J. Biochem. 132, 705–711 (2002)

Identification of the Ribosomal Proteins Present in the Vicinity of Globin mRNA in the 40S Initiation Complex

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Received April 26, 2002; accepted August 7, 2002

The interaction of ribosomal proteins with mRNA in the 40S initiation complex was examined by chemical cross-linking. 40S initiation complexes were formed by incubating rat liver [^{3}H]Met-tRNAi, rat liver 40S ribosomal subunits, rabbit globin mRNA, and partially purified initiation factors of rabbit reticulocytes in the presence of guanylyl(β, γ-methylene)-diphosphonate. The initiation complexes were then treated with 1,3-butadiene diepoxide to introduce crosslinks between the mRNA and proteins. The covalent mRNA-protein conjugates were isolated by chromatography on an oligo(dT) cellulose column in the presence of sodium dodecyl sulfate, followed by sucrose density gradient centrifugation. Proteins cross-linked to the mRNA were labeled with Na^{125}I, extracted by extensive ribonuclease digestion, and analyzed by two-dimensional and diagonal polyacrylamide gel electrophoresis. Three ribosomal proteins, S6, S8, and S23/S24, together with small amounts of S3/S3a, S27, and S30, were identified as the protein components cross-linked to the globin mRNA protein complex, and were shown to attach directly to the mRNA. It is suggested that these proteins constitute the ribosomal binding site for mRNA in the 40S initiation complex.

Key words: 40S initiation complex, 1,3-butadiene diepoxide, crosslinks, globin mRNA, ribosomal protein.

The translation process is carried out by ribosomes where diverse ligands, such as tRNA, mRNA, and protein factors, participate in the formation of peptide bonds. During protein synthesis, tRNAs interact with both ribosomal subunits and mRNA passes through the interface between the subunits. It is now recognized that rRNA plays a central role in protein synthesis and ribosomal proteins are thought to function mainly in maintaining the ribosome structure. Recent studies have also suggested that a number of ribosomal proteins function in, for example, DNA repair, RNA-processing and development outside ribosomes (*1*). Many approaches have been taken to study the morphology and function of ribosomes. Electron microscopy of negatively stained ribosomes and ribosomal subunits shows the whole image of the particles (*2–4*). Individual ribosomal proteins and their relative positions are determined by immune electron microscopy (*5, 6*) and neutron scattering (*7–9*). Recently, the structure of the 30S subunit from a thermophilic bacterium, *Thermus thermophilus*, has been solved by X-ray crystallography at 3.3 Å and 3 Å (*10, 11*). The structure of the 50S subunit from a halophilic archaebacterium, *Haloarcura marismortui*, was solved to 2.4 Å resolution (*12*). Besides the sizes of their rRNAs, there are considerable differences in the numbers of ribosomal proteins and associated protein factors between prokaryotes and eukaryotes. Such complexity of eukaryotic ribosomes should indicate the presence of a regulatory system for protein biosynthesis in eukaryotes far more complex than that in prokaryotes. Although our understanding of ribosomal function has been greatly enhanced by the 3-D structures, knowledge is still limited to prokaryotic ribosomes, and it seems unlikely that the crystallographic structure of a eukaryotic ribosome will be available in the immediate future because of their extreme complexity. Therefore, biochemical analysis of eukaryotic ribosomes is still one of the major ways to understand the function of eukaryotic ribosomes, and biochemical information thus obtained will be of help in interpreting the 3-D structure–function relationship of such complex biological systems.

There are functional differences, especially in the initiation process between prokaryotes and eukaryotes. Our purpose is to map proteins that interact specifically with natural mRNAs. The regulation of mRNA translation at the initiation stage of protein biosynthesis is increasingly recognized as one of the most important control mechanisms of eukaryotic gene expression. In lysates of rabbit reticulocytes (*13, 14*) and extracts of interferon-treated cells (*13, 15*), it has been shown that the phosphorylation of initiation factor 2 (eIF2) is responsible for different rates of translation under different physiological conditions. On the other hand, the efficiency of translation of individual mRNAs appears to depend on the efficiency of their binding to ribosomal subunits (*16*) or to initiation factor eIF2 (*14*)

