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Nuclease mapping of the secondary structure of the 49-nucleotide 3' terminal cloacin fragment of *Escherichia coli* 16S RNA and its interactions with initiation factor 3

Eric Wickstrom

Department of Chemistry, University of South Florida, Tampa, FL 33620, USA

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ABSTRACT

Escherichia coli translational initiation factor 3 (IF3) may be crosslinked to the 3' end of 16S RNA in 30S ribosomal subunits. In order to determine the sequence to which IF3 may bind in vivo, samples of 5'-^{32}P labelled 3' terminal 49-nucleotide fragment of 16S RNA were incubated 5 min. at 37° in 40 mM Tris-HOAc, pH 7.4, 100 mM NaCl, 1 mM $Mg(OAc)_2$, 1 mM $ZnSO_4$, with or without IF3, then reacted a further 5 min with nuclease S1, RNase T1, or RNase A. Base pairing between the 5' and 3' legs of the fragment occurs in the absence of IF3, but is disrupted by IF3 binding. IF3 appears to protect some residues near the 5' end of the fragment (U1495, A1499, A1500, A1502, and A1503) from nuclease S1, and potentiates S1 attack on others (G1494, G1497, C1501, G1504, G1505, U1506, G1517, G1529, G1530, and C1533). A series of equimolar reactions at increasing dilution imply an association constant range of $1.4\text{-}7.0 \times 10^7$ M^{-1}.

INTRODUCTION

Protein synthesis initiation factor 3 (IF3) of Escherichia coli has been observed to greatly stimulate the binding of natural and synthetic mRNAs to 30S ribosomal subunits (1-3). The amino acid sequence of IF3 (4) and the nucleotide sequence of its gene (5) are known. A variety of mechanisms have been proposed for the function of IF3 in the binding of mRNA to 30S ribosomal subunits. It has been proposed that IF3 may denature mRNA ribosome binding sites to single strands (6), in order to catalyze Shine-Dalgarno basepairing between the ribosome binding site and the 3' end of 16S RNA (7). Such a function has also been proposed for IF3 and ribosomal protein S1 acting in conjunction (8). On the other hand, it is just as reasonable to propose that IF3 alters the 30S subunit conformation so as to favor mRNA:rRNA basepairing, without direct IF3/mRNA interaction (9,10). IF3 has been crosslinked to the 3' terminus of 16S RNA

in 30S subunits (11), and to several 30S subunit proteins (12-15) which, like the 3' terminus (16-18), are located in the cleft of the 30S subunit.

The protein-nucleic acid interactions of IF3 (6,19-22) indicate that IF3 is a strongly binding RNA helix destabilizing protein. The secondary structure in solution of the 3' terminal 49-nucleotide fragment of 16S RNA (Fig. 1) has been studied extensively (23-26) by physical techniques, leading to some disagreement with studies of 16S RNA secondary structure in 30S subunits (27,28) as to the extent of base pairing in the 3' terminal fragment. It therefore seemed likely that a nuclease mapping study of the secondary structure of the 3' terminal 49-nucleotide of 16S RNA, and a determination of its sites of interaction with IF3, would be of value in elucidating the actual function of IF3 in mRNA binding to 30S subunits.

MATERIALS AND METHODS

Preparation of Ribosomes and IF3

Tight couple 70S ribosomes, prepared from freshly grown *E. coli* MRE 600 cells, and washed twice in 1.0 M NH_4Cl (29), were the kind gift of Dr. Albert J. Wahba. IF3 was prepared as previously described (22,30). IF3 concentrations were estimated by the Coomassie blue assay (31), $E^{1\%}_{280} = 2.0$ (30), and the molecular weight of IF3, 20,668 (4).

Preparation of the 3' Terminal Cloacin Fragment of 16S RNA

The 49-nucleotide 3' terminal fragment of 16S RNA, residues 1494-1542 (32), was prepared according to Baan, et al. (33). 3600 A_{260} units of salt-washed tight couple ribosomes were cleaved with cloacin DF13 (34), the kind gift of Dr. Frits K. de Graaf. Final purification was by preparative slab gel electrophoresis on a 12% acrylamide gel (see below, Electrophoresis). RNA bands were detected by ultraviolet light shadowing over a fluorescent chromatography plate. In this system, the fragment travels slightly more slowly than xylene cyanol FF. The fragment was extracted into 0.5 M NH_4OAc, 10 mM EDTA, 0.5% $NaDodSO_4$, then precipitated with three volumes of EtOH, and redissolved in the latter buffer. Absorption spectra indicated a yield of 0.90 A_{260} units. The cloacin DF13 cleavage

leaves the 3' fragment with a 5' OH (33). The 5' OH was ^{32}P-labelled with [γ-^{32}P]ATP (New England Nuclear) using polynucleotide kinase (BRL) as described (35), and purified by preparative slab gel electrophoresis as above, and eluted as above. Labelled oligonucleotide was coprecipitated with carrier tRNA, and stored in 10% EtOH in water at -20°. Concentrations were estimated by assuming $E^{1\%}_{260} = 200$.

