The Shine-Dalgarno hybrid during initiation of translation and elongation

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It was unknown whether a synthetic Shine-Dalgarno (SD) oligonucleotide labelled with ^{32}P at its 5'-end ([^{32}P]oct) would be able to reach the anti-SD sequence of 16S rRNA at the early stages of translation only or during elongation. To verify this, [^{32}P]oct was incubated with 30S ribosomal subunits (RSUs), 70S ribosomes and polysomes, separately, while the SD/anti-SD binding was checked in them through sucrose gradients. The anti-SD sequence resulted highly available in 30S RSUs and sufficiently available in ribosomes. In both 30S RSUs and ribosomes, the addition of a model 002 mRNA in equimolar proportions displaced [^{32}P]oct for about 50 %. However, in ribosomes the presence of initiation factors (IFs) and fMet-tRNA influence neither the binding of [^{32}P]oct nor the competition coming from mRNA. In polysomes, [^{32}P]oct was unable to hybridize the anti-SD sequence, in agreement with the hypothesis that mRNA and 16S rRNA are involved in the SD/anti-SD interaction also during elongation.

Keywords: ribosomal machinery, mRNA/16S rRNA recognition, peptide bond formation, polysomal conformation and translational state.

Introduction. Initiation of protein synthesis in bacteria represents an intriguing process during which the small 30S RSU binds both mRNA and initiator fMet-tRNA to construct a complex which interacts with the large 50S RSU. Formation of the first peptide bond between fMet-tRNA in the *P* site (corresponding to the first mRNA codon) and aminoacyl-tRNA in the *A* site (corresponding to the second mRNA codon) marks the transition between initiation of translation and elongation. Three IFs (IF_1, IF_2, IF_3) and one GTP molecule are necessary to ensure efficiency and fidelity of the reactions [1, 2].

One of the early events is the recognition, by 30S RSU, of the mRNA sequence known as transition initiation region. Its body includes the initiation codon (in most cases AUG), a spacer of variable length and the purine-rich SD sequence (5'-AAGGAGGT-3') complementary to the 3'-end region of 16S rRNA (3'-TTCCTCCA-5') [3]. The base pairing between the SD sequence of mRNA and the anti-SD sequences of 16S rRNA is a crucial step in mRNA/ribosome recognition and, consequently, in the process of translation: mutant mRNAs with more extended SD pairing show an increased level of gene expression, but mutant mRNAs with a low extent of base pairing are

poorly translated [4, 5]. Alternatively, the capability of mRNAs to be expressed, when they do not carry the SD sequence, suggested that this sequence is not essential for translation [6] and that its main function is to ensure a high concentration of initiation codon near the ribosomal *P* site [7].

This information shed sufficient light on the role of the SD/anti-SD interaction at the early stages of translation, although it was still unclear if such an interaction would be limited to the early steps of translation initiation only or if 16S rRNA and mRNA would also remain in contact during elongation. The latter possibility was supported by two observations: in experiments with hydroxyl-radical footprinting the protection of the SD sequence by ribosome still persists in an elongation complex (70S/mRNA/fMet-tRNA/Phe-tRNA) [8]; the SD/anti-SD interaction influences the ribosomal frame-shifting [9]. On this basis, it was suggested that the SD base pairing might function by stabilizing the mRNA/ribosome interaction during elongation [9].

The present investigation demonstrated that [^{32}P]oct is able to reach the anti-SD sequence of 16S rRNA not only in isolated 30S RSUs but also in 70S ribosomes, whereas the same anti-SD sequence is inaccessible in polysomes.