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Abbreviations: DEB, 1,3-butadiene diepoxide; eIF2, eukaryotic initiation factor 2; GuoPP[CH_2]P, guanylyl(β, γ-methylene)-diphosphonate; Met-tRNAi, initiator tRNA; TEA, triethanolamine.

during the initiation of protein synthesis. Studies involving synthetic oligonucleotides (*17*) and systematic mutagenesis of a cloned preproinsulin gene (*18*) have suggested that nucleotide sequences flanking the initiator codon AUG determine the efficiency of binding of mRNA to ribosomal subunits. It is of importance to investigate the nature of the mRNA binding domain of the ribosome because of its probable importance in the regulation of protein synthesis. Several laboratories have reported mRNA binding sites on mammalian ribosomes (*19–21*). The experiments using polysomes are, however, thought largely to reflect the interaction between mRNA and ribosomes in the stage of polypeptide elongation. Westermann and Nygård (*22*) constructed a 40S initiation complex from native ribosomal subunits in rabbit reticulocyte lysates, and reported several ribosomal proteins (S1, S3/S3a, S6, and S11) by chemical cross-linking methods that may be regarded as mRNA-binding proteins functioning at the initiation step. We have been studying the mechanism and regulation of protein synthesis in rat liver, and, in the present study, we also tried to identify ribosomal mRNA-binding proteins using the 40S initiation complex in the rat liver system.

Using 1,3-butadiene diepoxide (DEB) as cross-linking reagent, we have now identified ribosomal proteins in the immediate vicinity of mRNA in the 40S initiation complex constructed from well-characterized initiator tRNA, globin mRNA, partially purified initiation factors and NH_4Cl-washed 40S subunits. We identified six ribosomal proteins, S3/S3a, S6, S8, S23/S34, S27, and S30, bound directly to mRNA in the 40S initiation complex. Of the six ribosomal proteins, two are the same as reported by Westermann and Nygård (*22*), and the others are newly identified proteins.

MATERIALS AND METHODS

Materials—1,3-Butandiene diepoxide was purchased from Tokyo Kasei (Tokyo). Oligo(dT) and benzoylated DEAE-cellulose were obtained from Collaborative Research (Waltham, USA) and Sigma (St. Louis, USA), respectively. L-[Methyl-^{3}H] methionine (2.6–3.1 TBq/mmol) and carrier-free $Na^{125}I$ (~629 GBq/mg) were obtained from Amersham (Amersham, UK) and New England Nuclear (Boston, USA), respectively.

Preparation of Initiation Factors—Initiation factors were prepared from the 0.5 M KCl-wash of rabbit reticulocyte lysate polysomes as described previously (*23*).

Preparation of 40S Subunits—NH_4Cl-washed ribosomes were prepared from rat liver as described previously (*23*). After high KCl-wash followed by puromycin treatment, 40S subunits were separated by sucrose gradient centrifugation according to the procedure of Blobel and Sabatini (*24*).

Preparation of Globin mRNA and Its Radioiodination—Globin mRNA was purified from rabbit reticulocyte lysate by the oligo(dT) cellulose procedure of Aviv and Leder (*25*), and radioiodinated with $Na^{125}I$ by the method of Commerford (*26*). Its specific activity was 2×10^6 cpm/μg.

Preparation of [^{3}H]Met-tRNAi—[^{3}H]Met-tRNAi was prepared by the method of Kerwar *et al.* (*27*) using benzoylated DEAE-cellulose and its specific activity was 790 cpm/μg.

Formation of 40S Initiation Complex—The 40S initiation complex was formed in a 100 μl reaction mixture that contained 20 mM triethanolamine-HCl (pH 7.6), 50 mM KCl, 4 mM 2-mercaptoethanol, 3 mM $Mg(OCOCH_3)_2$, 1 mM ATP, 0.24 mM GuoPP[CH_2]P, 0.3 A_{260nm} unit of rat liver 40S subunits, 0.05 A_{260nm} unit of globin mRNA (25,000 cpm/μg), 6 μl of initiation factors (equivalent to 144 μg of protein), and 83 μg of [^{3}H]Met-tRNAi. The reaction mixture was incubated at 30°C for 10 min, and further incubated at 37°C for 30 min in the presence of 5 mM DEB. The DEB-treated 40S initiation complex was separated by centrifugation on a sucrose density gradient prepared in 50 mM TEA-HCl (pH 7.6), 25 mM NaCl, and 5 mM $MgCl_2$. The sedimentation conditions are described in the figure legends.