Nuclease Reactions

Reactions were carried out in 2.0 µl of 40 mM Tris-HOAc, pH 7.4, 100 mM NaCl, 1.0 mM $Mg(OAc)_2$, 1.0 mM $ZnSO_4$, for 5 min. at 37°, unless otherwise indicated. Typically, 1-2 pmol of 5'-[^{32}P] oligonucleotide, 1-2 x 10^5 dpm, plus 10 µg mixed *E. coli* tRNA (Plenum), were reacted with 20 units of nuclease S1 (BRL), or 0.02 units of ribonuclease T1 (Sankyo), or 10 pg pancreatic ribonuclease A (Pharmacia). When IF3 was included, the protein was preincubated with the RNA for 5 min. at 37° before adding the nuclease. Base hydrolysis reactions were carried out in 2-3 µl of 25 mM Na_2CO_3, pH 9.2, for 5 min. at 90°, with the same amounts of RNA and labeled oligonucleotide as above. All reactions were terminated with one volume of 9 M urea, 10 mM EDTA, 0.05% xylene cyanol FF, 0.05% bromophenol blue.

Electrophoresis

Preparative electrophoresis was done on a 0.15 x 12 x 14 cm slab gel of 12% acrylamide, 0.6% bisacrylamide in 7.0 M urea, 50 mM Tris-H_3BO_3, pH 8.3, 1 mM EDTA, which was run for 1-2 hr at 200 V. Analytical electrophoresis was done on a 0.15 x 33 x 40 cm slab gel of 20% acrylamide, 0.67% bisacrylamide in the above buffer, or on a 0.04 x 33 x 40 gel of 12% acrylamide, 0.6% bisacrylamide in the same buffer. Gels were run at 900 V. Following electrophoresis, gels were autoradiographed with Kodak XRP-5 film backed by DuPont Cronex Lightning-Plus screens at -70° for 1-2 days.

RESULTS

Nuclease Mapping of the 49-Nucleotide Cloacin Fragment

Nuclease mapping of the cloacin fragment with nuclease S1, RNase T1, and RNase A, followed by denaturing gel electrophoresis and autoradiography (Fig. 2), gave a pattern of bands which were

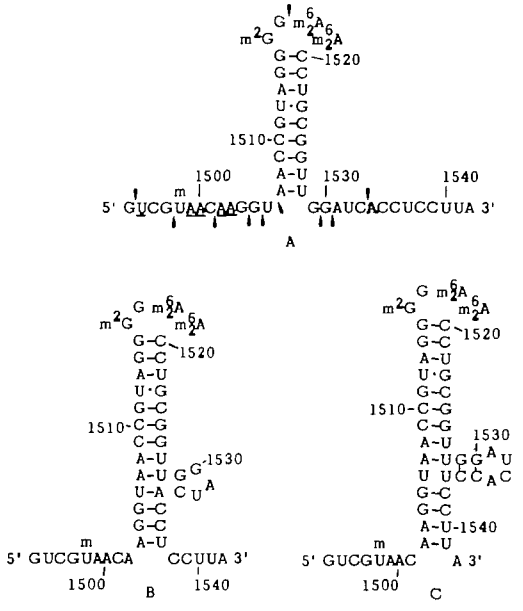


Fig. 1. A,B,C: alternative structures of the 3' terminal 49 nucleotides of 16S RNA, from (25). Nucleotides protected from nuclease S1 by IF3 are underlined in structure A; those attacked more readily by nuclease S1 in the presence of IF3 are indicated by arrows.

