NEAR NEIGHBORS OF IF3 BOUND TO 30S RIBOSOMAL SUBUNITS

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1. Introduction

The acquisition of an mRNA by the bacterial ribosome apparently involves the hydrogen bond interaction between nucleotide sequences at the 3′end of the 16S ribosomal RNA and complimentary sequences in the mRNA [1–3]. The 30S ribosomal protein S1 [4,5] as well as the initiation factor IF3 [6,7] are required to mediate this interaction. Since S1 has already been shown to be in the immediate neighborhood of the 3′terminus of the 16S RNA [8], we have attempted to determine whether or not IF3 is bound to the ribosome in the same neighborhood.

We show that IF3 bound to the 30S ribosomal subunit can be crosslinked to the 30S ribosomal protein S7, which is one of several proteins associated with the 3′region of the 16S RNA [9,10]. In addition, it is possible to covalently attach IF3 to the 3′terminus of 16S RNA if the 30S-IF3 complex is oxidized with periodate. We conclude that IF3 bound to the 30S ribosomal subunit is in the immediate neighborhood of the 3′region of the 16S RNA and its associated proteins such as S1.

2. Materials and methods

Frozen masses of *Escherichia coli* A19 were thawed and ground with aluminium oxide as described earlier [11]. The lysate was suspended in a buffer containing 10 mM Tris-acetate, pH 7.4, 10 mM magnesium acetate, 60 mM ammonium chloride, 0.1 mM ethylene diamine tetracetic acid, and 1 mM dithiothreotol. After clarification of the suspension by low speed centrifugation, it was centrifuged for 3 h at 40 000 rev/min (60 Ti rotor, Beckman) through 20% glycerol in a buffer similar to that used for extraction except that the magnesium concentration was reduced to 5 mM, and the ammonium chloride concentration was raised to 0.6 M. Ribosomal subunits were prepared from the resulting pellet as described previously [12].

Crosslinking experiments with tartaryl diazide (TDA) were carried out as described in detail earlier [13]. A typical cross linking experiment consisted of adding 50 A_{260} units of 70S ribosomes to 30 μg purified IF3 [20] and exhaustively dialyzing the mixture against a buffer containing 10 mM Hepes, 3 mM magnesium acetate, 50 mM potassium chloride, and 0.1 mM ethylene diamine tetracetic acid, pH 7.4. TDA was then added to a final concentration of 10 mM and the reaction was continued for 30 min at room temperature. The reaction mixture was then layered on a 5–25% sucrose gradient, in the same buffer, and the 30S subunits were recovered for further analysis by the diagonal method described earlier [13].

Oxidation of IF3–30S ribosomal subunit complexes was carried out in buffer containing 10 mM Hepes, 3 mM magnesium acetate, 40 mM sodium chloride, and 0.1 mM ethylene diamine tetracetic acid. The oxidation by 20 mM sodium periodate was performed at pH 6.2 for 45 min at 20°C in the dark. Following oxidation, the samples were dialyzed against two changes (one hour each) of the same buffer which was now adjusted to pH 8.0. Finally, the samples were reduced by the addition of 10 mM sodium borohydride to the buffer, and the mixture was kept for ten minutes on ice.

The purified IF3 preparations were made radioactive by reaction with [C^{14}] formaldehyde. The

labelling procedure found in [14] was followed for these preparations.

3. Results and discussion

Fig.1 shows a typical experiment in which the proteins from 30S subunits that were crosslinked by TDA in the presence and absence of IF3 are compared by the diagonal method [13]. Here, and easily identified pair of crosslinked proteins is S7 and IF3. Several other proteins are crosslinked to IF3; however, an unambiguous identification of their member proteins has not yet been completed.

Further comparison of the crosslinked proteins recovered from the samples with and without IF3 reveals that with the exception of the complexes containing the factor, the patterns are virtually identical. We were unable to detect any effects on the crosslink pattern by the addition of GTP, poly U, MS2 RNA, or IF1. Furthermore, the S7–IF3 neighborhood was a major factor-containing complex produced when either 30S subunits or 70S ribosomes were used. We conclude that the major features of the 30S protein neighborhoods are not dramatically altered by any of the enumerated components, including IF3.

Fig.2 illustrates the results obtained in two kinds of control experiments that were done to determine the site-specificity of the S7–IF3 neighborhood. First, the pattern of crosslinked proteins was analyzed after the 30S proteins had been incubated with IF3 in the presence and absence of low concentrations of lithium chloride. The latter addition was used as suggested by C. Gualerizi (personal communication) to prevent formation of a stable factor–ribosome complex and, indeed, no S7–IF3 complexes could be recovered when it is present in the crosslinking mixture. Nevertheless, the addition of lithium chloride