Isolation and Purification of Protein Cross-Linked Globin mRNA—Protein cross-linked to globin mRNA was dissociated from the DEB treated 40S initiation complex by the addition of SDS at a final concentration of 1%. The cross-linked mRNA was isolated by chromatography on oligo-(dT)-cellulose and further purified by centrifugation on a 5–20% sucrose density gradient prepared in 20 mM TEA-HCl (pH 7.6), 100 mM NaCl. Centrifugation was performed in a Beckman SW 40Ti rotor at 198,000 ×*g* for 13 h at 4°C.

Identification of Proteins Cross-Linked to Globin mRNA—Proteins cross-linked to the mRNA were labeled with $Na^{125}I$ by the chloramine T method as described (*28*). The RNA in the labeled complex was digested completely by RNase, and the proteins were then resolved in basic-acidic and acidic-SDS two-dimensional gel electrophoresis systems (*29, 30*). Forty S ribosomal proteins were extracted with 67% acetic acid in the presence of 33 mM magnesium acetate and the extracted solution was stored at 4°C as a protein-stock solution. To analyze ribosomal proteins bound to mRNA, radioiodinated ribosomal proteins were mixed with an aliquot of the protein stock solution (equivalent to 100 μg), and the ribosomal proteins were then precipitated by the addition of 10 volumes of acetone. This protein-precipitation step was necessary to remove SDS from the cross-linked proteins. In the basic-acidic gel system, electrophoresis in the first dimension was done at 80 V for 19 h using a 6% acrylamide gel at pH 8.6. The second dimension of electrophoresis was carried out in a 15% acrylamide gel at 80 V for 24 h following dialysis of the first dimension gel against 8 M urea–0.35 M acetic acid for 90 min. Electrophoresis in the acidic-SDS system was done by the method of Mets and Bogorad (*31*). The first dimension of electrophoresis was carried out in a 4.5% gel at 150 V for 5 h. After electrophoresis, the gel was soaked in a solution containing 1% SDS, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 10% glycerol, and incubated at 90°C for 20 min. In the second dimension, electrophoresis was done in a 15% gel at 100 V for 6 h.

RESULTS

Effect of Diepoxybutane on the 40S Initiation Complex—Figure 1A shows the formation of the 40S initiation complex as examined by sucrose density gradient centrifugation. Complex formation was factor-dependent since neither globin mRNA nor initiator tRNA was bound to the 40S subunits in the absence initiation factors (data not shown). As is seen in Fig. 1B, DEB treatment of the 40S initiation complex did not affect the binding of globin mRNA or initiator tRNA.

Isolation and Purification of Globin mRNA Carrying Cross-Linked Ribosomal Proteins—Globin mRNA carrying