Materials and Methods. RSUs, ribosomes and polysomes were isolated from the MRE 600 strain of *Escherichia coli* [10] with minor modification of the procedures described earlier [11–13]. To purify polysomes, a 60-ml bacterial culture grown to 0.5–0.6 OD at 560 nm was chilled and harvested through a 3-min centrifugation at 5,000 rev./min. The precipitated bacteria were reversed in 0.4 ml of 25 % sucrose diluted in 0.01 M Tris-HCl at pH 8.1. To this sample a 0.1-ml volume of 0.25 M Tris-HCl at pH 8.1, containing 2.7 mg/ml ethylen-diamino-tetraacetic acid (EDTA), was added. After 45-sec shaking, it was transferred into a cold centrifuge tube containing a mixture of 100 ml of 0.1 M $MgSO_4$, 50 ml of 1 mg/ml DNAse, 200 ml of 1 % sodium deoxycholate (in 0.1 M Tris-HCl at pH 8.1) and 5 % Brij 58 (in 0.1 M Tris-HCl at pH 7.2). Then, the bacterial cells were lysed within 2 min, the lysate was concentrated at 7,000 rev./min for 5 min and the supernatant, holding the polysomes, was run for 19 hrs at 13,000 rev./min through a 15–30 % sucrose gradient performed in a mixture of 5 mM Tris-HCl at pH 7.2, 10 mM $MgSO_4$ and 60 mM KCl.

IFs, aminoacyl-tRNAs and T7 RNA polymerase (RNApol). The IFs of translation and the aminoacyl-tRNAs were prepared from *E. coli* essentially according to [1, 12]. The RNApol was purified from the BL 21 strain carrying the *pAR* 1219 plasmid which includes its gene controlled by the *tac* promoter [14]. The sample was inoculated in 12 ml of defined medium (M9TB). In addition to 1 g/l NH_4Cl, 3 g/l KH_2PO_4, 6 g/l Na_2HPO_4, 10 g/l trypton and 5 g/l NaCl, this contained 40 mg/ml ampicillin, 0.4 % maltose, and 1 mM $MgSO_4$. A hyperproduction of RNApol was induced by 0.4 mM isopropyl-thio- -D-galactoside. After growing to 0.5 OD at 600 nm, the bacteria were harvested at 7,000 rev./min for 10 min, washed twice with 20 ml of 20 mM Tris-HCl at pH 8 (enriched with 20 mM NaCl plus 1 mM Na_2EDTA) and put at –80 ^{o}C. Once frozen, they were resuspended in 15 ml of 50 mM Tris-HCl at pH 8.0 containing 20 mM NaCl, 2 mM Na_2EDTA, and 6 mM -mercaptoethanol (ME). The cell lysis was reached by adding 1.5 mg/ml lysozyme. The lysate, treated with 24 ml of 0.1 M phenyl-methyl- sulfonyl-fluorure and 14 ml of 0.1 M benzamide (to inhibit possible proteases), was left for 20 min at room temperature. Then, it was mixed with 180 ml of 4 % sodium deoxycholate, prepared in 1 mM $MgCl_2$, 3 ml of 2 M $(NH_4)_2SO_4$ and 5 mg/ml DNAse, and maintained for another 20 min at room temperature. The final sample was treated with 30 ml of a 50 mM Tris-HCl buffer at pH 8 comprising 20 mM NaCl, 2 mM Na_2EDTA, and 6 mM ME. The RNA and DNA macromolecules were precipitated with 1.5 ml of 10 % Polymin P. The suspension was stirred in ice for 20 min and centrifuged at 15,000 rev./min for 15 min. Its supernatant was precipitated again with 1.5 ml of 10 % Polymin P, stirred in ice for 15 min and centrifuged at 15,000 rev./min for 15 min. The new supernatant was mixed with $(NH_4)_2SO_4$ to reach a concentration of 33 %. Thereafter, the solution was centrifuged for 10 min at 12,000 rev./min, while the pellet was resuspended in 10 ml of 20 mM potassium-phosphate buffer at pH 7.7 combined with 100 mM NaCl, 1 mM Na_2EDTA, 6 mM ME and 5 % glycerol. The sample was dialysed overnight against 600 ml of the same buffer at 4 ^{o}C. The dialysed material

