Volume 12 Number 23 1984 Nucleic Acids Research

Cross-linking of mRNA to initiation factor eIF-3, 24 kDa cap binding protein and ribosomal proteins S1, S3/3a, S6 and S11 within the 48S pre-initiation complex

Peter Westermann* and Odd Nygård[1]

Central Institute of Molecular Biology, Academy of Sciences of GDR, 1115 Berlin-Buch, GDR, and [1]The Wenner-Gren Institute, University of Stockholm, S-113 45 Stockholm, Sweden

Received 17 September 1984; Revised and Accepted 6 November 1984

ABSTRACT

Native small ribosomal subunits from rabbit reticulocytes contain all initiation factors necessary for the formation of the mRNA-containing 48S pre-initiation complex. The complex formed in the presence of Met-tRNA$_f$ and ^{125}I-labelled globin mRNA was cross-linked with diepoxybutane, and the covalent mRNA-protein complexes were isolated under denaturating conditions. The proteins of the covalent complex were identified as the 110, 95 and 66/64 kDa subunits of eIF-3. In addition, the 24 kDa cap binding protein and the ribosomal proteins S1, S3/3a, S6 and S11 were found covalently linked to the mRNA. Ribosomal proteins S3/3a and S6 were also involved in the ribosomal mRNA-binding domain of reticulocyte polysomes.

INTRODUCTION

The native small ribosomal subunits ($40S_N$) found in rabbit reticulocytes contain most of the initiation factors known to participate in the initiation of protein biosynthesis (1). Initiation factor eIF-3 is present as a 1:1 complex whereas the proportions of other factors e.g. eIF-2 are less than stoichiometric (1). As a result of the factor content, $40S_N$ subunits supplemented with derived 60S subunits and pH5-enzymes are active in mRNA translation (2). The binding of mRNA to the $40S_N$ particles during initiation is one of the steps determining the efficiency by which the individual messages are translated (3). The mRNA binding step is likely to be regulated by the various components involved in formation of the initiation complex i.e. initiation factors (4-7), cap binding proteins (7-9), ribosomal RNA (10) and ribosomal proteins (11). Reduced attachment of $40S_N$ particles to mRNA has been demonstrated after ethionine treatment of rats. The effect was attributed to a reduced amount of eIF-3 attached to the $40S_N$ subunit (12). In the pre-initiation complex, cap binding proteins (13,14) and 18S rRNA (15,16) have been shown to be positioned in close proximity to the mRNA. Protein phosphorylation has been suggested to play an essential role in regulating the functional activity of eIF-2 (17) and the

ribosomal protein S6 (11,18).

The mRNA sequence involved in the binding reaction is protected against nuclease digestion within the pre-initiation complex (19,20). The sequence contains the 5' untranslated part, including the cap region, the AUG initiation codon and one to three adjacent codons of the translated part of the mRNA (19,20). In order to identify the proteins located in the close vicinity of mRNA, 48S pre-initiation complexes containing reticulocyte $40S_N$ subunits and globin mRNA. were cross-linked with diepoxybutane and the proteins covalently linked to the mRNA identified by gel electrophoresis. The location of the mRNA within the initiation complex is discussed in accordance with the positions of the mRNA binding proteins estimated by electron microscopy.

MATERIALS AND METHODS

Preparations

Rabbit reticulocytes were prepared as previously described (21). Native small ribosomal subunits ($40S_N$) were isolated from the reticulocytes according to Sundquist and Staehelin (1). Polysomes were prepared from reticulocyte lysates by centrifugation through a 2M sucrose cushion in Buffer A (25mM KCl, 3mM $MgCl_2$ and 20mM triethanolamine (TEA)-HCl, pH 7.6). Globin mRNA was purified by chromatography on oligo-dT cellulose (22) and labelled with ^{125}I as described (23). Initiator tRNA, Met-$tRNA_f$, was prepared by charging yeast tRNA with methionine in the presence of Escherichia coli synthetases (24).