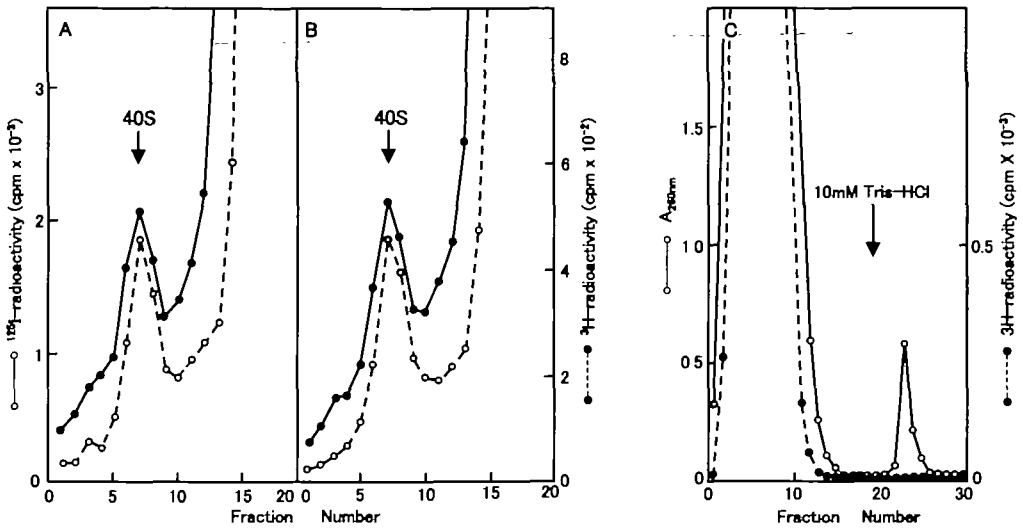

Fig. 1. **Effects of DEB treatment on the formation of 40S initiation complex and isolation of cross-linked globin mRNA.** The 40S initiation complex was formed as described in "MATERIALS AND METHODS." In A and B, the sample solution containing the initiation complex was divided into two halves, which were incubated at 37°C for 30 min: A is a control, DEB-untreated initiation complex; in B, the 40S initiation complex was treated with DEB at a final concentration of 5 mM. The samples were layered onto 5–30% hyperbolic sucrose gradients and centrifuged in a Hitachi RPS-50 rotor at 235,000 ×*g* for 3 h at 2°C. The samples were collected into 17 fractions and their radioactivities were measured. ●, [^{3}H]-radioactivity; ○, [^{125}I]-radioactivity. In C, the 40S initiation complex was prepared on a 25 times scale as that described in "MATERIALS AND METHODS" using unlabeled globin mRNA. Following DEB treatment, the 40S initiation complex was isolated by centrifugation. The globin mRNA in the complex was dissociated by the addition of an equal volume of 1 M NaCl, 0.2% SDS, 2 mM EDTA, and 20 mM Tris-HCl (pH 7.5). The solution was heated at 56°C for 10 min, chilled in an ice bath and then applied onto an oligo(dT) cellulose column. The non-absorbed material was eluted by continuous washing with 0.5 M NaCl, 0.1% SDS, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.5). The cross-linked globin mRNA was eluted with 0.1% SDS, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.5). One milliliter-fractions were collected. Aliquots (50 μl) were taken from the fractions and their ^{3}H radioactivities were measured. ○, A_{260nm}, ●, [^{3}H]-radioactivity.

cross-linked ribosomal proteins, was dissociated from the DEB-treated 40S initiation complexes and isolated by oligo(dT) column chromatography. The elution pattern is shown in Fig. 1C. Radioactive [^{3}H]Met-tRNAi was not detected in fractions eluted with the second buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1% SDS), suggesting that little cross-linking occurred between initiator tRNA and globin mRNA. The cross-linked mRNA thus isolated was further purified by sucrose density gradient centrifugation in the presence of SDS. The sedimentation pattern of the cross-linked mRNA was perfectly superimposed on that of free marker globin mRNA (data not shown). This result indicates that neither degradation nor aggregation of globin mRNA occurred during the treatment with DEB. This was further confirmed by the observation that the recovery of globin mRNA from DEB-treated and untreated initiation complex was identical. The recoveries of DEB-treated globin mRNA were 104.6 and 99.5% compared with untreated control in duplicate experiments.

Identification of Ribosomal Proteins Cross-Linked to Globin mRNA—The protein moiety of the cross-linked mRNA was radioiodinated with Na^{125}I and its RNA moiety was then digested completely with RNase A and RNase T1. To identify the individual proteins cross-linked to the mRNA, we analyzed the labeled proteins in two different gel systems, basic-acidic and acidic-SDS, according to Uchiumi *et al.* (*29, 30*) and Mets and Bogorad (*31*), respectively. A number of similar experiments reported so far (*21, 32, 33*) in which ribosomal proteins were cross-linked to rRNA, tRNA and mRNA have established that ribosomal proteins cross-linked to RNA always migrate slightly toward the origin of 2D-gels as compared with unmodified proteins. This small shift may be attributed to nucleotide(s) or components of nucleotides still attached to the proteins despite extensive nuclease digestion, which will give the proteins a negative charge as in the case of the phosphorylated form of protein S6. Figure 2 shows a representative of three different experiments, the results of which were virtually identical. As is shown in Fig. 2, proteins S6, S8, and S23/S24 were identified together with small amounts of protein S3/S3a in the basic-acidic system, and proteins S6, S8, S23/S24, S27, and S30 were identified in the acidic-SDS system. Taking this shift into account, we could unambiguously assign three radioactive spots seen in the basic-acidic system of Fig. 2A as protein spots of S6, S8, and S24/24. The same was the case in the acidic-SDS system of Fig. 2C in which four radioactive spots except one correspond to the protein spots of S6, S8, S27, and S30. Although there are three candidates (S22, S23/24, and S25) for the remaining spot, S23/24 was the most probable candidate for the spot considering the results in the acidic-basic system (Fig. 2, A and B).