consistent with the nucleotide sequence (32) and probable secondary structures (25,26) of the 49-nucleotide fragment (Fig. 1). The blank lane in Fig. 2 showed some fragmentation at C1501, U1506, U1512, and C1533; however, no greater fragmentation was seen in lane 1, where the fragment was incubated with IF3, but without nuclease. In the base hydrolysis OH lane, the least cleavage was seen at m_2^6A1518 and m_2^6A1519, in agreement with their strong stacking (26). Nuclease S1 (lane 2), RNase T1 (lane 7), and RNase A (lane 9) mapping indicated significant accessibility to G1494-G1497, A1499, A1500, and C1501. Moderate accessibility was seen to A1502-G1505, followed by significant accessibility to U1506. A1507-G1511, A1513-G1515, and their presumed complements C1520-U1522 and C1524-U1528 showed hardly any accessibility, while both U1512 and G1523 showed moderate accessibility. G1517 showed significant accessibility, while m^2G1516, m_2^6A1518, and m_2^6A1519 showed none. G1529, G1530, and C1533 showed some

Figure 2a.

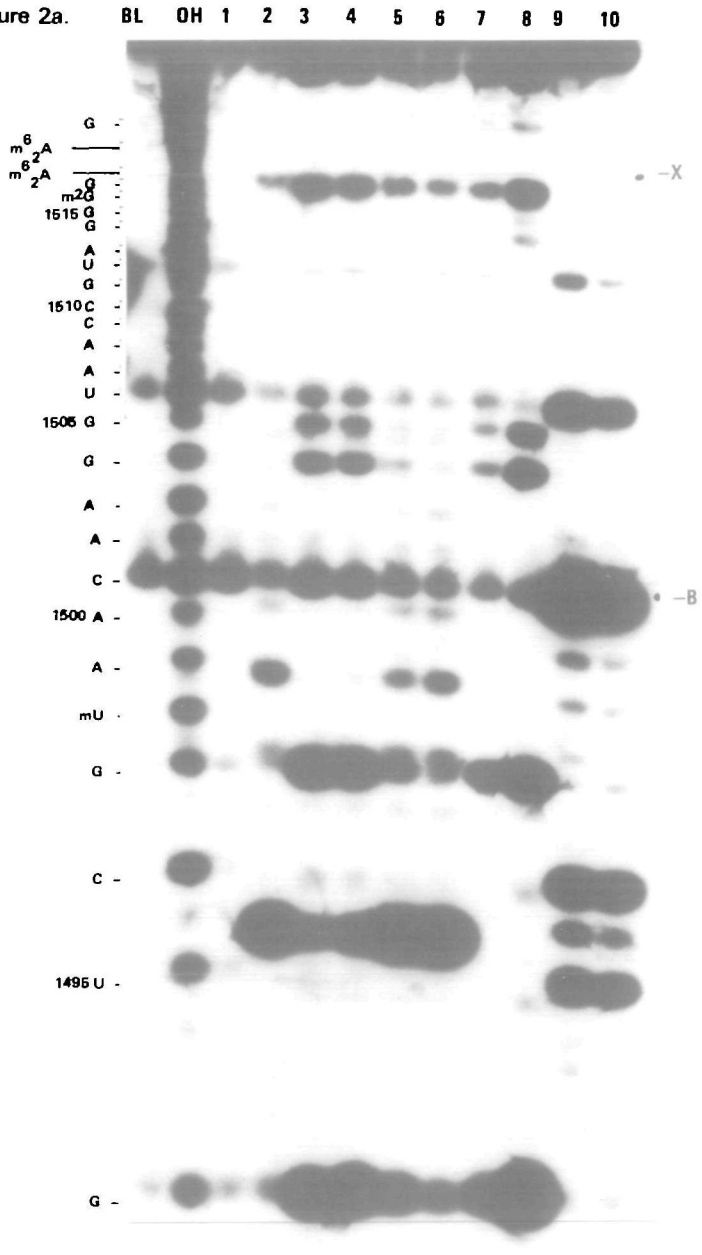
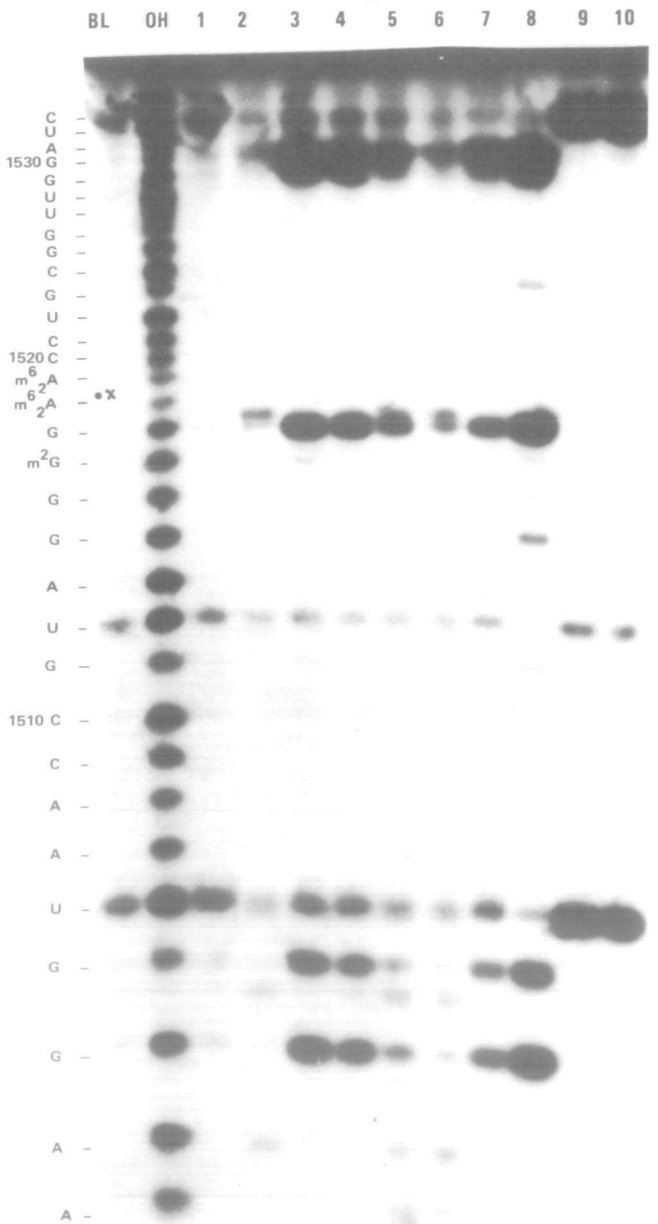


