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Ribosomal localization of the mRNA in the 30S initiation complex as revealed by UV crosslinking

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Translation initiation complexes consisting of 30S ribosomal subunits, ^{32}P-labelled mRNA (002 mRNA), fMet-tRNA and the three initiation factors were subjected to UV-crosslinking to determine the protein and rRNA neighbors of the bound mRNA by immunochemical methods and by nucleic acid hybridization techniques. The mRNA was found to be crosslinked to a specific region of the 16S rRNA spanning from nucleotide 418 to 615 and to ribosomal proteins S1 and S21 (the main targets), S3, S10, S12 and S14; a low level of crosslinking was also detected with S2, S7, S13, S18 and S19.

Translation initiation; Initiation complex; Ribosome; mRNA; UV-crosslinking

1. INTRODUCTION

Several approaches have been used to address the important problem of the topographical localization of the ribosome-bound mRNA. Worth mentioning among these are the direct localization of ribosome-bound mRNA by electron microscopy [1,2] which have suggested that the mRNA makes a U-turn on the ribosome, a view also supported by fluorescent energy transfer studies between 3'- and 5'-ends of model mRNAs [3,4] and several types of photoaffinity labelling, crosslinking and site-directed crosslinking studies (for reviews see [5,6]).

A potential problem in the interpretation of these results is, however, the striking dishomogeneity of the experimental approaches employed in different laboratories which have used oligo- or polynucleotides instead of natural mRNAs and other non-physiological conditions (e.g. very high Mg^{2+} concentrations, absence of one or more of the initiation factors, use of non-natural forms of initiator tRNA). In this article we report on the topographical localization of the model 002 mRNA which has the consensus TIR sequence upstream of the AUG initiation triplet [7] crosslinked by UV-irradiation in a homogeneous 30S initiation complex (i.e. containing fMet-tRNA and initiation factors IF1, IF2 and IF3).

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2. MATERIALS AND METHODS

2.1. *Buffers*

Buffer A, 10 mM Tris-HCl, pH 7.7, 60 mM NH_4Cl, 1 mM dithiothreitol.

Buffer B, 10 mM Tris-HCl, pH 7.5, 1% SDS, 0.1 M NaCl, 1 mM EDTA.

Buffer C, 50 mM Tris-HCl, pH 7.5, 25% formamide, 0.7 M NaCl, 10 mM EDTA.

Buffer D, 10 mM potassium phosphate, pH 7.5, 90% formamide, 0.2% lauroylsarcosin, 10 mM EDTA.

Buffer E, 75 mM sodium citrate, pH 7.0, 0.75 M NaCl, 50% formamide, 1 × Denhardt's solution, 20 mM sodium phosphate, pH 6.8, 0.2% SDS, 0.1 mg/ml sonicated DNA (from salmon testes, Sigma), 0.1 mg/ml yeast RNA (Boehringer).

2.2. *Sequence of 002 mRNA*

The model 002 mRNA [7] used in this study has the following sequence: GAAUUCGGGCCCUUGUUAACAAUUAAGGAGGUAUACUAUGUUUACGAUUACUACGAUCUUCUUCACUUAAUGCGUCUGCAGGCAUGCAAGCU$(A)_{20}$.