In a similar cross-linking experiment in which the 80S initiation complex was treated with DEB, we could not find any ribosomal proteins attached to globin mRNA (data not shown). It seems likely that the protein binding sites were shielded from the reagent upon association of 60S subunits, consistent with the fact that the above proteins are located at the interface of the two subunits. From these results, it was concluded that proteins S6, S8, and S23/S24, seen in

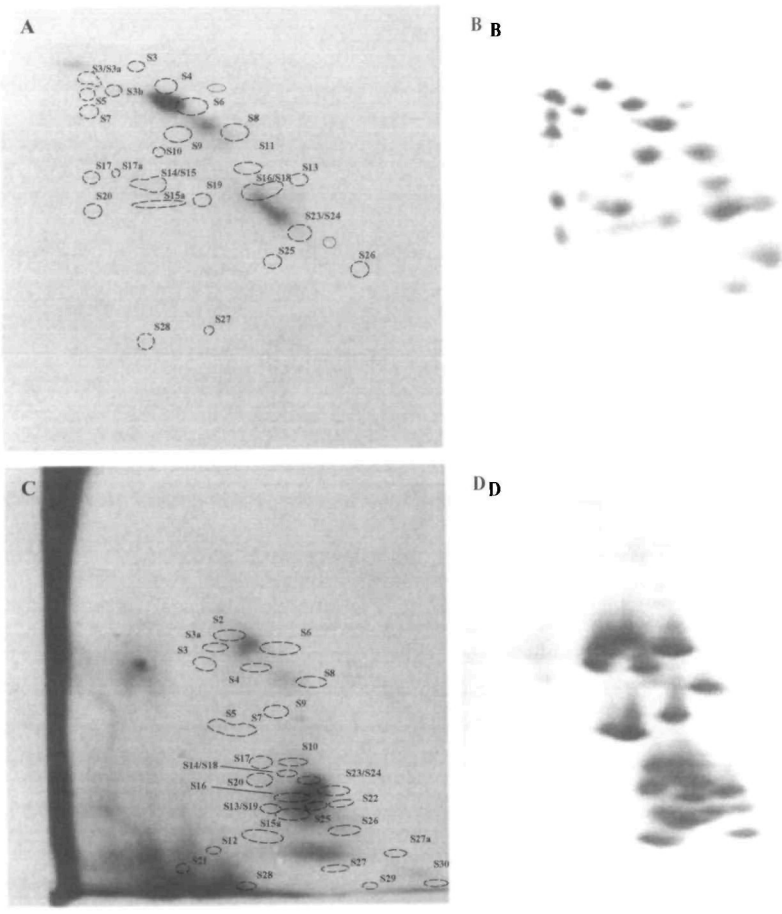

Fig. 2. **Identification of ribosomal proteins cross-linked to globin mRNA.** Purified globin mRNA carrying covalently bound ribosomal proteins was precipitated by ethanol, and dissolved in 6 M urea. The proteins were labeled with $Na^{125}I$ by the chloramine T method (*28*) and the globin mRNA was then digested completely with RNase A and RNase T1. After the addition of carrier ribosomal proteins, the labeled proteins were analysed by two dimensional electrophoresis in basic-acidic and acidic-SDS systems and then detected by autoradiography. A, basic-acidic system; B, schematic diagram of A; C, acidic-SDS system; D, schematic diagram of C. The positions of the stained spots are identified by broken lines.

