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Ribosomal Proteins Cross-Linked to the Initiator AUG Codon of a mRNA in the Translation Initiation Complex by UV-Irradiation

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Eukaryotic ribosomal proteins constituting the binding site for the initiator codon AUG on the ribosome at the translation initiation step were investigated by UV-induced cross-linking between protein and mRNA. The 80S-initiation complex was formed in a rabbit reticulocyte cell–free system in the presence of sparsomycin with radiolabeled Ω-fragment as a template, which was a 73-base 5′-leader sequence of tobacco mosaic virus RNA having AUG at the extreme 3′-terminal end and extended with ^{32}pCp. Two radioactive peaks were sedimented by sucrose gradient centrifugation, one being the 80S initiation complex formed at the 3′-terminal AUG codon, and the other presumably a "disome" with an additional 80S ribosome bound at an upstream AUU codon, formed when Ω-fragment was incubated with sparsomycin [Filipowicz and Henni (1979) *Proc. Natl. Acad. Sci. USA* 76, 3111–3115]. Cross-links between ribosomal proteins and the radiolabeled Ω-fragment were induced *in situ* by UV-irradiation at 254 nm. After extensive nuclease digestion of the complexes, ribosomal proteins were separated by two-dimensional gel electrophoresis. Autoradiography identified the proteins S7, S10, S25, S29, and L5 of the 80S initiation complex and S7, S25, S29 and L5 of that in the disome as ^{32}P-labeled proteins. Together with the results of cross-linking experiments of other investigators and recently solved crystal structures of prokaryotic ribosomes, the spatial arrangement of eukaryotic ribosomal proteins at the AUG-binding domain is discussed.

Key word: initiation complex, initiator AUG codon, protein synthesis, ribosomal proteins, translation initiation.

Translation initiation is now recognized as an important regulatory step of gene expression (*1*), and its dysregulation may be responsible for certain viral infections (*2*) and tumorigenesis (*3*). During the initiation of eukaryotic protein biosynthesis, binding of mRNA, initiation factors and initiator tRNA to the small ribosomal subunit takes place at an early stage to form the 40S initiation complex (sometimes called 43S pre-initiation complex). Then the large subunit associates with the complex, and "80S initiation complex" (composed of a complete ribosome and other components) is formed. This process seems essential in protein synthesis, as the initiation step in prokaryotic cells proceeds by a virtually identical route. Affinity labeling of the prokaryotic ribosome with photo-reactive AUG analogues showed that the binding site for the initiator AUG occupies the interface between the small and large subunits, and ribosomal proteins constituting the binding site have been identified (*4*).

Crystal structures of the prokaryotic 50S and 30S ribosomal subunits have recently been solved at 2.4–3 Å (*5*–*7*) and that of the complete 70S ribosome at 5.5 Å resolution (*8*). A low-resolution cryo-electron microscopic structure of yeast 80S ribosome was also reported (*9*). Although our understanding of ribosomal function has been greatly enhanced by the 3-D structures, detailed knowledge is still largely limited to prokaryotic ribosomes and, because of the extreme complexity, it will take some time for a high-resolution crystal structure of a eukaryotic ribosome to be available. Therefore, a biochemical approach 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. In eukaryotic systems, little is known about the species of ribosomal proteins actually interacting with the specific regions of mRNA in the initiation complexes. Recently, we showed that three ribosomal proteins, S6, S8 and S23/S24, together with small amounts of S3a/S3, S27 and S30, were directly cross-linked to ^{125}I-labeled globin mRNA in the 40S initiation complex by chemical cross-linking (*10*). In the present study we extended the previous experiments using the rabbit reticulocyte system and a radiolabeled Ω-fragment in which 3′-terminal AUG had been extended to AUGC with [5′-^{32}P]pCp, and examined the proteins directly interacting with this limited region by the cross-linking technique in the 80S initiation complex. UV-irradiation can induce very short-range cross-linkings as compared with bifunctional chemical crosslinkers, which have spacers of various lengths. Here, we show ribosomal proteins S7, S10, S25, S29 and L5 are present in the close vicinity of initiator AUG in mRNA within the 80S initiation complex.