Cross-linking of complexes of $40S_N$ and globin mRNA

The 48S pre-initiation complex was formed by incubating 0.5mg tRNA charged with methionine, 50µg globin mRNA and 2µg ^{125}I-labelled globin mRNA with 1.7 A_{260} units of $40S_N$ in 0.5 ml Buffer A containing 10mM 2-mercaptoethanol, 1mM spermidine, 5mM creatine phosphate, 100µg creatine phosphokinase, 1mM ATP and 0.2mM GTP for 5 min at 37° C. Samples were transferred to an ice bath and the incubation was continued for 30 min in the presence of 20mM diepoxybutane. Alternatively, the incubation was continued without diepoxybutane. After incubation the suspensions were layered on to 10 to 30% (w/v) linear sucrose gradients in Buffer A and centrifuged for 14 h at 22 000 rpm in an SW41 rotor (Beckman Instr.) at a temperature of 2° C. Fractions containing cross-linked 48S pre-initiation complexes or unbound, cross-linked globin mRNA (Fig. 1A) were precipitated with 2.5 volumes of ethanol at -20° C. The pre-initiation complexes and

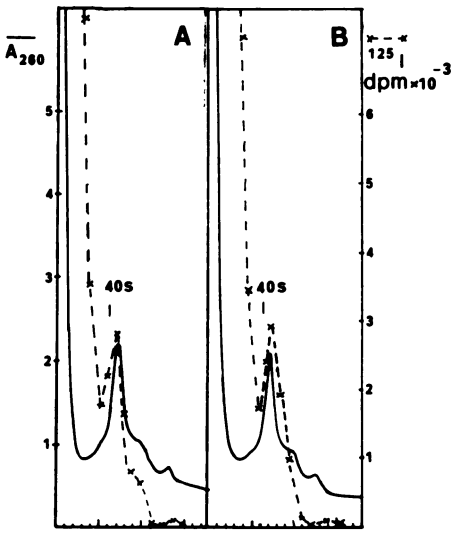

Fig. 1. Binding of globin mRNA labelled with ^{125}I to $40S_{IF}$ from rabbit reticulocytes. Incubation was with (A) or without (B) 20mM diepoxybutane. Centrifugation was from left to right.

unbound mRNA isolated from the non-cross-linked incubations (Fig. 1B) were cross-linked with 20mM diepoxybutane for 30 min at 0° C and precipitated with ethanol at -20° C.

Cross-linking of polysomes from rabbit reticulocytes

Reticulocyte polysomes were resuspended in Buffer A at a final concentration of 13 A_{260} units per ml and incubated for 30 min at 0° C in the presence of 20mM diepoxybutane. The cross-linked mRNP was isolated as described below.

Identification of proteins cross-linked to globin mRNA

The precipitated cross-linked complexes were dissolved in 50mM TEA-HCl, pH 8.0, 1mM EDTA, 1% (w/v) sodium dodecylsulfate (SDS), labelled by incubation with 0.1mCi N-succinimidyl-3-(4-hydroxy-5-^{125}iodophenyl)-propionate (25) and precipitated with 2 volumes of cold ethanol. The precipitates were collected by centrifugation and solubilized in 0.5M LiCl, 1% (w/v) lithium dodecylsulfate (LiDS),20mM TEA-HCl, pH 7.6 and incubated for 5 min at 56° C. The samples were layered onto 10 to 25% (w/v) linear sucrose gradients containing 50mM LiCl, 0.1% (w/v) LiDS, 20mM TEA-HCl, pH 7.6 and centrifuged for 15 h at 45 000 rpm using an SW 60 rotor (Beckman Instr.) at a temperature of 5° C. Fractions containing globin mRNA (Fig. 2A) were precipitated with 2 volumes of ethanol using 50µg tRNA as carrier. The precipitated material was collected, solubilized, recentrifuged (Fig. 2B) and precipitated. The RNA was degraded by incubation with 12µg RNase A and 60 units RNase T1 in 0.1M Tris-HCl, pH

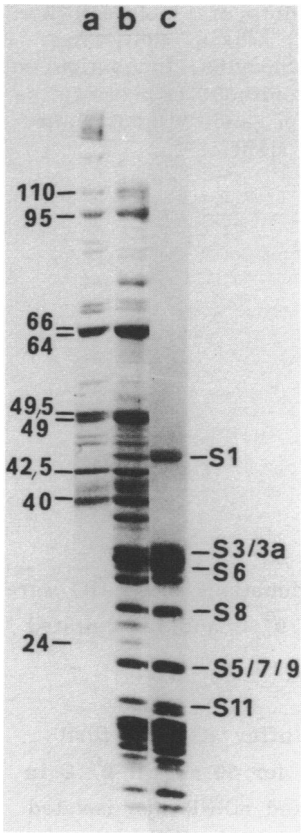

Fig. 2. Comparison of the polypeptides of initiation factor eIF-3 (36) and the 24kDa cap binding protein (lane a) with proteins from native small ribosomal subunits (lane b) and derived small ribosomal subunits (lane c) by polyacrylamide gel electrophoresis in the presence of SDS.

