IDENTIFICATION OF PROTEIN S 1 AT THE MESSENGER RNA BINDING SITE OF THE ESCHERICHIA COLI RIBOSOME

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SUMMARY: Poly-4-thiouridylic acid acts as messenger RNA for polyphenylalanine synthesis in an *in vitro* protein synthesizing system. When a complex consisting of ribosomes, poly-4-thiouridylic acid and Phe-tRNA is irradiated at 300 to 400 nm, covalent bonds between this messenger RNA and protein S 1 are formed.

INTRODUCTION: Previous experiments (1 - 6) have indicated that protein S 1 of the small ribosomal subunit participates in binding of poly U to ribosomes of *E. coli*. This protein has also been shown to bind poly U in solution (4), suggesting that it might comprise the mRNA binding site on the ribosome. However, no direct proof for a close association on the ribosome between this protein and mRNA has been obtained so far.

Recently, affinity labeling techniques have been used successfully to identify proteins at the donor- and the acceptor-site of ribosomes by employing derivatives of tRNA as well as analogues of antibiotics and GDP (7 - 14). We have, therefore, searched for a polynucleotide which can act as mRNA and which at the same time is suitable for affinity labeling. Bähr *et al.* (15) have shown that poly (s^4U) [1] codes for polyphenylalanine in an *in vitro* protein synthesizing system. Vormbrock et al. (16) have demonstrated that trinucleotides containing 4-thiouridine stimulate Phe-tRNA binding to ribosomes. In addition, Frischauf and Scheit (17) have employed poly-4-thiothymidylic acid and 4-thiouridinetriphosphate as photoaffinity labels for the study of DNA-dependent RNA polymerase.

In this study we have investigated poly (s^4U) in its function as an analogue

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[1] poly (s^4U) = poly-4-thiouridylic acid.

of poly U. Complexes were formed between ribosomes, [^{3}H]poly (s^4U) and Phe-tRNA and then subjected to irradiation at 300 to 400 nm. Subsequently the ribosomes were degraded and the radioactivity incorporated into 30 S proteins was determined. Radioactive label was found attached to protein S 1. Small amounts of label were also recovered in other proteins.

MATERIAL AND METHODS: Ribosomes were obtained from E. coli D 10 (18). Poly (s^4U) and [^{3}H]poly (s^4U) were synthesized as described (19). Rabbit antibody against protein S 1 was purified according to Stöffler and Wittmann (20). Poly U was purchased from Miles Laboratories.

Binding was carried out in 60 mM NH_4Cl, 100 mM Tris-HCl (pH 7.2), 20 mM magnesium acetate, 4 mM dithioerythritol containing per 1 ml: 8.5 mg ribosomes, 0.3 mg Phe-tRNA and 0.018 mg [^{3}H]poly (s^4U) at a specific activity of 5.9×10^7 cpm/mg of poly (s^4U). The mixture was incubated for 20 min at 25° C and irradiated for 30 min at 0° C with a Philips high pressure mercury lamp SP 500 W using filter UG 1 (Schott, Mainz/GFR). 30 S ribosomal subunits were prepared and digested with ribonucleases A and T 1 in 3 M urea, 0.1 M EDTA (pH 7.4) and 10 mM dithioerythritol for 2 hrs at 37° C (11). Proteins were dialyzed against 10 % and 1 % acetic acid and lyophilized.

SDS gel electrophoresis was carried out according to Weber et al. (21). Alkaline hydrolysis of the RNA-protein complex was carried out in 0.3 M NaOH for 20 min at 37° C. The immunological characterization employed sucrose gradient centrifugation of soluble antigen-antibody complexes and has been described previously (11, 12).

RESULTS: Irradiation of poly (s^4U) at 300 to 400 nm results in photo-oxidation leading to an intermediate which can subsequently react with a nucleophilic group on a protein (17). Formation of a covalent bond between mRNA and ribosome should manifest itself in a strong inhibition of mRNA movement and con-

sequently of polypeptide synthesis.

To begin with we have therefore investigated the effect of irradiation of a ribosome - poly (s^4U) - [^{14}C]Phe-tRNA complex on its capacity to synthesize polyphenylalanine in an in vitro protein synthesizing system. The result is shown in Fig. 1. Complexes were irradiated for the time indicated, aliquots were removed and added to a mixture containing supernatant proteins and all other components required for in vitro protein synthesis. As shown in Fig. 1, the activity of the complex for poly Phe synthesis decreases rapidly upon irradiation. In contrast, a control carried out in parallel in which poly U was used as mRNA showed essentially no reduction in poly Phe formation. This indicates that irradiation blocks specifically a step involving poly (s^4U) rather than causing a nonspecific damage to the ribosome or Phe-tRNA.

In order to obtain direct proof for covalent bond formation ribosomal complexes were formed using radioactive [^3H]poly (s^4U) and unlabeled Phe-tRNA.