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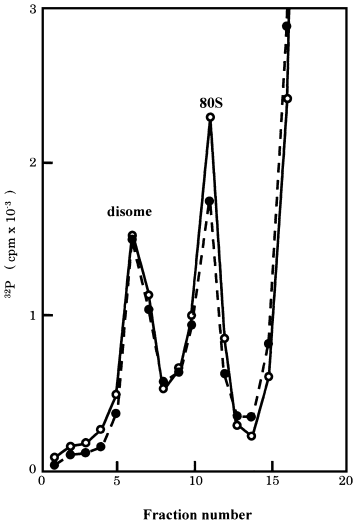

Fig. 1. **Formation of the initiation complex and effect of UV-irradiation.** A rabbit reticulocyte lysate (10 μl) was preincubated with 100 μM sparsomycin at 30°C for 15 min, then ^{32}P-labeled Ω fragment (*ca*. 30,000 cpm) was added and the mixture was further incubated at 30°C for 15 min. After the mixture had been irradiated by UV lamp for 25 min, initiation complexes were separated by sucrose gradient centrifugation. The conditions for irradiation, centrifugation and measurement of radioactivity are described under "MATERIALS AND METHODS." Open circles, without irradiation; filled circles, irradiated for 25 min. 80S, 80S initiation complex; disome, two 80S ribosomes, one being the 80S initiation complex, the other an additional 80S particle bound to the 5′ leader sequence of Ω fragment (*18*).

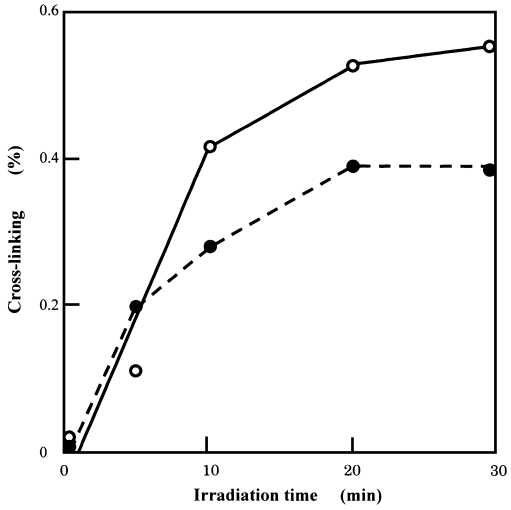

Fig. 2. **Effect of the UV-irradiation time on the cross-linking between ribosomal proteins and Ω fragment.** Initiation complexes were formed and separated under the conditions described in the legend of Fig. 1. The complexes were irradiated for the indicated times under the conditions described in "MATERIALS AND METHODS." The amounts of ^{32}P-labeled Ω-fragment and proteins were estimated by Millipore filtration assay by the method of Cazillis *et al*. (*14*). The values from the unirradiated lysate were used as a control and were subtracted from irradiated ones. The efficiency of cross-linking is expressed as the percentage of added radioactivity that was retained on the filter in the 80S or "disome" complex (*18*). Closed circles, 80S complex; open circles, disome complex.

MATERIALS AND METHODS

Materials—Nuclease-treated (*i.e.*, exogenous mRNA-dependent) rabbit reticulocyte lysates were prepared by the procedure of Pelham and Jackson (*11*). [γ-^{32}P]ATP (>220 TBq or 7,000 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, California). Polynucleotide kinase and T4 RNA ligase were from Takara Shuzo (Tokyo).

Preparation of TMV-Ω Fragment—The Ω-fragment was prepared from a ribonuclease T1 digest of TMV-RNA as described in Ref. *12*.

3′-End Labeling of RNA—The labeling at the 3′ end of RNA was performed in a 10 μl reaction mixture containing 25 mM Tris-HCl pH 8.3, 5 mM $MgCl_2$, 5 mM DTT, 10 μg/ml bovine serum albumin, 10% dimethylsulfoxide, 50 μM ATP, 0.5–1.0 unit of T4 RNA ligase and [5′-^{32}P]pCp prepared from 3.7 MBq (100 μCi) of [γ-^{32}P]ATP according to England *et al.* (*13*). Labeled samples were analyzed or purified on 8% acrylamide slab gels containing 7 M urea, 90 mM Tris-borate pH 8.3 and 2.5 mM EDTA.