2.3. *Initiation complex formation and crosslinking reaction*

The incubation mixture contained, in 1 ml of buffer A, 10 mM Mg acetate, 1 mM GTP, 600 pmol *E. coli* MRE600 30S ribosomal subunits, 600 pmol (70,000 dpm/pmol) 002 mRNA [7] labelled by incorporation of [α-^{32}P]ATP and 600 pmol each of IF1, IF2, IF3 and fMet-$tRNA_f^{Met}$. After incubation at 37°C for 5 min, the crosslinking reaction was carried out by UV-irradiation (10 min on ice at a distance of 5 cm) with a germicidal lamp (Original Hanau-Sterisol F 1140). The crosslinked complexes were precipitated (1 h at 0°C) with one volume of ethanol, dried, resuspended in 250 μl of buffer A containing 0.3 mM Mg acetate and purified by centrifugation (3 h at 280,800 × *g* in a Beckman SW60Ti rotor) through a 4 ml 10–30% sucrose gradient in the same buffer. The fractions containing the crosslinked complex were pooled, adjusted to 10 mM with respect to Mg acetate, precipitated overnight with 2 volumes of ethanol at −20°C and resuspended in 500 μl buffer B. After incubation for 10 min at 37°C, the mixture was diluted with 2 ml of buffer C and loaded onto a Poly(U)-Sepharose column (bed volume 1 ml) which was washed with 10 ml of buffer C. Since 002 mRNA contains at its 3'-end a stretch of 26 adenines [7], the crosslinked complexes containing the mRNA were retained by this

column from which they were eluted with 3 ml of buffer D collecting 200 μl fractions. The radioactivity associated with the eluted fractions was determined and the peak fractions were pooled, diluted with 1 volume of water and precipitated with 2.5 volumes of ethanol overnight at −20°C after addition of 0.5 mg of carrier tRNA and of sodium acetate to a final concentration of 0.3 M. The resulting precipitate was dissolved in 250 μl of 10 mM Tris-HCl, pH 7.8, containing 4 M urea and centrifuged (16 h at 118,400 × g in the Beckman SW60Ti rotor) through a 4 ml 10–30% sucrose gradient in the same buffer; the fractions containing the 16S mRNA complexes were pooled, precipitated as described above, dried and dissolved in 100 μl of buffer A containing 10 mM Mg acetate.

2.4. *Dot-blot analysis*

DNA aliquots (1 μg each) corresponding to M13 clones of different 16S RNA regions [8] were spotted on nitrocellulose which was subsequently treated with 1.5 M NaCl, 0.5 M NaOH (5 min), with 0.5 M Tris-HCl, pH 7.4 (30 s) and with 0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl (5 min); the filters were then dried, baked in a vacuum oven (2 h at 80°C) and prehybridized in buffer E followed by hybridization with about 2.5 pmol of the 16S–[^{32}P]mRNA crosslinked complex in 10 ml of buffer E (6 h at 42°C). After washing twice with 50 ml of 1 × SSC containing 0.1% SDS (1 h at 60°C), the filters were incubated (1 or 2 h at 37°C) with 100 U RNase T1 (Boehringer) in 10 ml 1 × SSC. The filters were finally washed (60°C for 1 h) with 50 ml of 1 × SSC containing 0.5% SDS and 5 mM $CuSO_4$, dried and subjected to autoradiography.

2.5. *Identification of the ribosomal proteins crosslinked to mRNA*

The ribosomal proteins crosslinked to the radioactive 002 mRNA were identified by the quantitative immunological test as previously described [9].

3. RESULTS AND DISCUSSION

As seen from the dot blot analysis shown in Fig. 1, the most extensive UV-crosslinking of the 002 mRNA was found in spot 4 which corresponds to a segment of the 16S rRNA spanning nucleotides 418 to 674. However, since DNA homologous to nucleotides 615–674 of the 16S rRNA is also present in the construct blotted in spot 5, which remained completely unlabelled, the region of the 16S rRNA crosslinked to the mRNA in the 30S initiation complex can be narrowed to that comprised between nucleotides 418 and 615. Based on our current knowledge, in this rRNA region, the 530 loop is the most likely candidate for an interaction with the mRNA. In fact, this universally conserved segment of the 16S RNA constitutes a functional pseudoknot [10] implicated in and probably stabilized by protein S12 binding [11,12], involved in A-site decoding [13], streptomycin sensitivity [14,15], mRNA binding [16] and possibly in monitoring translational reading frame [17]. Furthermore, based on a number of considerations summarized by Brimacombe [18], it is now suggested that the methylated G at position 527, which belongs to the 530 loop region, is in a tight cluster with almost all other modified bases of the rRNA. Thus, nearly all the elements of the 16S rRNA thought to be involved in or to influence the mRNA binding and decoding (i.e. the 530 loop [16], the 3′-end (A1542) [21–23], the anti-SD sequence (1534–1541) [2] and the nucleotide 1400 region [19,20]) would be located in close proximity to each other in the platform/cleft region of the 30S subunit.