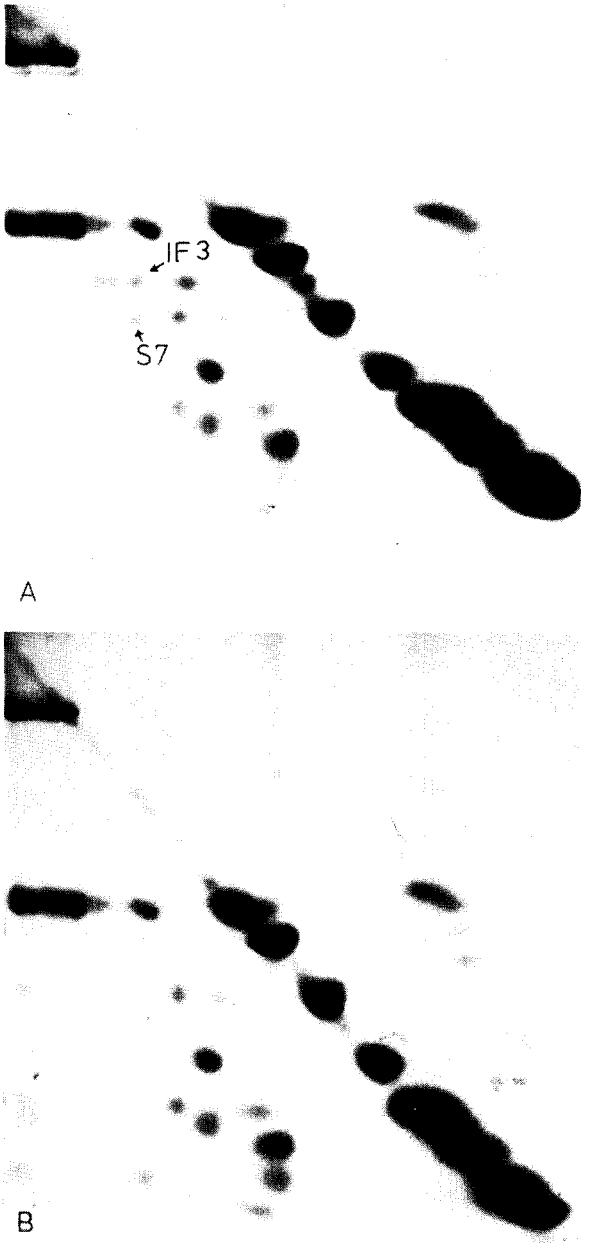

Fig.1. Electrophoretic fractionation of TDA-crosslinked protein extracted from 30S ribosomal subunits incubated in the presence (A) and absence (B) of IF3. Details are found in Materials and methods and [13]. The protein was first electrophoresed in a 15% discontinuous SDS-containing polyacrylamide gel. Afterwards the gel was treated with sodium periodate to cleave the crosslinks between the proteins. Finally, the proteins were fractionated in the second dimension, which was also a 15% discontinuous SDS containing gel. Proteins crosslinked to each other and then released by cleavage are found on the gel below the diagonal formed by the uncrosslinked proteins. Proteins which are released from a single complex are found on the same vertical line, while a protein released from more than one complex is found as a series of spots on a horizontal line.

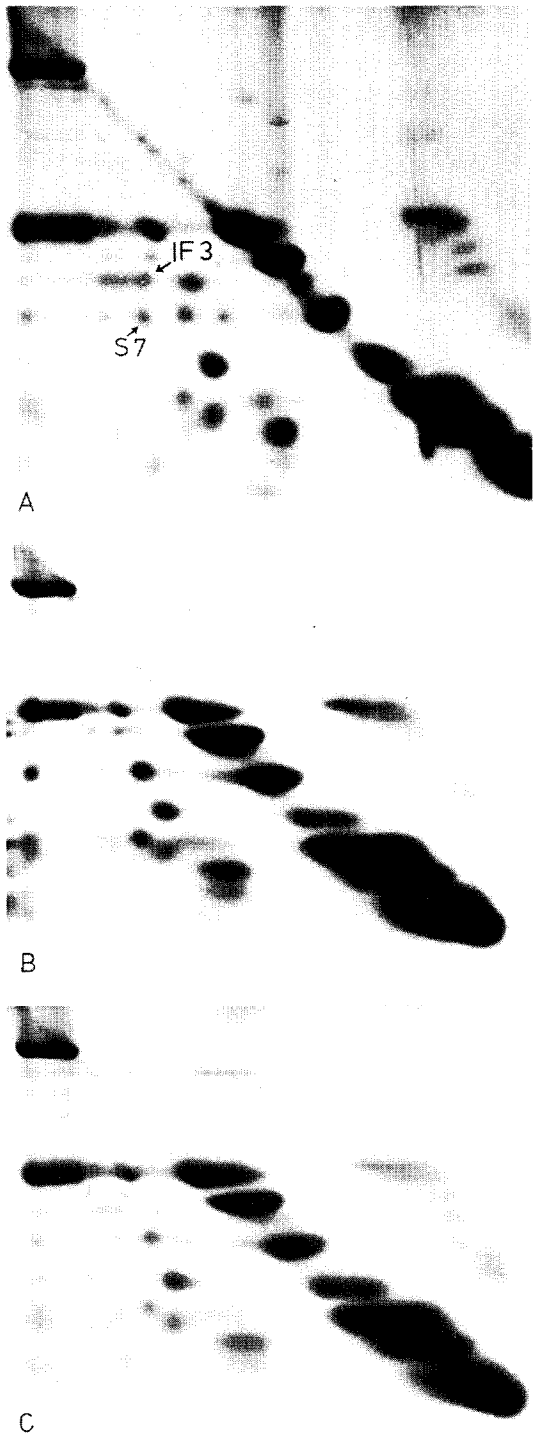

Fig.2. Electrophoretic fractionation of TDA-crosslinked protein extracted from 30S ribosomal subunits incubated with IF3 and crosslinked in the following media: (A) normal incubation buffer (see Materials and methods); (B) buffer containing 0.8 M Li Cl; (C) buffer containing 0.2 mM ATA.