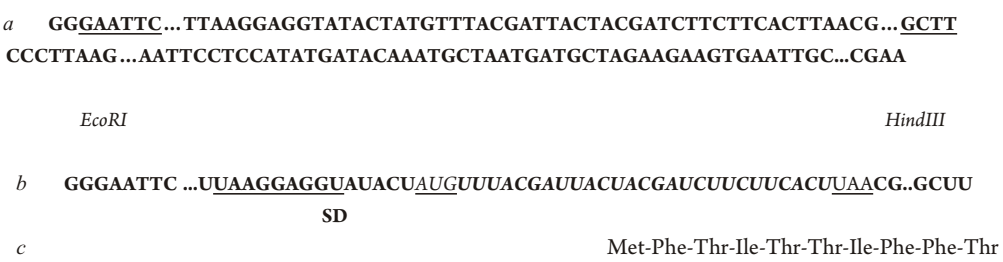

Fig. 1. Model 002 gene integrated in the plasmid pTZ18. (*a*) Double stranded DNA: the restriction sites for *EcoRI* and *HindIII* are underlined. (*b*) mRNA transcript: the SD sequence, the initiation codon AUG and the stop codon UAA are underlined; the «reading frame» is in italics. (c) Encoded peptide constituted by 10 residues. The dots, in (*a*) and (*b*), replace a number of upstream and downstream nucleotides not shown

was centrifuged at 10,000 rev./min for 10 min and, in turn, the supernatant was mixed with an equal volume of 20 mM potassium-phosphate at pH 7.7 holding 1 mM Na_2EDTA, 6 mM ME, and 5 % glycerol. The preparation was then percolated through a cation exchanger SP-Sepharose Fast Flow column equilibrated with 20 mM potassium-phosphate buffer at pH 7.7 (in which 10 mM NaCl, 1 mM Na_2EDTA, 6 mM ME and 5 % glycerol were diluted). Elution of RNApol was obtained using 20 mM potassium-phosphate buffer at pH 7.7 (including 200 mM NaCl, 1 mM Na_2EDTA, 6 mM ME, and 5 % glycerol), while its yield was monitored by measuring the OD at 280 nm in the collected fractions. Finally, after overnight dialysis against 200 ml of 20 mM potassium-phosphate buffer at pH 7.7 (mixed with 10 mM NaCl, 1 mM Na_2EDTA, 6 mM ME, and 5 % glycerol), the homogeneity of the enzyme protein was checked on a 7.5 % sodium dodecylsulphate-acrylamide gel.

Transcription and purification of 002 mRNA. Through a minor modification of the procedure proposed by [7], the transcript was made on a plasmid DNA which carried the T7 promoter (Fig. 1). The *in vitro* reaction lasted 180 min at 37 °C in 1-ml volume of 40 mM Tris-HCl at pH 8.1. In addition to 40 mg/ml plasmid DNA and 47 mg/ml RNApol [14], this buffer contained: 22 mM $MgCl_2$, 5 mM spermidine, 5 mM dithiothreitol, 0.1 mg/ml bovine serum albumine and, at an equimolar 3.75 mM concentration, ATP, GTP, CTP, and UTP. The reaction was stopped with 50 ml of 0.5 M EDTA. After adding 1 ml of 1 M NaCl, the polymerized polyribonucleotide was percolated through an oligo(dT)-cellulose column equilibrated with 20 mM Tris-HCl at pH 7.4 and 1 mM EDTA. Fractions of 1 ml were collected and tested for OD at 260 nm, while the polymer, dissolved in them, was precipitated at –20 °C in the presence of 3 M sodium acetate (1/10 of the volume) plus 100 % ethanol (2.5 volumes). The solution was centrifuged at 10,000 rev./min for 30 min while, after adding 70 % ethanol, the yielded mRNA was recentrifuged at 10,000 rev./min for 30 min. Following ethanol evaporation, it was dissolved in water, treated with 0.1 % diethyl-pyrocarbonate, frozen at –20 °C and checked for its purification degree on a 10 % polyacrylamide gel with 7 M urea.