Figure 2b.



accessibility, but A1531 and U1532 showed little. An extended electrophoresis run, intended to maximize resolution at the 3' end (Fig. 3), indicated little nuclease accessibility to A1534-U1537, but easy accessibility to C1538-A1541. Structure B of Fig. 1 seems to fit these data most closely.

IF3 Protection and Dose Dependence

Preincubation of the fragment with IF3 before addition of nuclease S1 strongly potentiated attack at G1494, G1497, C1501, G1504, G1505, U1506, G1517, G1529, G1530, and C1533, but inhibited attack at U1495, A1499, A1500, A1502, and A1503. Both attack and protection were dose dependent, as may be seen in lanes 3-6, where the IF3 concentration declines from over 4 times the fragment concentration to 1/7 of the fragment concentration. Lane 6, with the least IF3, is difficult to distinguish from lane 2, with no IF3. IF3 also potentiates RNase T1 attack at G1494, G1497, G1504, G1505, G1511, G1514, G1515, m^2G1516, G1517, G1523, G1529, and G1530. No discernable effect was seen on C1496 or mU1498 in the region where some IF3 protection was observed.

Equimolar IF3-Oligonucleotide Reactions

In order to make an estimate of the strength of IF3 interaction with the fragment, equimolar mixtures of IF3 and fragment were prepared at increasing dilution, then reacted with nuclease S1 (Fig. 4). Parallel reactions were carried out at the same dilutions, without IF3, to control for concentration effects on S1 attack. The results in lanes 1 and 2, where IF3 and fragment were both 0.7 μM, agreed with those in Fig. 2. Lane 4, where IF3 and fragment were both 0.14 μM, still showed

Fig. 2. Nuclease mapping of IF3 interactions with the cloacin fragment. Reactions and electrophoresis were carried out as described in Materials and Methods. The gel was 20% acrylamide, 1.5 mm thick. Oligonucleotide concentration was 0.7 μM. Lane BL: oligonucleotide incubated in buffer without IF3 or nuclease; lane OH: base hydrolysis ladder; lane 1: incubation with IF3 but without nuclease; lane 2: incubation with nuclease S1; lanes 3-6: preincubation with 3 μM, 1 μM, 0.4 μM, and 0.1 μM IF3, respectively, before nuclease S1 addition; lane 7: ribonuclease T1 hydrolysis; lane 8: preincubation with 3 M IF3 before RNase T1 addition; lane 9: pancreatic RNase A hydrolysis; lane 10: preincubation with 3 M IF3 before RNase A addition. X: xylene cyanol FF; B: bromophenol blue. Part A: second loading; part B: first loading.