It is noteworthy that the main mRNA crosslinking target on the 16S RNA identified in this paper is compatible with one of the two positions (i.e. nucleotide 532) found covalently linked to the mRNA by Rinke-Appel et al. [16]; however, other regions of the 16S RNA (i.e. position 1050 [24], 1390–1400 [16,25,26] and the 3′-terminus [24,26]) which have been implicated in mRNA–ribosome interaction in other studies were found to be crosslinked to the mRNA either very weakly or not at all (spot 11 of Fig. 1, for instance, corresponds to nucleotides 1385–1542). In discussing this discrepancy, it should be borne in mind that the absence of crosslinking cannot be regarded as evidence for the lack of contact between two molecules since several parameters (local environment, nucleotide sequence, etc.) may affect the efficiency of crosslinking at

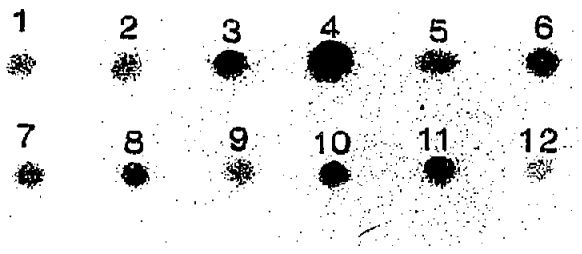

Fig. 1. Identification of the 16S rRNA region crosslinked to 002 mRNA in a complete 30S initiation complex. The dot blot analysis of the region of 16S rRNA crosslinked in situ to [^{32}P]002 mRNA by UV irradiation was carried out as described in section 2. The individual spots correspond to 11 different M13 clones [8] carrying DNA inserts encoding the following 16S rRNA regions: 1, 1–46; 2, 47–213; 3, 214–417; 4, 418–674; 5, 615–734; 6, 735–895; 7, 896–929; 8, 930–1139; 9, 1140–1207; 10, 1208–1384; 11, 1385–1542; 12 corresponds to a control clone containing a region unrelated to 16S rRNA (22 M13 mp10).

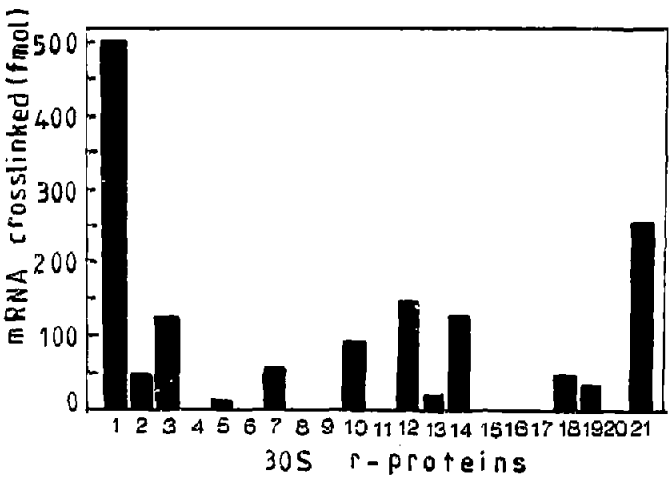

Fig. 2. Identification of the ribosomal proteins crosslinked to 002 mRNA by UV irradiation in a complete 30S initiation complex. The ribosomal proteins crosslinked to [^{32}P]002 mRNA by UV irradiation were identified immunologically as previously described [9].