Formation of Initiation Complex—The initiation complex was constructed using a rabbit reticulocyte lysate. In brief, 10 μl of rabbit reticulocyte lysate was preincubated with 100 μM sparsomycin at 30°C for 15 min, the ^{32}P-labeled Ω-fragment was added and the mixture was further incubated at 30°C for 15 min. For preparative purposes, 50 μl aliquots of the lysate were used.

UV-Irradiation—The lysates containing initiation complexes were irradiated with a mercury lamp (Mineralight, UVP Inc., Upland, California) for 25 min at a distance of 2 cm. The samples were kept on ice during the irradiation. The irradiation intensity was 18,600 μW/cm^2 at 254 nm. The extent of cross-linking of ^{32}P-labeled Ω-fragment to proteins was estimated by Millipore filtration assay by the method of Cazillis *et al.* (*14*).

Separation of Initiation Complex—The UV-irradiated samples were diluted with buffer A (50 mM Tris-HCl pH 7.6, 25 mM KC1 and 5 mM $MgCl_2$) to 100 μl. The solution was overlaid onto 5 ml of 15–30% linear sucrose density gradient in buffer A. After the sedimentation at 235,000 × *g* for 90 min in a Hitachi RPS-50 rotor at 2°C, the gradient solution was collected into 17 fractions and Cerenkov radiation of each fraction was measured. The ^{32}P-radioactive fractions were pooled separately and precipitated with 2 volumes of ethanol after the addition of 0.1 volume of 1 M potassium acetate.

Extraction of Ribosomal Proteins—The initiation complexes precipitated with ethanol were dissolved in 6 M urea and digested with RNase A (50 μg/ml) at 30°C for 15 min. The samples were dialyzed against 10% acetic acid for 12 h. Following addition of carrier ribosomal proteins (70 μg), proteins were precipitated by addition of 5 volumes of acetone.

Gel Electrophoresis of Ribosomal Proteins—Ribosomal proteins were separated by two-dimensional gel electrophoresis using two different gel systems (a basic-acidic and a basic-SDS system) described in Ref. *15*. After the electrophoresis, gels were stained with Coomassie brilliant blue, dried on filter papers, and radioactive spots were identified by autoradiography on Fuji X-ray films.

Gel Electrophoresis of RNA—The electrophoresis of RNA was performed on an 8% polyacrylamide gel slab (0.1 cm