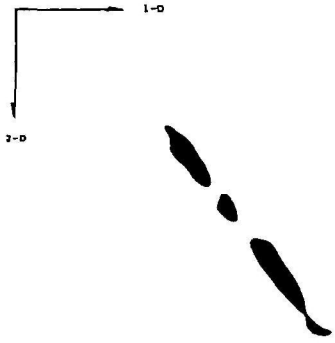

Fig. 3. **Diagonal gel electrophoresis of cross-linked ribosomal proteins.** The ^{125}I-labeled ribosomal proteins, prepared as described in the legend to Fig. 2, were subjected to the first dimensional electrophoresis in an SDS-polyacrylamide gel (0.3 cm × 12 cm) according to the method of Laemmli (*34*). After electrophoresis, the gel was soaked in 15 mM $NaIO_4$ for 5 h to cleave any protein-protein cross-links present according to Lutter *et al.* (*46*). The periodate-treated gel was placed onto the top of a slab gel (0.1 cm × 15 cm × 15 cm) of the same composition as the first dimension gel, and electrophoresed in the second dimension. Proteins were detected by autoradiography.

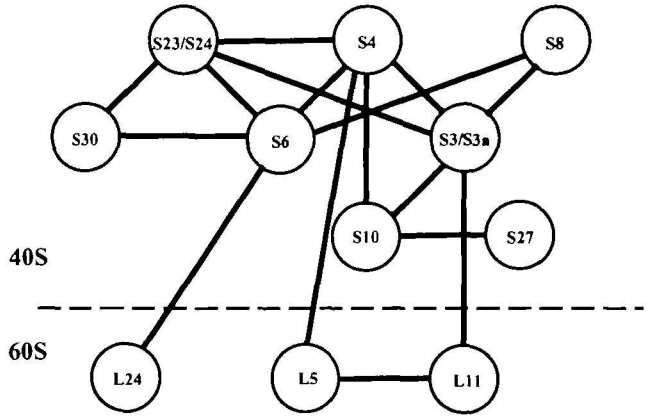

Fig. 4. **Model of the spatial arrangement of ribosomal mRNA-binding proteins.** Proteins are represented by circles. The shaded circles correspond to proteins crosslinked to mRNA in the present study. Solid lines indicate cross-links within 40S subunits and between 40S and 60S subunit interface proteins (*43–45*).

both gel systems, are present in the immediate neighborhood of the initiator region of mRNA in the 40S initiation complex.

Diagonal Gel Electrophoresis of the Cross-Linked Proteins—Next we subjected the cross-linked proteins to diagonal gel electrophoresis to examine whether the identified proteins were bound directly to the globin mRNA. The cross-linked proteins were first electrophoresed in an SDS gel according to the method of Laemmli (*34*), and the gel was then oxidized with $NaIO_4$ to cleave the cross-links. The oxidized proteins were then subjected to SDS slab gel electrophoresis in the second dimension. Any protein-protein

conjugate, if present, would give a spot off the diagonal due to cleavage of the cross-link. As seen in the autoradiograph in Fig. 3, no radioactive spots were present below the diagonal indicating that the proteins identified in Fig. 2 are cross-linked directly to the initiator region of globin mRNA.

DISCUSSION

We have formerly shown that ribosomal proteins S3a, S6, L5, and L6 bind covalently to poly A^+ RNA when rat liver polysomes are irradiated with UV light (*21*). We observed that the protein L5-5S RNA complex interacts with the initiator region of the mRNA in the 80S initiation complex (*19*). Further studies using the Ω fragment of tobacco mosaic virus RNA, a 70-nucleotide stretch within the 5′ leader sequence with the initiator codon AUG at the 3′ terminus (*35*), have shown that this interaction occurs between protein L5 and the initiator region of the mRNA containing the initiator codon AUG (Takahashi *et al.*, manuscript in preparation).