7.6. After incubation for 1h at 37° C, 20µg 40S ribosomal protein was added and the incubation mixtures were made 1% (w/v) in SDS and 0.2M in NaOH. The incubation was continued for 1h at 37° C. The proteins were precipitated by the addition of 10% (w/v) TCA. The precipitate was collected by centrifugation, washed with ethanol and dissolved in 0.1M Tris-OH, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol for electrophoresis according to Laemmli (26) or in 6M urea containing 10mM KCH_3COO, pH 4.2 and 10mM 2-mercaptoethanol for two-dimensional electrophoresis as described previously (27). The gels were dried and exposed to XR-1 film (Orwo, Wolfen) for 2 days at -70°C using intensifying screens.

RESULTS

Native small ribosomal subunits ($40S_N$) from rabbit reticulocytes

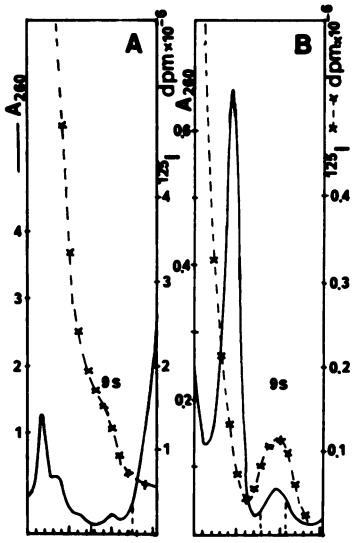

Fig. 3. Isolation of cross-linked globin mRNA-protein complexes by centrifugation of the labelled 48S pre-initiation complex in the presence of LiCl and LiDS (A). The covalent complex was precipitated using tRNA as a carrier and further purified by re-centrifugation (B).

contain all factors needed for the binding of mRNA. As seen in Fig. 2, the $40S_N$ particles contain additional non-ribosomal proteins. All subunits of eIF-3 and the 24kDa cap binding protein could be identified among the proteins from the native particles, whereas the derived subunits were devoid of these proteins (Fig. 2). This is in agreement with previous observations (1). After supplementation with energy and Met-$tRNA_f$ about one quarter of the $40S_N$ bind ^{125}I-labelled mRNA and appear as 48S pre-initiation complexes (Fig. 1B) demonstrable by gradient centrifugation. The proportion of $40S_N$ particles appearing as pre-initiation complexes was not altered by cross-linking the initiation complex with diepoxybutane prior to centrifugation (cf. Fig. 1A and B). This indicates that the extent of initiation complex formation in this system was limited by the availability of initiation factor(s).

When the globin mRNA was isolated from the cross-linked ^{125}I-labelled 48S complexes under denaturating conditions (Fig. 3), only proteins covalently linked to the mRNA were recovered. The proteins were identified by gel-electrophoresis following degradation of the mRNA. As seen in Fig. 4, lane a, the presence of three of the eIF-3 subunits (molecular masses 110, 95 and 66/64 kDa) and the 24kDa cap binding protein could be demonstrated by SDS gel electrophoresis. In addition, the ribosomal proteins S3/S3a and S6 were bound to globin mRNA within the 48S pre-initiation complex. The designation of the ribosomal proteins was confirmed by two-dimensional gel-electrophoresis. In this gel system additional ribosomal

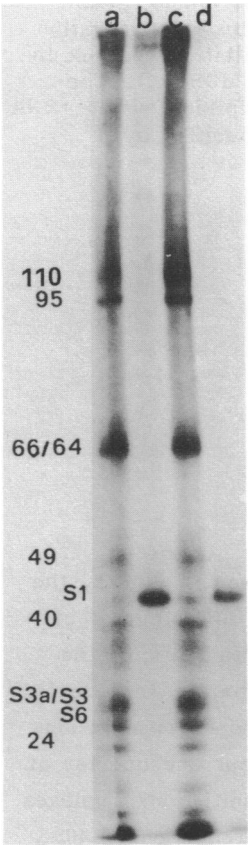

Fig. 4. Proteins cross-linked to mRNA within the 48S pre-initiation complex (lanes a and c) and to free mRNA (lanes b and d). Cross-linking was performed before (lanes a and b) or after (lanes c and d) the isolation of the 48S pre-initiation complex by gradient centrifugation. Proteins isolated from the labelled complexes were detected by autoradiography and identified by co-electrophoresis of purified eIF-3 and 40S subunit proteins.

proteins (S1 and S11) could be identified as bound to the mRNA (Fig. 5A).

