RPS19 remained in the nucleolus (lower panel).

Discussion

Many ribosomal proteins have extraribosomal functions in addition to their roles in protein synthesis on ribosomal particles [8]. Among the 80 mammalian ribosomal proteins, RPS19 is especially interesting. It is the only ribosomal protein whose expression has been correlated to a human disease. Mutations in RPS19 were found among the patients of a kind of aplastic anemia, DBA [2]. It is entirely unknown how RPS19 is involved in erythropoiesis. However, its extraribosomal function may be important for

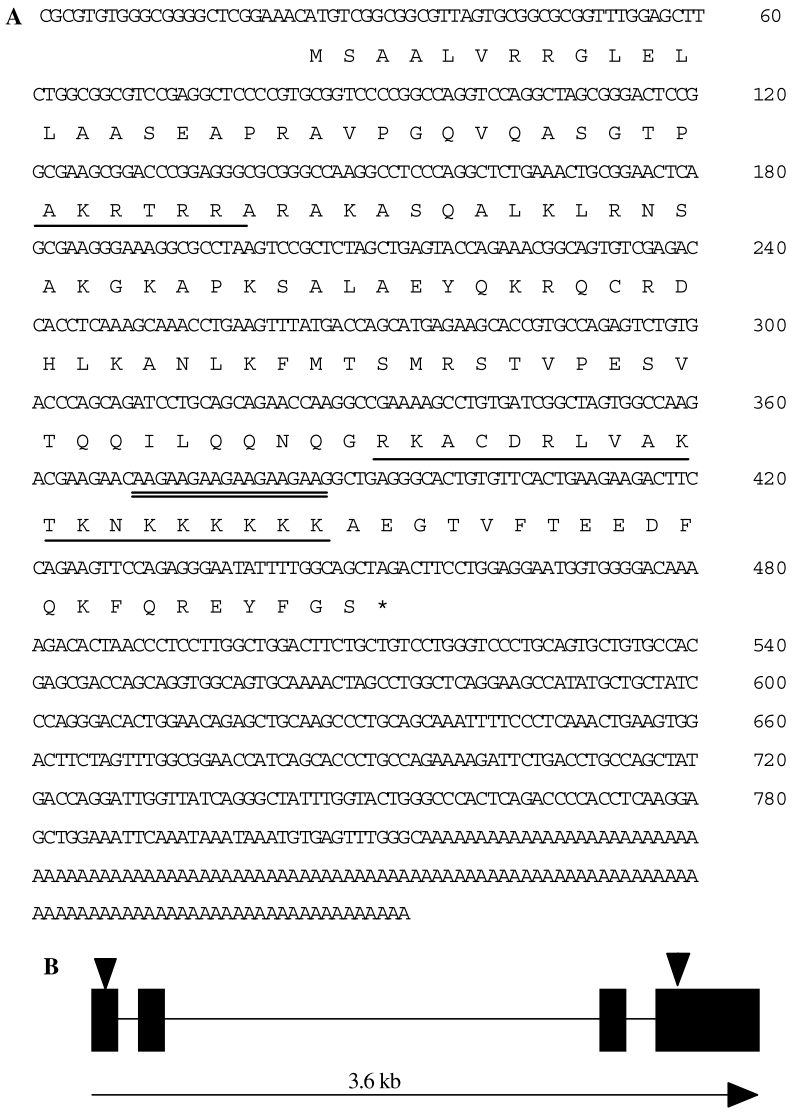
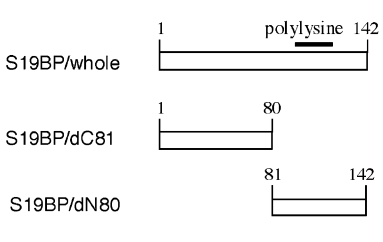


Fig. 2. Nucleotide sequence of S19BP. (A) Nucleotide sequence and deduced amino acid sequence of mouse S19BP. The AAG-repeated region is double-underlined. Two putative nuclear localization signal motifs indicated by solid lines. (B) The gene structure of mouse S19BP. Exons are shown as filled boxes. Arrowheads show the positions corresponding to the translation start and stop sites.



pACT2 fusion	HIS and ADE2	β-gal
S19BP/whole	+	+
S19BP/dC81	-	-
S19BP/dN80	+	+
none	-	-

Fig. 3. Binding of S19BP deletion mutants to RPS19. The C-terminus deletion mutant (S19BP/dC81) and the N-terminus deletion mutant (S19BP/dN80) were obtained from S19BP cDNA. HIS3 and ADE expression and β-gal expression are indicated in Table 1.

the etiology of the anemia, since no other ribosomal protein genes tested so far were mutated in patients with the anemia [7]. Another interesting finding is that fibroblast growth factor 2 binds to free RPS19 [12], although its functional significance is unclear. It is also known that the dimer of RPS19 migrates out of apoptotic cells to mediate the chemotactic movement of monocytes [13]. It is important to elucidate the mechanism behind the extraribosomal function of RPS19 not only for studying cellular physiology but also for uncovering the etiology of and developing a treatment for the disease. We tried to study this mechanism by analyzing the protein that interacts with RPS19.