In the present study, we extended our investigation of the mRNA binding site using the cross-linking reagent diepoxybutane. This reagent provides a powerful tool for studying close neighborhood relationships between proteins and RNA because of the short distance (4 Å) between its two reactive groups and because of its mild reaction conditions. Care was taken to avoid contamination of the protein-crosslinked ribosomal RNA fragments in the mRNA fraction. These fragments may be generated by fragmentation of the 18S rRNA during SDS treatment of the 40S initiation complexes and could remain in the mRNA fraction prepared by buoyant density fractionation only. To avoid this problem, we used oligo(dT)-cellulose chromatography followed by sucrose density gradient centrifugation to purify the cross-linked mRNA. Ribosomal proteins cross-linked to globin mRNA were identified in two different gel systems because the negative charges of nucleotides, which remained undigested in spite of extensive RNase treatment, were expected to shift the cross-linked proteins slightly towards the anode as compared with spots of carrier proteins. Three proteins, S6, S8, and S23/S24, were identified in both gel systems as shown in Fig. 2. However, protein S23/S24 was seen only in the basic-acidic system and proteins S27 and S30 were noted only in the acidic-SDS system. This result may be due to the different abilities of these proteins to enter the second dimension of gels because they are carrying fragments of RNase digested globin mRNA. It is of particular interest that S27 has zinc finger domains (*36, 37*) and that ribosome and ribosome subunits contain zinc and iron as well. Zinc finger domains are known to bind not only to DNA but also to RNA (*38*). This suggests that S27 might have the potential to bind to mRNA. The ribosomal proteins identified by the present study are summarized in a spatially arranged model (Fig. 4). As to the proteins appearing in this model, several interesting results have been reported in relation to their functional roles in ribosomes. Affinity labeling and cross-linking experiments have revealed that proteins S3a and S6 are involved in the interaction of poly(U) (*20*), poly$(A)^+$ RNA from rat liver polysomes (*21*), eIF2 (*39, 40*) and initiator tRNA (*41*). Protein S6 was cross-linked to eIF3 with iminothiolane (*42*). Immune electron microscopic studies have indicated that the neck region of the 40S subunit contains proteins S3a and S6 (*40*). Uchiumi *et al.* found protein pairs of S3a-L11 and S6-L24 (*43*), indicating the proteins we identified here are located at the interface of the small and large ribosomal subunits. Using various cross-linking reagents, protein pairs of S3a-S8, S3a-S23/S24, S6-S8, S6-S23/S24, S6-S30, and S23-S30 have been identified (*44, 45*). These results suggest that all the identified proteins in the present study are in close proximity and partly overlap with the binding site of eIF2, eIF3, and initiator tRNA, and located at the contact region of the 40S and 60S subunits.

Westermann and Nygård (*22*) reported that ribosomal proteins S1, S3/S3a, S6, and S11 (together with subunits of eIF3 and cap binding protein) are cross-linked to globin mRNA by DEB within the native 40S initiation complex of rabbit reticulocytes. To identify mRNA-binding proteins, they labeled proteins with radioiodinated Bolton-Hunter's reagent (*22*). This reagent, however, does not seem to be ideal for the purpose because of its relatively large molecular size (nearly 400 Da) and high reactivity to lysine residues, which are usually present in large amounts in basic ribosomal proteins. Proteins heavily labeled with this reagent will have altered molecular weights and net charges that may cause ambiguity in identification of the individual labeled proteins in 2-D gels. In the present study, we identified the mRNA binding proteins by the chloramine T method that directly radioiodinates target proteins to minimize these adverse effects on the mobility of labeled proteins in 2-D gels. It may also be important to confirm that the cross-links are directly between ribosomal proteins and mRNA, and are not secondary links between ribosomal proteins and those cross-linked to mRNA. This evidence seems lacking in the previous experiment (*22*).

Our results confirm the crosslinking of two of these proteins, S3/S3a and S6, and identify two additional proteins, S8 and S23/S24. The two remaining proteins, S1 and S11, found to be crosslinked to mRNA by Westermann and Nygård, were not detected in our system. This may be due to the differences between their system and ours. They used native factor carrying 40S subunits prepared from rabbit reticulocytes and did not add exogenous initiation factors, whereas we used purified (factor-free) 40S subunits from rat liver and supplemented them with partially purified rabbit reticulocyte initiation factors. The purified subunits may have a slightly altered conformation and hence interact with mRNA in a mode somewhat different from that of the native subunits. On the other hand, native subunits are known to be associated with a number of uncharacterized non-ribosomal proteins of unknown function, which might interfere with the cross-linking reaction by secondary interaction with ribosomal proteins and complicate the results. It is also possible that slight differences between the amino acid sequences of rat and rabbit ribosomal proteins alters the reactivity of individual proteins to the cross-linking reagent.

The present results, together with those of Westermann and Nygård (*22*), suggest that ribosomal proteins S3/S3a, S6, S8, S23/S24, S27, and S30 constitute the mRNA binding domain of 40S subunits during the initiation stage of translation.

We are grateful to Dr. Toshio Uchiumi (Institute of High Polymer Research, Faculty of Textile Science and Technology, Shinshu University) for valuable advice and technical help.

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