Binding of [^{32}P]oct to 16S rRNA. In agreement with [15], the synthetic 5'-AAGGAGGT-3' oligonucleotide (furnished by *Eugentec* without phosphate group at the 5'-end) was labelled with ^{32}P released by [-^{32}P]ATP (*Amersham*) under the action of T4 kinase (*GibcoBRL*). The reaction mixture had a volume of 10 ml comprising: 70 mM Tris-HCl at pH 7.6, 10 mM $MgCl_2$, 0.1 M KCl, 1 mM ME, 3 ml [-^{32}P]ATP (3,000 Ci/mmol), 10 units of T4 kinase and 200 pmoles unlabelled oligonucleotide. The transfer of ^{32}P developed within 45 min at 37 °C while, after enzyme inactivation which lasted 10 min at 68 °C (followed by freezing at –20 °C), the yield of [^{32}P]oct was quantified through denaturing polyacrylamide gel electrophoresis [15]. The interaction of [^{32}P]oct with 16S rRNA was verified through the sucrose gradient method. Each sample carried 200 pmoles of [^{32}P]oct and 100 pmoles of 30S RSUs or 70S ribosomes or polysomes (when specified, it contained 200 pmoles of mRNA, 100 pmoles of IFs and 100 pmoles of fMet-tRNA or Phe-tRNA). The reactions were performed in 20 mM Tris-HCl at pH 8.1 enriched with 150 mM KCl and 15

mM $MgCl_2$. Once incubated at 37 °C for 10 min and 10 °C for 30 min, the samples were loaded onto 10–30 % sucrose gradients. These were made in 10 mM Tris-HCl at pH 7.7, enriched with 10 mM Mg-acetate and 60 mM NH_4Cl. Centrifugation lasted 2 hrs in the cold at 40,000 rev./min, using the Kontron TST 60.4 rotor. The radioactivity carried by the fractions was measured in the Rackbeta LKB liquid scintillation counter using the Cherenkov radiation channel. Absorbance was determined at 260 nm in the Beckman DU 640 spectrophotometer after adequate dilution with 10 mM Tris-HCl at pH 7.7 to which 10 mM Mg-acetate and 60 mM NH_4Cl were added.

Results and Discussion. It emerged that, in 30S RSUs, the amount of [^{32}P]oct bound to the anti-SD sequence of 16S rRNA gradually increased as a function of its concentration. In fact, the addition of 400 pmoles of [^{32}P]oct caused a 60 % SD/anti-SD hybridization (Fig. 2). In 70S ribosomes, the amount of [^{32}P]oct bound to the anti-SD sequence of 16S rRNA decreased constantly with respect to that observed in 30S RSUs. In this case, the addition of 400 pmoles of [^{32}P]oct caused a 40 % SD/anti-SD hybridization (Fig. 2). Such a difference accounted for a higher availability of the anti-SD sequence of 16S rRNA in 30S RSUs and for its lower availability in 70S ribosomes.

*Competition between [^{32}P]oct and 002 mRNA in hybridizing the anti-SD sequence of 16S rRNA in 30S RSUs***.** The hybridization of [^{32}P]oct with the anti-SD sequence of 16S rRNA was confirmed when in 30S

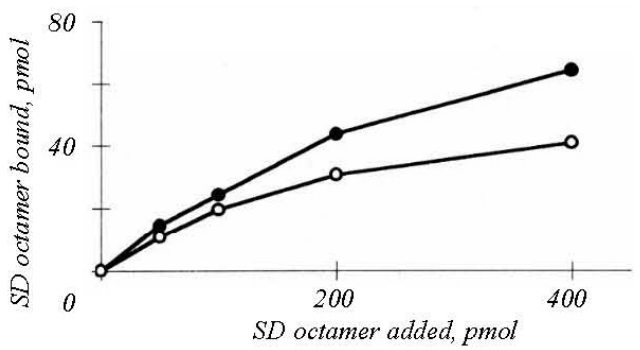

Fig. 2. Kinetics of binding between [^{32}P]oct and 30S RSUs *vs*. that of binding between [^{32}P]oct and 70S ribosomes. One hundred pmoles of 30S RSUs or 70S ribosomes were incubated with 50 to 400 pmoles of [^{32}P]oct. The radioactivity bound to 30S RSUs (filled circles) and 70S ribosomes (empty circles) was detected after their centrifuging through sucrose gradient. The values represent the mean of three experiments