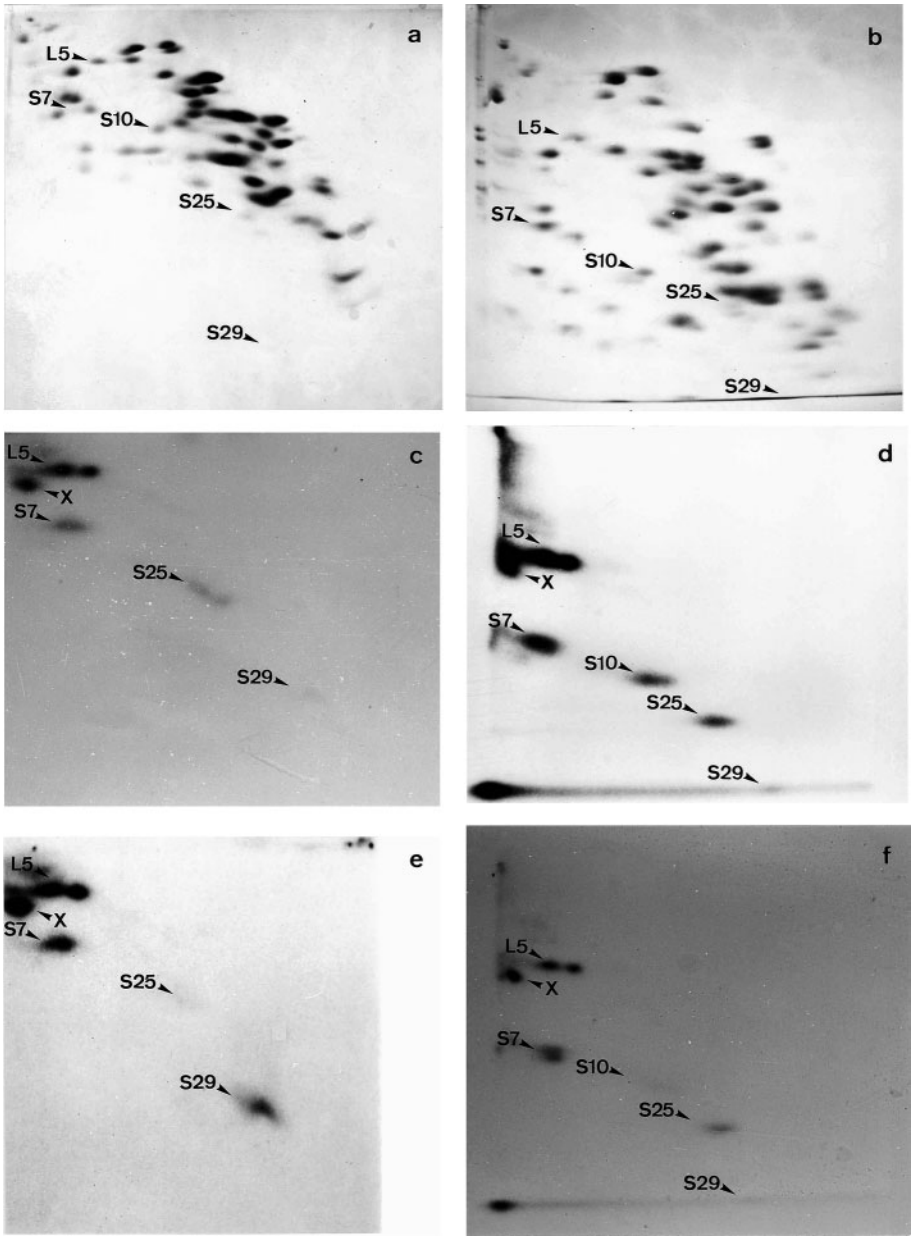

Fig. 3. **Two-dimensional gel electrophoresis of cross-linked ribosomal proteins.** Initiation complexes were formed with 50 μl of lysate and 3–5 × 10^6 cpm of ^{32}P-labeled Ω-fragment. The separation and irradiation of the complexes were done as described under "MATERIAL AND METHODS." The proteins cross-linked with ^{32}P-labeled Ω-fragment were extracted with acetic acid after extensive digestion with RNase A. The samples were mixed with carrier ribosomal proteins and subjected to the basic-acidic and basic-SDS systems of two-dimensional gel electrophoresis by the method of Ref. *15*. (a) Staining pattern of ribosomal proteins in the basic-acidic system, (b) staining pattern of ribosomal proteins in the basic-SDS system, (c) cross-linked proteins of the 80S complex in the basic-acidic system, (d) cross-linked proteins of the 80S complex in the basic-SDS system, (e) cross-linked proteins of the disome complex in the basic-acidic system, (f) cross-linked proteins of the disome complex in the basic-SDS system. Basic ribosomal proteins were numbered according the proposed uniform nomenclature (*23*).

× 15 cm × 15 cm) containing 7 M urea by the method of Donis-Keller *et al*. (*16*).

Sequencing of RNA—Sequence analyses of RNA by two-dimensional gel electrophoresis were performed by the method of Lockard *et al*. (*17*).

Measurement of Radioactivity—The radioactivity was measured in a Triton X-100 toluene-DPO scintillator. Cerenkov radiation was measured when samples were to be recovered for further analyses.

RESULTS AND DISCUSSION

Ribosome Binding to TMV Ω-Fragment and Effect of UV-Irradiation—Figure 1 shows the sedimentation profile of reticulocyte lysate incubated with ^{32}P-labled Ω-fragment and sparsomycin, an elongation inhibitor. As the figure shows, two radioactive peaks were sedimented. In the presence of sparsomycin, the 5′ leader sequence and the first AUG codon of the TMV RNA and Ω-fragment can bind two 80S ribosomes forming "disome" (*18–20*). This was first observed for the wheat germ system, and also recognized for the rabbit reticulocyte lysates (*21*). In both systems, nearly equal amounts of the 80S complex and the disome were formed (*18*, *21*). Detailed analysis showed that the second particle was an 80S initiation-like complex, rather than the 40S, formed on an AUU codon upstream of the 3′-AUG (*20*). These observations are consistent with the result from recently solved crystal structures that sparsomycin stabilizes 80S initiation complex by interacting with both the 3′-CCA region of tRNA and the 60S subunit (*22*). Therefore, the two radioactive peaks can be assigned respectively to the 80S initiation complex and the disome. This was further confirmed by the presence of 5S RNA in both peaks, which were identified by direct RNA sequence analysis (data not shown). An RNA band corresponding to 5.8S RNA