In order to ascertain the specificity of the mRNA-protein interactions, non-cross-linked 48S pre-initiation complexes were isolated by gradient centrifugation and subsequently treated with diepoxybutane. In this case also the 110, 95 and 66/64 kDa subunits of eIF-3 were found cross-linked to mRNA together with the 24kDa cap binding protein and the ribosomal proteins S3/S3a and S6 (Fig. 4 lane c). The similarities in the protein patterns obtained by the two different procedures indicate that the 48S complex is stable during centrifugation and that no proteins were loosely bound to mRNA during the complex formation. Interestingly, the mRNA not bound to the $40S_N$ particles was found cross-linked to the largest 40S ribosomal protein (S1, molecular mass 42kDa) (Figs. 4 lanes b and d, 5B). This suggests that the S1 protein was bound either to the 48S pre-initiation

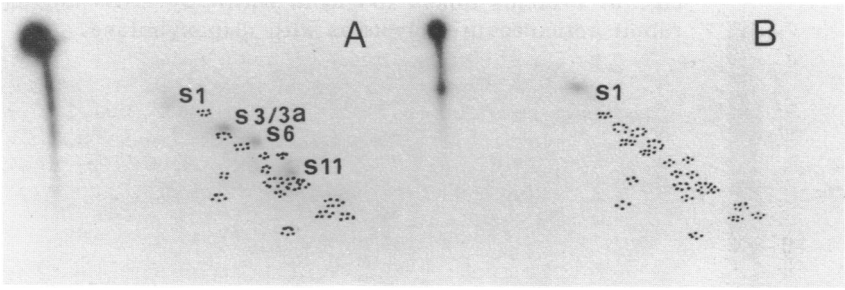

Fig. 5. Two-dimensional gel electrophoresis of ribosomal proteins cross-linked to globin mRNA within the 48S pre-initiation complex (A) and to unbound globin mRNA (B). The protein spots were identified by autoradiography and correlated to the stained pattern of the 40S subunit marker proteins (dotted pattern).

complex or, after leaving the $40S_N$ particle, became bound to free mRNA.

For comparison with the 48S pre-initiation complex, the ribosomal domains in contact with mRNA in polysomes were studied by cross-linking. As seen in Fig. 6 the proteins that could be cross-linked to the polysome bound mRNA were identified as the ribosomal proteins S3/S3a and S6. In addition, the 78kDa and 52kDa mRNP proteins were found cross-linked to the mRNA.

DISCUSSION

Protein synthesis can be regulated by the selective translation of specific messages. This type of control mechanism is supposed to be involved in cellular differentiation and virus infection (28,29). The mRNA selection is thought to take place mainly at the level of protein synthesis initiation (30). A key role in this process has been attributed to proteins identified as mRNP proteins (31), initiation factors (32), cap binding proteins (33) and ribosomal protein S6 (11). One way of studying the interaction between proteins and RNA is to investigate their structural arrangement within the initiation complex by chemical cross-linking. Diepoxybutane has proved a useful reagent for this type of investigation (10,34,35). By cross-linking 48S reticulocyte pre-initiation complexes the 110, 95 and 66 or 64 kDa subunits of eIF-3 become covalently linked to globin mRNA. This indicates that eIF-3 is in direct contact with mRNA through at least three of its 9 subunits (36). The same three subunits can also be cross-linked to the RNA in a complex only consisting of eIF-3 and

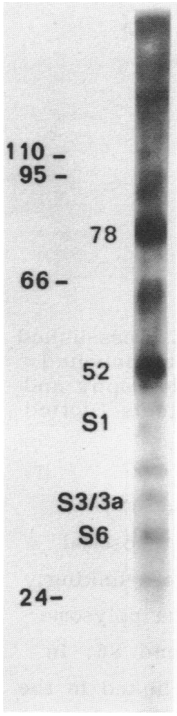

Fig. 6. Proteins linked to globin mRNA by cross-linking rabbit reticulocyte polysomes with diepoxybutane.