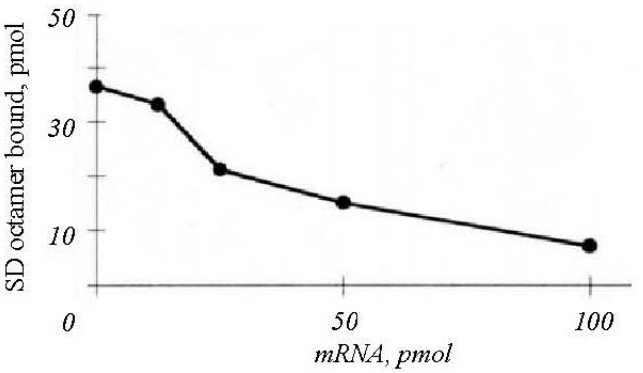

Fig. 3. Competition between [^{32}P]oct and 002 mRNA for binding with the anti-SD sequence of 16S rRNA. Fifty pmoles of 30S RSUs were incubated with 100 pmoles of [^{32}P]oct, in the presence of 0 to 100 pmoles of mRNA. The radioactivity bound to 30S RSUs was detected after centrifuging through sucrose gradient. The experiment was repeated twice

RSUs it competed with mRNA for the same site. A clear demonstration of this was observed when the amount of bound [^{32}P]oct decreased as a function of the mRNA pmoles added to the 30S-RSU samples: the hybridization showed values of about 40 % in the absence of mRNA and values lower than 10 % in the presence of 100 pmoles of mRNA (Fig. 3).

This result was expected, since the first step in the initiation of translation is the building of an aggregate constituted by 30S RSU which carries mRNA and fMet-tRNA.

Full availability of the anti-SD sequence in 30S RSUs. The access to the anti-SD sequence of 16S rRNA was further confirmed through three experiments aimed at verifying the direct level of binding radioactivity originating in [^{32}P]oct. When the 30S-RSU sample was incubated with [^{32}P]oct only, its 11–17th sucrose gradient fractions exhibited an extremely high specific labelling (Fig. 4, *A*). When the 30S-RSU sample was incubated with [^{32}P]oct in the presence of mRNA, its 11–17th sucrose gradient fractions exhibited a lower level of specific labelling due to competition (Fig. 4, *B*). Thirdly, when the 30S-RSU sample was incubated with [^{32}P]oct in the presence of mRNA accompanied by IF_1, IF_2, IF_3, and fMet-tRNA, its 11–17th sucrose gradient fractions exhibited a level of specific labelling which was lower (Fig. 4, *C*) than that observed in Fig. 4, *A*, but higher than that observed in Fig. 4, *B*. Thus, the presence of IFs and fMet-tRNA compensated to a significant extent the effect of competition exerted by