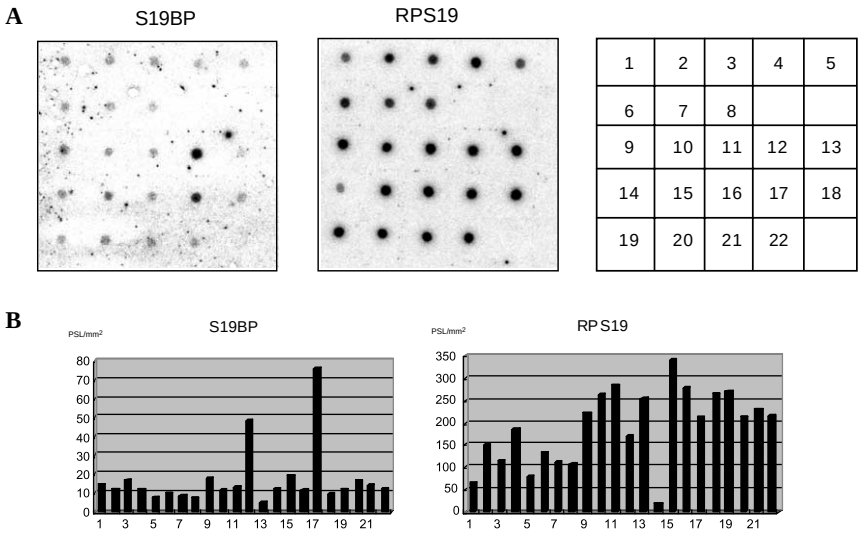


Fig. 4. Tissue specificity of S19BP and RPS19 expression. (A) A mouse RNA master blot (Clontech) was hybridized with ^{32}P-labeled cDNA probes, S19BP (left panel) or S19 (center panel). Numbers show: 1, brain; 2, eye; 3, liver; 4, lung; 5, kidney; 6, heart; 7, skeletal muscle; 8, smooth muscle; 9, pancreas; 10, thyroid; 11, thymus; 12, submaxillary gland; 13, spleen; 14, testis; 15, ovary; 16, prostate; 17, epididymis; 18, uterus; 19, 7-day embryo; 20, 11-day embryo; 21, 15-day embryo; 22, 17-day embryo. (B) The levels of mRNAs were quantified by imaging the dot blots. PSL: Photo stimulated luminescence.

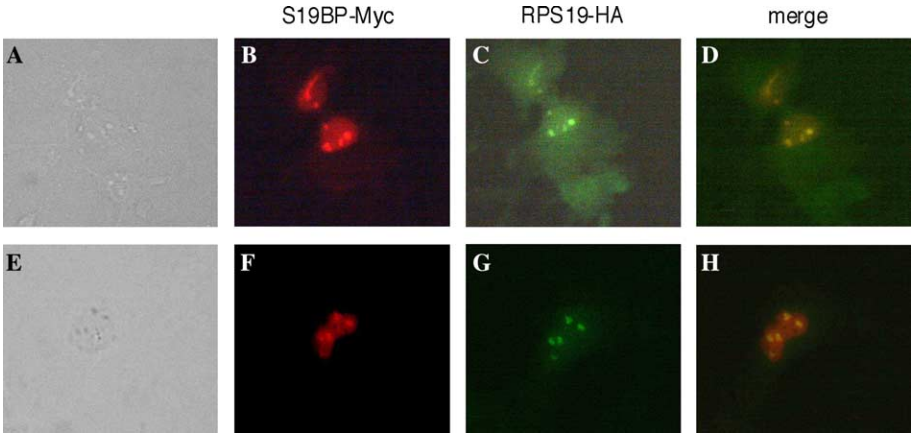


Fig. 5. Subcellular localization. Cos-7 cells were co-transfected with expression vectors containing RPS19 and S19BP tagged with HA- or myc-epitopes. Indirect immunofluorescent analysis was performed as described under Materials and methods. After 6 h (A–D) or 12 h (E–H) of co-transfection, cells were stained with TRITC-conjugated antibody to detect S19BP-myc tagged protein (B,F) or with FITC-conjugated antibody to detect S19-HA tagged protein (C,G). (A,E) Phase-contrast images and (D,H) merged images.

We have isolated clones of such a protein, S19BP, which is a small basic protein containing two basic amino acid clusters (Fig. 2, underlined). The clusters are likely to be nuclear or nucleolar localization signals. The last cluster contains a short polylysine stretch. The number of lysine residues in the stretch varies from six to seven. The difference in amino acid number may affect the activities of the protein, its interaction with other molecules, and also its distribution. Known functional sequence motifs were not found in S19BP besides the two basic amino acid clusters. Secondary structural predictions of S19BP showed a high content of α-helix. It may take a helix–turn–helix structure to bind nucleic acids, but this possibility remains to be tested experimentally.

The expression pattern of S19BP is basically that of a housekeeping gene expressed ubiquitously, but a significantly higher level of expression was observed in some tissues. Double-labeling showed that S19BP co localized in the nucleolus with RPS19 and was partly distributed in the nucleoplasm. One may suspect that S19BP functions in ribosome biosynthesis, for instance as a chaperon working in the assembly process, because of its localization as well as ubiquitous expression. But the tissues that showed especially high levels of S19BP, e.g., the submaxillaly gland and epididymis, are not likely to be more active in protein synthesis and ribosome biogenesis. In fact, expression of RPS19 is not significantly greater in these tissues than in others. The nucleolus has now been revealed to be involved in many cellular functions [14]. It is interesting in this

connection that a loss of RPS19 from the nucleolus has been reported to cause DBA [15]. It is possible that RPS19 cooperates with S19BP to mediate or to control various reactions in the nucleolus.

A BLAST search of public databases on the web detected S19BP homologs in many species. S19BP is a fairly conservative protein that is at least common to vertebrates. Its human gene was located at 22p where DBA has not been mapped. Another causative gene has been suggested to exist [11], but not yet identified. It is possible that S19BP is related to the etiology of DBA. Further study will be required to clarify its molecular mechanism of action.

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