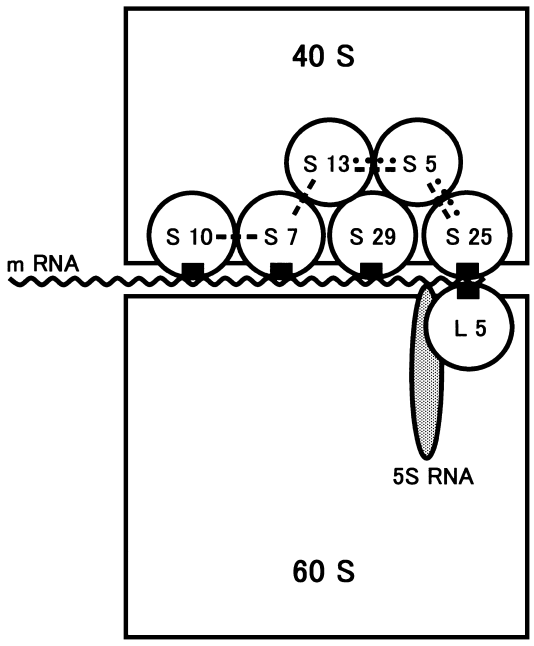

Fig. 4. **A model for mRNA-binding domain in the initiation complex.** Protein pairs shown with a broken line and a dotted line have been described in Refs. *27* and *28*, respectively. Proteins harboring a filled box are those cross-linked to mRNA by UV-irradiation.

was also found by electrophoresis of RNA components in these fractions (data not shown). As shown later, detection of a large subunit protein, L5, in these fractions further supported this interpretation.

Figure 1 also shows that UV-irradiation (for 25 min) did not affect the sedimentation pattern significantly. Both of the irradiated complexes (80S and disome) were sedimented at the same density in the respective experiments. Further, in the irradiated sample, aggregation was not seen in a region heavier than the disome. The results indicated that irradiation did not induce any detectable conformational change or non-specific aggregation of the initiation complexes, although the amount of 80S initiation complex was slightly decreased by the irradiation.

Efficiency of Cross-Linking between ^{32}P-labeled Ω-Fragment and Ribosomal Proteins—We estimated the efficiency of cross-linking by the filter-binding assay method of Cazillis *et al*. (*14*). As shown in Fig. 2, the binding of the Ω-fragment increased as a function of irradiation time and reached a plateau after about 20 min. We, therefore, chose 25 min for the time of irradiation. Under these conditions, 0.4 and 0.55% of the added radioactivity was recovered in the 80S complex and "disome" complex, respectively, at the plateau.

Identification of Proteins Cross-Linked to the Initiator Region of mRNA—The proteins bound to the initiator region of mRNA were analyzed by two-dimensional gel electrophoresis with carrier ribosomal proteins after the cross-linked initiation complexes had been extensively digested with RNase A. To ensure the identification of the proteins, we used two different gel systems (basic-acidic and basic-SDS) described in Ref. *15*. Figure 3, a and b, shows the staining patterns of carrier 80S ribosomal proteins from a rabbit reticulocyte lysate. Although the radiolabeled proteins shifted slightly to the northwest relative to the unlabeled protein spots due to the negative charges introduced by covalently bound nucleotide fragment (*24*), autoradiograms for the 80S complex showed that radioactive spots co-migrated with proteins S7, S25, S29, L5 and X in the basic-acidic gel system (Fig. 3c) and with S7, S10, S25, S29, L5 and X in the basic-SDS gel system (Fig. 3d). In the disome fraction, the same set of ribosomal proteins, S7, S25, S29, L5 and X, was identified except for S10 (Fig. 3, e and f). Since only the 3′-end had been labeled with ^{32}P, radioactive protein spots should have been derived from the 80S initiation complex formed on the 3′-end AUG codon, and proteins in the second 80S particle could not be detected. Therefore, it is reasonable that the same set of labeled ribosomal proteins was found for both fractions. It was shown that the Ω-fragment is long enough to accommodate only two 80S particles, and these should be arranged side-by-side on the message (*18*, *20*). The presence of the second particle in the close vicinity might have reduced the UV-reactivity of S10 and Ω-fragment in the 80S initiation complex on the AUG. The presence of protein S10 solely in the 80S complex electrophoresed in a basic-SDS gel system and its absence in the "disome" complex were reproducibly noted in repeated experiments. This result also excluded the possibility that proteins identified here were the result of artifactual binding of the radiolabeled oligonucleotides to the proteins during the preparation. From these results, we concluded that these proteins constituted an AUG-binding domain in the 80S initiation complex. A labeled protein, marked X, at near the origin of the gels (Fig. 3) did not correspond to any known ribosomal protein (probably one of the initiation factors) and was not characterized further.