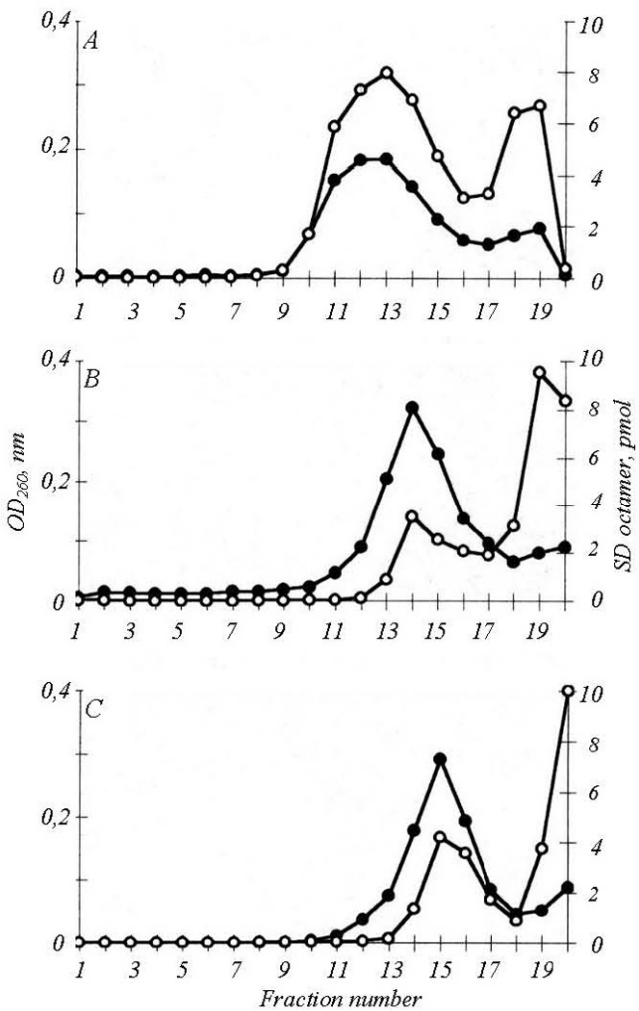

Fig. 4. Interaction of [^{32}P]oct with 16S rRNA and its competition with 002 mRNA in the absence and in the presence of IFs and fMet-tRNA. A 10 30 % sucrose gradient was performed in Nierenberg buffer containing, in 10 mM Tris-HCl at pH 7.7, 10 mM magnesium acetate and 60 mM NH_4Cl at pH 7.7. Centrifuging through this gradient lasted 2 hrs at 40,000 rev./min in the rotor TST 60.4 of the Kontron. (*A*) Incubation of 100 pmoles of 30S RSUs with 200 pmoles of [^{32}P]oct. (*B*) Incubation of 50 pmoles of 30S RSUs with 100 pmoles of [^{32}P]oct and 100 pmoles of unlabelled mRNA. (*C*) Incubation of 50 pmoles of 30S RSUs with 100 pmoles of [^{32}P]oct, 100 pmoles of unlabelled mRNA and 50 pmoles of unlabelled IF_1, IF_2, IF_3, and fMet-tRNA. The fractions were 200 ml each: the first corresponded to the bottom of the centrifuge tube; the last corresponded to its top. Filled circles: OD at 260 nm. Empty circles: amount of the captured [^{32}P]oct. The experiments were repeated three times for (*A*) and two times for (*B*) and (*C*)

mRNA in hybridizing the almost fully available anti-SD sequence of 16S rRNA.

Partial availability of the anti-SD sequence in 70S ribosomes. The access to the anti-SD sequence of 16S rRNA, in the entire 70S ribosomes, was appreciably reduced when compared to the situation characterizing the 30S RSUs. This emerged from a new analysis showing that, if the 70S-ribosomal sample was incubated with [^{32}P]oct only, its 7–15th sucrose gradient fractions carried a modest amount of specific radioactivity (Fig. 5, *A*). Instead, the 7–15th 70S sucrose gradient fractions exhibited a smaller amount of specific radioactivity if, in hybridizing the anti-SD sequence of 16S rRNA, a competition occurred between [^{32}P]oct and mRNA (Fig. 5, *B*). On the other hand, the level of specific radioactivity in the 7–15th 70S sucrose gradient fractions was even lower when the 70S ribosomes were incubated with [^{32}P]oct in the presence of mRNA and fMet-tRNA (Fig. 5, *C*). This level did not change too much when the 70S ribosomes were incubated with [^{32}P]oct in the presence of mRNA accompanied by fMet-tRNA and Phe-tRNA (Fig. 5, *D*). In sum, in all four cases, the availability of the anti-SD sequence of 16S rRNA had to be considered as partial in 70S ribosomes.

Inaccessibility of the anti-SD sequence in polysomes. At variance with the differential specific labelling of 30S RSUs (Fig. 4) and 70S ribosomes (Fig. 5), the 2–9th sucrose gradient fractions, having polysomes, did not exhibit any radioactivity if incubated with [^{32}P]oct (Fig. 6). This demonstrated that the polysomal structure did not expose the anti-SD sequence of 16S rRNA to the labelled octamer.