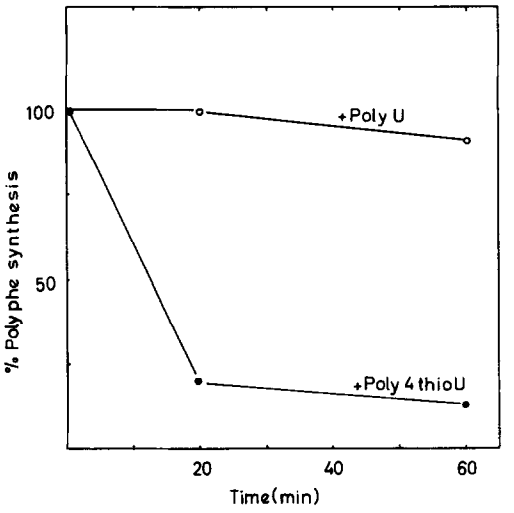

Fig. 1: Effect of irradiation on [^{14}C]Phe incorporation. The incubation mixture contained per ml: 2.5 mg ribosomes, 0.8 mg [^{14}C]Phe-tRNA (specific activity 2.7 x 10^5 cpm/mg tRNA), 0.15 mg poly U or poly (s^4U) in 60 mM NH_4Cl, 100 mM Tris-HCl (pH 7.2), 20 mM magnesium acetate, 4 mM dithioerythritol. The sample was incubated and irradiated as described in Materials and Methods. 25 µl were removed at the times indicated and mixed with 75 µl of a solution containing S 30 supernatant (23), ATP, GTP and an energy generating system (23). In the sample containing poly U, 100 % poly Phe synthesis represents 2,139 cpm of [^{14}C]Phe incorporated. In the poly (s^4U) containing sample, 100 % is equivalent to 1,285 cpm of [^{14}C]Phe incorporated.

Mixtures were irradiated for 30 min at 0° C. Ribosomes were then dissociated and the 30 S subunits isolated by sucrose gradient centrifugation. Following denaturation by urea and EDTA the samples were digested with ribonucleases A and T 1. Poly (s^4U) is highly sensitive to ribonuclease A. The ribosomal proteins were then subjected to SDS gel electrophoresis; the result is given in Fig. 2 A. The bulk of radioactivity migrates more slowly than the largest ribosomal protein S 1 as seen by comparison with the stained pattern of ribosomal proteins. No peak of radioactive material was found in the control in which poly (s^4U) was irradiated before being bound to ribosomes (Fig. 2 A) or in the non-irradiated control sample (data not shown). The radioactivity represents labeled protein since this peak was abolished by incubating the mixture with trypsin before electrophoresis. The sample has been digested extensively

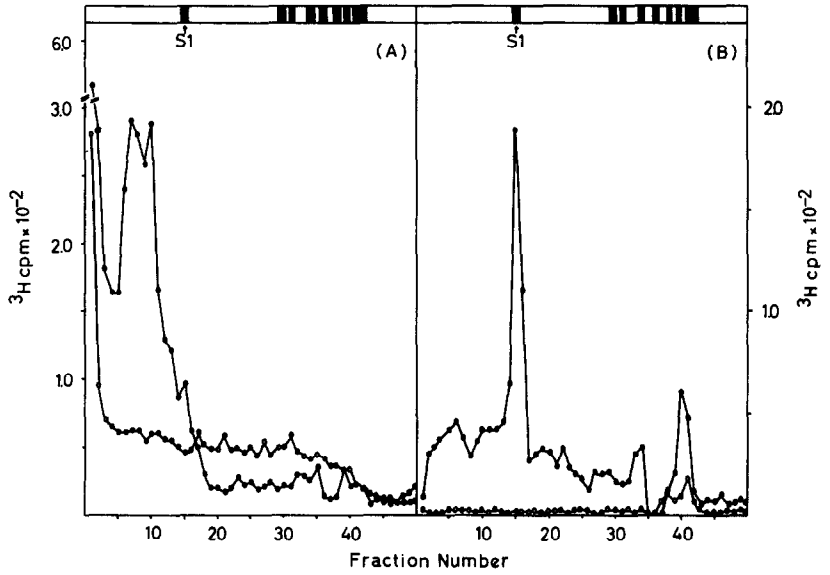

Fig. 2: Identification of labeled protein by SDS gel electrophoresis. 30 µg of labeled 30 S protein was mixed with 60 µg of unlabeled 30 S protein. Samples were heated for 90 s at 100° C in 0.1 M phosphate buffer (pH 7.1), 0.1 M dithioerythritol, 2.5 % SDS before layering on the gel. Electrophoresis was carried out in 6 mm cylindrical gels for 8 h at 10 mA per tube. Gels were cut into 2 mm slices and the radioactivity determined as described previously (11, 12). Closed circles(●——●) show the pattern of radioactive proteins obtained from the irradiated ribosome - [^{3}H]poly (s^4U) - Phe-tRNA complex. Open circles (o——o) represent the control experiment in which [^{3}H]poly (s^4U) was irradiated before binding to the ribosome.
(A) gives the pattern obtained before alkaline digestion,
(B) after alkaline digestion of the RNA-protein complex.

with ribonuclease. Therefore it seems unlikely that the large decrease in electrophoretic mobility is due solely to attachment of a long stretch of poly (s^4U) to a single protein. It appears more intriguing to assume that a short stretch of polynucleotide is attached covalently to several proteins which in turn protect it against degradation by ribonuclease.