at these concentrations has no effect on the pattern of crosslinking between the 30S proteins (fig.2). Such results indicate that random collisions between IF3 and 30S subunits in the presence of TDA would not produce detectable amounts of the S7–IF3 complex.

A second control consisted of studying the effects of aurintricarboxylic acid (ATA) on the formation of the S7–IF3 crosslink. ATA is a specific inhibitor of initiation and depresses the binding of IF3 to ribosomes (15). The data in fig.2 show that it also dramatically reduces the recovery of the S7–IF3 complex. We estimate that at most 20% as much S7–IF3 complex can be obtained in the presence of ATA as compared to the recovery of this complex in its absence. We conclude that the functional interaction between IF3 and the 30S subunit takes place at a site close to or including S7.

Other authors [16,17] have stressed the association of IF3 with 30 S proteins other than S7, and, most important, have observed a crosslink between IF3 and S12. In contrast, we have consistently failed to observe such a neighborhood. There are many potential sources for this discrepancy, but one is particularly relevant. TDA (6 Å) is much shorter than the reagents used by the other investigators. Since S7 is, by far, the protein most extensively crosslinked to IF3 by TDA, we are inclined to believe that IF3 is positioned very close to it.

S7 is associated with nucleotide sequences belonging to the 3′ region of 16S RNA [9,10]. Therefore, we next performed experiments to determine whether or not IF3 could be crosslinked to the 3′ end of the 16S RNA after oxidation of the ribosomes with periodate which is a specific reagent for the 3′ ribose [8,18,19]. In order to facilitate the analysis of these experiments, the IF3 preparations were labelled radioactively in vitro. The results of a typical series of experiments with [C^{14}] IF3 are shown in fig.3.

A significant fraction of the 16S RNA molecules recovered from 30S subunits that had been oxidized

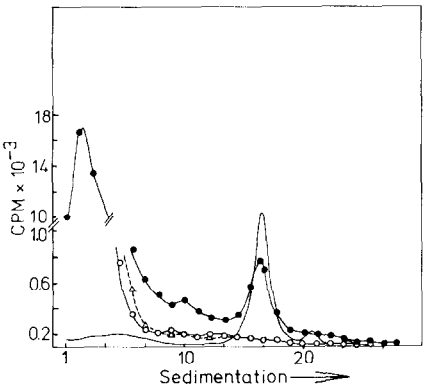

Fig.3. Sucrose gradient fractionation of the RNA extracted from 30S ribosomal subunits incubated with [C^{14}]IF3 and oxidized with periodate under the following conditions: (–•–), standard buffer (see Materials and methods); (–○–), buffer containing 0.2 mM ATA; (–△–), not oxidized, but otherwise treated under standard conditions. The solid line represents the A_{260} of the material recovered from the gradient. The sucrose gradient contained 0.1% SDS, and was centrifuged for 16 h at 35 000 rev/min in the SW41 (Beckmann) at 5°C. The buffer in the gradient contained in addition to SDS: 50 mM sodium acetate, 10 mM EDTA, 0.5 M LiCl, and 3 M urea, pH 5.8.

in the presence of [C^{14}]IF3 are labelled by the factor. If the experiment is performed in the presence of ATA (fig.3) or low concentrations of lithium chloride (data not shown), little or no radioactivity is recovered on the 16S RNA. In addition, oxidation is required for the attachment of IF3 to the RNA (fig.3).

The binding of IF3 to the 30S subunits is stoichiometric [20]. However, oxidation of the subunits with periodate reduces their binding capacity for IF3 by about 50%. Since only one fourth of the [C^{14}]IF3 eventually bound to the subunits can be crosslinked to the oxidized RNA, a usual result of an experiment such as that in fig.3 is to recover approximately 10% of the 16S RNA with an associated IF3 molecule. When the 16S RNA–IF3 complex is treated with nuclease [8], the [C^{14}]IF3 can be recovered by electrophoresis on SDS gels as a single peak containing approx. 90% of the C^{14}-label on the gel.

Our finding that IF3 bound to the 30S subunit can be crosslinked to both S7 and the 3′ end of the 16S RNA does not by itself mean that these components provide the binding sites for the factor. The present results only indicate that IF3, S7, the 3′ end of the 16S RNA and, by implication, both S1 as well as S21 are part of a ribosomal neighborhood which functions in the formation of a preinitiation complex with mRNA.

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