The assumption that in bacteria the SD/anti-SD hybrid might be maintained not only at the early stages of translation but also during elongation had to be further verified [16]. If we consider that (i) the SD interaction influences the ribosomal frame-shifting [8], (ii) the SD base pairing stabilizes the mRNA/ribosome interaction also during the first round of translation elongation [9], (iii) the hydroxyl-radical footprinting shows that protection of the SD sequence by the ribosome still persists in the «70S/mRNA/fMet-tRNA/Phe-tRNA» elongation complex [9], we may state that this background information was consistently in harmony with the findings provided herewith. For the sake of clarity, the first result showed that the anti-SD sequence of 16S rRNA was highly available in 30S RSUs, since about 65 % of these were able to bind [^{32}P]oct. In this case, the presence of equimolar amounts of 002 mRNA, carrying the SD sequence, led

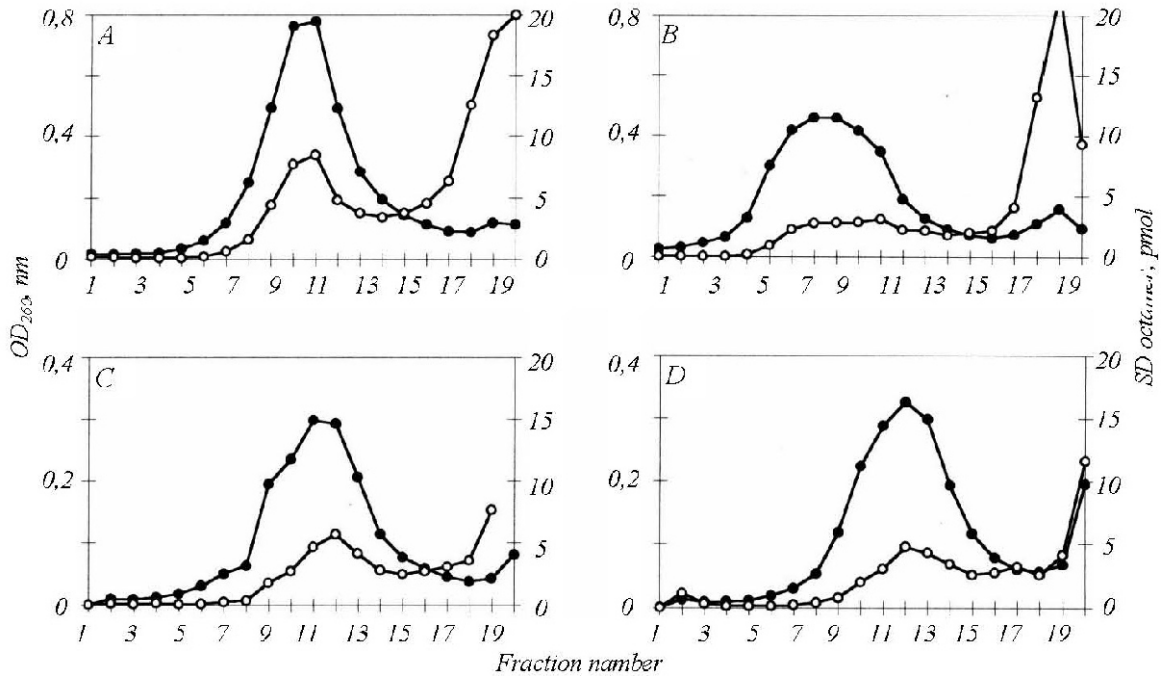

Fig. 5. Interaction of [^{32}P]oct with 70S ribosomes and its competition with 002 mRNA. The analysis through sucrose gradient was performed as for Fig. 4. (*A*) Incubation of 100 pmoles of 70S ribosomes with 200 pmoles of [^{32}P]oct. (*B*) Incubation of 100 pmoles of 70S ribosomes with 200 pmoles of [^{32}P]oct and 200 pmoles of unlabelled mRNA (competition of [^{32}P]oct with mRNA for the binding with the 70S ribosomes). (*C*) Incubation of 100 pmoles of 70S ribosomes with 200 pmoles of [^{32}P]oct, 200 pmoles of unlabelled mRNA and 100 pmoles of unlabelled fMet-tRNA (competition of [^{32}P]oct with mRNA for the binding with the 70S ribosomes in the presence of fMet-tRNA). (*D*) Incubation of 100 pmoles of 70S ribosomes with 200 pmoles of [^{32}P]oct, 200 pmoles of unlabelled mRNA and 100 pmoles of unlabelled fMet-tRNA and Phe-tRNA (competition of [^{32}P]oct with mRNA for the binding with the 70S ribosomes in the presence of fMet-tRNA and Phe-tRNA). Filled circles: OD at 260 nm. Empty circles: amount of the captured [^{32}P]oct. The experiments were repeated three times for (*A*) and two times for (*B*), (*C*), and (*D*)

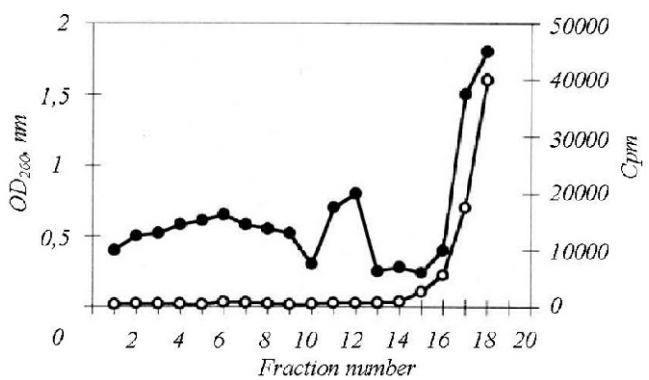

Fig. 6. Interaction between [^{32}P]oct and polysomes. 100 pmoles of polysomes were incubated with 200 pmoles of [^{32}P]oct. The analysis through sucrose gradient was performed as for Fig. 4 and 5. Filled circles: OD at 260 nm. Empty circles: radioactivity of captured [^{32}P]oct. The experiment was repeated twice