Semliki forest virus mRNA (37). The intimate contact between mRNA and eIF-3 also explains why $40S_N$ particles depleted of eIF-3 by ethionine treatment (12) fail to bind to mRNA. The 66kDa subunit has also been located in the near neighborhood of 18S rRNA (10). Thereby, the factor might mediate the interaction between mRNA and 18S rRNA. A 22S complex containing globin mRNA, 18S rRNA and a 66 kDa protein has also been isolated from rabbit reticulocyte polysomes (38). The interaction between mRNA and $40S_N$ includes the 5' terminal cap within the untranslated part of the mRNA (19,20). The cap structure can be directly cross-linked to the cap binding proteins after oxidation of the terminal 7-methylguanosine (39). In our experiments a 24 kDa protein was also bound to the mRNA. This protein could be isolated together with eIF-3 (36), suggesting identity with the 24kDa cap binding protein. In addition, the mRNA binding domain on the 40S ribosomal subunit was found to include proteins S1, S3/3a, S6 and S11. Proteins S3 and S3a have previously been identified as belonging to the mRNA-contacting domain in 80S ribosomes by affinity labelling using a

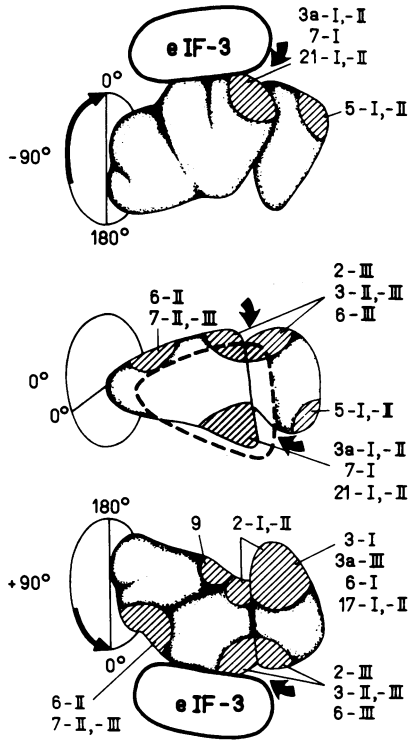

Fig. 7. Schematic representation of the site of mRNA binding to the 48S pre-initiation complex (arrows). The antigenic binding sites of proteins S2, S3, S3a, S5, S6, S7, S17 and S21 are designated by Roman numerals (44,45). eIF-3 has been visualized by electron microscopy as a flat prism attached to the "body" part of the particle (G. Lutsch, unpublished results).

poly(U) analogue (40). Proteins S3, S3a and S6 were also found cross-linked to mRNA within polysomes after UV irradiation (41). From these results it can be concluded that the ribosomal binding sites for mRNA in the 48S pre-initiation complex and in the polysomes are similar if not identical. Furthermore, the ribosomal proteins S3a and S6 belong to the binding domains of eIF-3 (42), eIF-2 (43) and Met-tRNA$_f$ (35), indicating that these components of the pre-initiation complex are positioned in close proximity in such a way that both eIF-3 and mRNA come into contact with S3, S3a and S6 on the ribosomal surface.

The cross-linking data can be correlated with the arrangement of the ribosomal proteins within the 40S particle. The positions of proteins S3, S3a,

S6 and S7 have been determined by immune electron microscopy (44,45. cf. Fig. 7). From these data it can be concluded that the mRNA is bound to the area between "head" and "body" of the particle, very likely at the groove within this area. The neighbouring eIF-3 is attached to the "body" part of the particle. This location of eIF-3 has been confirmed by electron microscopy (G. Lutsch, unpublished results. See Fig. 7).

The 78 and 52 kDa mRNP proteins, which can be cross-linked to polysomal mRNAS by UV irradiation (46,47) or by diepoxybutane treatment, seem to be absent from the 48S pre-initiation complex and are obviously not needed for mRNA binding, although they remain bound to mRNA during initiation complex formation with mRNP (48).

*To whom correspondence should be addressed

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