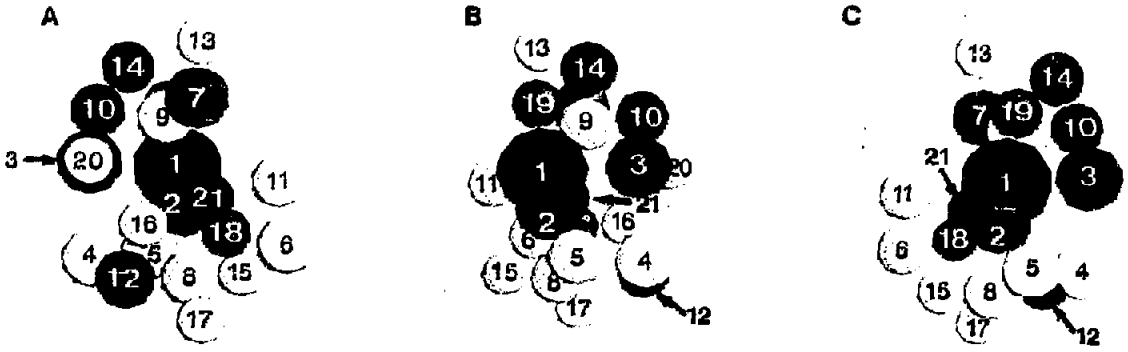

Fig. 3. Location of the ribosomal protein crosslinked to 002 mRNA in the 30S initiation complex in a computer-generated three-dimensional model of the 30S ribosomal subunit. (a) view of the subunit interface side, (b) and (c) views of the cytoplasmic side in two orientations. The black, gray and white spheres represent proteins with high, low and negligible yields of mRNA crosslinking, respectively. The 3D model of the 30S ribosomal subunit is essentially that described by Schüler and Brimacombe [28].

a given site. Nevertheless, our result is not entirely unexpected since various lines of evidence have suggested that, during formation of the 30S initiation complex, in the presence of initiation factors, the mRNA is shifted from the anti-Shine–Dalgarno region where it is originally bound (standby site) towards another (presumably the decoding) site of the ribosome [9,27]. It has already been noticed that the crosslinking pattern of mRNA to the 30S ribosomal proteins becomes more homogeneous going from 30S mRNA binary complex in the absence of factors to 30S mRNA-fMet-tRNA initiation complex in the presence of all three initiation factors [9]. Thus, technically, our present results are likely to reflect the high level of homogeneity of the 30S initiation complex obtained under optimized and physiological conditions in which over 80% of the input 30S subunits, fMet-tRNA and mRNA end up in a 30S initiation complex. None of these conditions were met in the studies mentioned above in which either 70S ribosomes, elongator tRNA and short mRNAs were used [16] or '30S initiation complexes' were prepared with deacylated $tRNA^{fMet}$ and without initiation factors [24,26].

The individual ribosomal proteins crosslinked by UV irradiation to the 002 mRNA in the 30S initiation complex were also identified immunologically and the extent of the reaction was quantitatively determined using the sensitive and reproducible procedure previously described [9]. The results obtained are shown in Fig. 2. It can be seen that S1 and S21 are the main targets of the crosslinking reaction but significant crosslinking was also obtained with S3, S19, S12, S14 and, to a much lesser extent, with S2, S7, S13, S18 and S19. These results are in fairly good agreement with those obtained with the same initiation complex following crosslinking with diepoxybutane [9]. It should be noted, however, that S12 is crosslinked almost exclusively by UV-irradiation while S5 and S9, crosslinked by diepoxybutane, are not crosslinked by UV-irradiation.

The location of these crosslinked proteins is presented in the computer-generated model of the 30S subunit based on the three-dimensional map of the mass centers of the ribosomal proteins derived from neutron scattering data [28]. As seen in Fig. 3, the location of the crosslinked proteins suggests a winding path of the mRNA around the cleft of the ribosomal subunit in a fashion at least compatible with that recently proposed by Shatsky et al. [29].

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