to the binding of about 15 % of [^{32}P]oct. The second result showed that in the entire 70S ribosomes, the anti-SD sequence of 16S rRNA was still available, although to a lesser extent, since about 50 % of 70S ribosomes was able to bind [^{32}P]oct. Moreover, the presence of IFs plus fMet-tRNA induced a small increase of bound [^{32}P]oct in 70S ribosomes while the presence of fMet-tRNA plus Phe-tRNA (the first tRNA needed for elongation) induced a slight percentage increase of bound [^{32}P]oct in them. Thus, in these conditions, the SD sequence at the 5'-end of mRNA was not yet detached from the 3'-end of 16S rRNA. The third result concerned the polysomes. In them the anti-SD sequence of all ribosomes was shown to be fully inaccessible. This fact generated two hypotheses: (i) in polysomes, the anti-SD sequence of the first – leader ribosome – would continue to be involved in hybridization with the SD sequence of mRNA; (ii) in polysomes, the anti-SD sequence of the successive ribosomes would be masked by conformational changes abolishing its capability to attract [^{32}P]oct. The latter alternative might have, in the prokaryotic world,

some bearing with the cell-cycle dependent conformational changes of the eukaryotic polysomal structure [17]. Similarly, conformational changes of polysomes in prokaryotes might cause non-availability of the anti-SD sequence carried by 16S rRNA to be recognized by the SD sequence carried by mRNA.

Conclusion. Although with quantitative differences, the access to the anti-SD target of 16S rRNA was shown to be always «open» in both 30S RSUs and 70S ribosomes, while the anti-SD target of 16S rRNA appeared to be completely «closed» in polysomes. On this basis, we assumed that the SD/anti-SD hybrid is maintained from the time of formation of the first peptide bond up to the time of release of the encoded polypeptide. Such an important suggestion deserves attention also in connection with the idea that the same mRNA – with its SD sequence – might regulate accessibility. This does not exclude that any variation of polysomal conformation, correlative with mRNA length [17], would influence the speed of the ribosomal movement along it [18] and, ultimately, the translational rate essentially [17].

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