In order to further degrade such an RNA-protein complex a partial alkaline hydrolysis of the polynucleotide was carried out. The result is presented in Fig. 2 B. Most of the radioactivity is now found in a sharp peak which comigrates with protein S 1. There are several small peaks of radioactivity migrating in the region of small ribosomal proteins. As expected no incorporation of radioactivity is found in the control experiments (Fig. 2 B).

Independent evidence for the identification of the major labeled protein as being S 1 comes from immunological studies. Specific IgG antibody directed against S 1 was added in excess to total labeled 30 S protein obtained by ribonuclease digestion. Under these conditions high molecular weight soluble antigen-antibody complexes are formed which sediment rapidly in sucrose density gradients (11, 12). Fig. 3 gives a comparison of the sedimentation profiles obtained with anti-S 1 immunoglobulin and with immunoglobulin from a non-immunized animal. 86.5 % of the total radioactive material can be shifted into the rapidly sedimenting fraction by antibodies to S 1. This independently confirms the results of the electrophoretic analysis.

DISCUSSION: An identical binding site for poly (s^4U) and poly U is suggested by the fact that both polynucleotides act as mRNAs for poly Phe synthesis and by direct competition experiments at limiting ribosome concentrations (data not shown). The higher stimulation of poly Phe synthesis by poly U as compared to poly (s^4U) (see legend Fig. 1) is probably due to a difference in the chain length of the polynucleotide (15).

The results presented in this paper demonstrate that the mRNA poly (s^4U) and protein S 1 are in immediate contact on the ribosome. A role for S 1 in binding

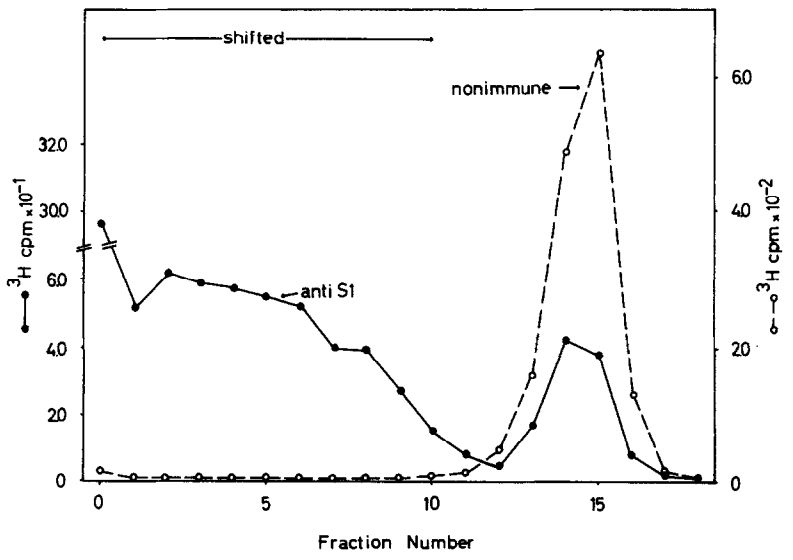

Fig. 3: Sucrose gradient centrifugation of soluble antigen-antibody complexes. 8 µg of total 30 S protein was mixed with 1.2 mg of immunoglobulin in 0.2 ml of a solution containing 0.5 M LiCl, 50 mM Tris-HCl (pH 8.2) and 1 mM dithiothreitol and incubated for 30 min at 0° C. Sucrose gradient centrifugation was carried out as described previously except that centrifugation was for 14 h at 24,000 rpm in an SW 40 rotor (11, 12). Fraction number 0 represents radioactivity recovered from the bottom of the centrifuge tube by resuspension in 1 ml of 5 % SDS. Anti-S 1 antibody shifted 86.5 % of the total radioactivity into the rapidly sedimenting fraction; when non-immune antibody was used 1.1 % of the toal radioactivity was found in this fraction.

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of poly U to ribosomes has been suggested by several authors (1 - 6). Recent experiments by Van Duin and Van Knippenberg (22) demonstrate that this protein is required for translation of poly U. Ribosomes actively translating either poly U (22) or MS 2-RNA (6) contain close to one copy of S 1 per ribosome. All these data together strongly suggest that S 1 indeed constitutes at least part of the mRNA binding site on the ribosome. The minor proteins labeled with [^{3}H]poly (s^4U) - which might also contribute to this site - are now being identified.

Nevertheless, the study of a homopolymer as mRNA leaves several questions unanswered. It would be desirable to conduct similar studies with oligonucleotides containing an initation triplet as well as studies with derivatives of natural mRNAs. These studies are presently under way in our laboratory.

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