A Model of the AUG-Binding Domain—We summarized the present results in a model of the spatial arrangement of the AUG-binding domain on ribosomes (Fig. 4). The crosslinks of S7-S10, S13-S7, S5-S13, S5-S25 and S5-S29 reported by Gross *et al*. (*25*) and those of S13-S15 and S5-S25 by Tolan and Traut (*26*) are included in the model, and they support the proximity of S7, S25 and S29 detected here. Uchiumi *et al*. (*15*) reported the chemical cross-linking between S25 and L5 in 80S ribosomes and suggested that these two proteins are located at the contact region of the large and small subunits. This strongly supports the present finding that both S25 and L5 constitute the AUG-binding domain. Synetos *et al*. reported that S25 and S7 together with S10 and S14/S15 of rat liver ribosomes were labeled by a photoaffinity probe of pactamycin and suggested that these proteins are located on the ribosomal region where the binding of mRNA or initiation factors occurs (*27*). Cross-linking experiments of eIF3 to ribosomes by Westermann and Nygard (*28*) and by Tolan *et al*. (*29*) identified S2, S3, S3a, S4, S6, S7, S8, S9, S14/S15, S23/S24, S25 and S27 as the proteins cross-linked to eIF3. Of these proteins, S25 is identified as an AUG-binding protein (the present study), S3a and S6 are cross-linked to poly $(A)^+$ RNA in rat liver polysomes by UV-irradiation (*30*). Proteins S6, S8 and S23/S24 were cross-linked to the initiator region of globin mRNA by diepoxybutane-induced cross-linking of the 40S initiation complex prepared from rat liver 40S subunits and initia-

tion factors purified from rabbit reticulocyte lysate (*10*). These results indicate that a part of the ribosomal binding site for elF3 overlaps with that of mRNA, particularly for the mRNA region related to the initiation step. This is consistent with a view that elF3 participates in binding of mRNA to 40S subunits (*31*). In the prokaryotic system, Pongs *et al*. examined AUG-binding proteins of *E. coli* ribosomes (*4*). They tentatively identified protein EL5, one of three known 5S RNA-binding proteins in *E. coli*, as an AUG-binder (*32*). This protein shares a limited sequence homology with yeast 5S RNA binding protein YL3 (*33*), a homologue of rat liver protein L5 (*34*), suggesting an evolutionary relationship between 5S RNA-binding proteins of prokaryotic and eukaryotic cells. These are also partially homologous to the *Halobacterium* 5S RNA binding protein HL19 (*35*). It is attractive, therefore, to postulate that the probable function of rabbit reticulocyte protein L5 as an AUG-binding protein at the initiation step is conserved among eukaryotic and prokaryotic organisms. There are no prokaryotic counterparts for S7, S10, and S25 (*36*), suggesting that these proteins are responsible for some eukaryote-specific events in the translation.

The present results were interpreted by considering the recently solved crystal structures of prokaryotic ribosomes. The cross-link between 5S RNA-binding L5 and mRNA in the 80S initiation complex seems to be compatible with the structural data that prokaryotic 5S RNA-binding protein, L5p, is located near the P site (*8*). S29 identified in the present study is a homologue of prokaryotic S14 (*36*). In the *T. thermophilus* 30S ribosomal subunit, three proteins, S3, S4, and S5, form a ring around the incoming mRNA (*37*). Since S4 and S14 are in a very close contact (*6*), formation of cross-linking between eukaryotic equivalent S29 and mRNA seems reasonable. In conclusion, we identified the ribosomal binding site for the initiator AUG codon, which is located at the interface between subunits as a compact functional unit.

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