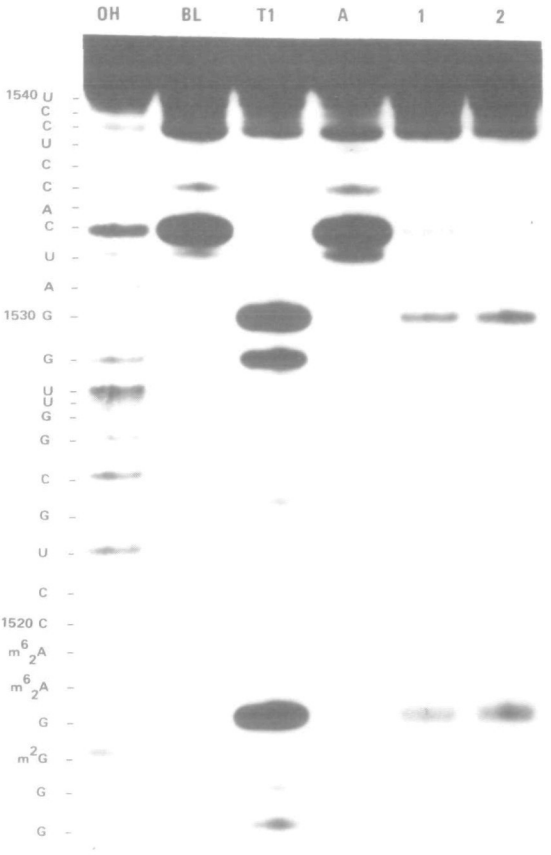


Fig. 3. Nuclease susceptibility of the oligonucleotide 3' end. Oligonucleotide concentration was 1 μM. The gel was 12% acrylamide, 0.4 mm thick. Lane OH: base hydrolysis; lane BL: buffer only; lane T1: RNase T1 hydrolysis; lane A: RNase A hydrolysis; lanes 1,2: nuclease S1 hydrolysis for 5 min. and 10 min.

significant IF3 protection and potentiation, compared with lane 3. However, lane 6, where IF3 and fragment were both 0.028 μM, showed only modest IF3 interactions. Hence, it appeared that the input concentration where IF3 and fragment would be half bound and half dissociated was greater than 0.028 μM, but less than 0.14 μM. In such a case, the concentrations of free IF3, free RNA, and IF3/RNA complex would be equal, and equal to the

dissociation complex. Thus, the dissociation constant falls within the range 14-70 nM, while the association constant has the range 1.4-7.0 x 10^7 M^{-1}.

Salt Dependence of IF3 Interactions

In an effort to assess the salt dependence of the IF3 interactions with the cloacin fragment observed under standard conditions, similar nuclease S1 mapping reactions were carried out in the absence of NaCl, with the usual 0.1 M NaCl, and with 0.2, 0.3, and 0.4 M NaCl (Fig. 5). Parallel reactions without IF3 were done at each salt concentration. For those nucleotides protected by IF3, the degree of protection appeared fairly constant with increasing salt, while the degree of nuclease S1 attack in the absence of IF3 increased with increasing salt. For those nucleotides whose susceptibility to nuclease S1 was potentiated by IF3, potentiation decreased quickly with increasing salt, but so did nuclease S1 attack in the absence of IF3. The presence or absence of IF3 did not seem to make any difference above 0.2 M NaCl, as far as potentiation was concerned.

DISCUSSION

Fig. 1 shows three possible secondary structures for the cloacin fragment (25). Structure A agrees with the kethoxal modification results (27) and nuclear magnetic resonance measurements (23), while structures B and C agree with absorbance temperature jump (24) and melting (25) measurements. The nuclease mapping results presented above agree with structure B, particularly since C1538-A1541 appeared quite exposed, while A1503-U1506 and A1534-U1537 appeared rather protected.

Preincubation of the cloacin fragment with IF3 had a significant effect on its nuclease accessibility. The nuclease mapping results presented in Figs. 2 and 3 suggest IF3 binding to and protection of some residues at the 5' end of the fragment, with widespread destabilization of the rest of the fragment's secondary structure. In the presence of IF3, the fragment appears to adopt structure A. This phenomenon may explain the discrepancies between the kethoxal data (27) and the absorbance data (24,25). The 30S subunits used for kethoxal modification

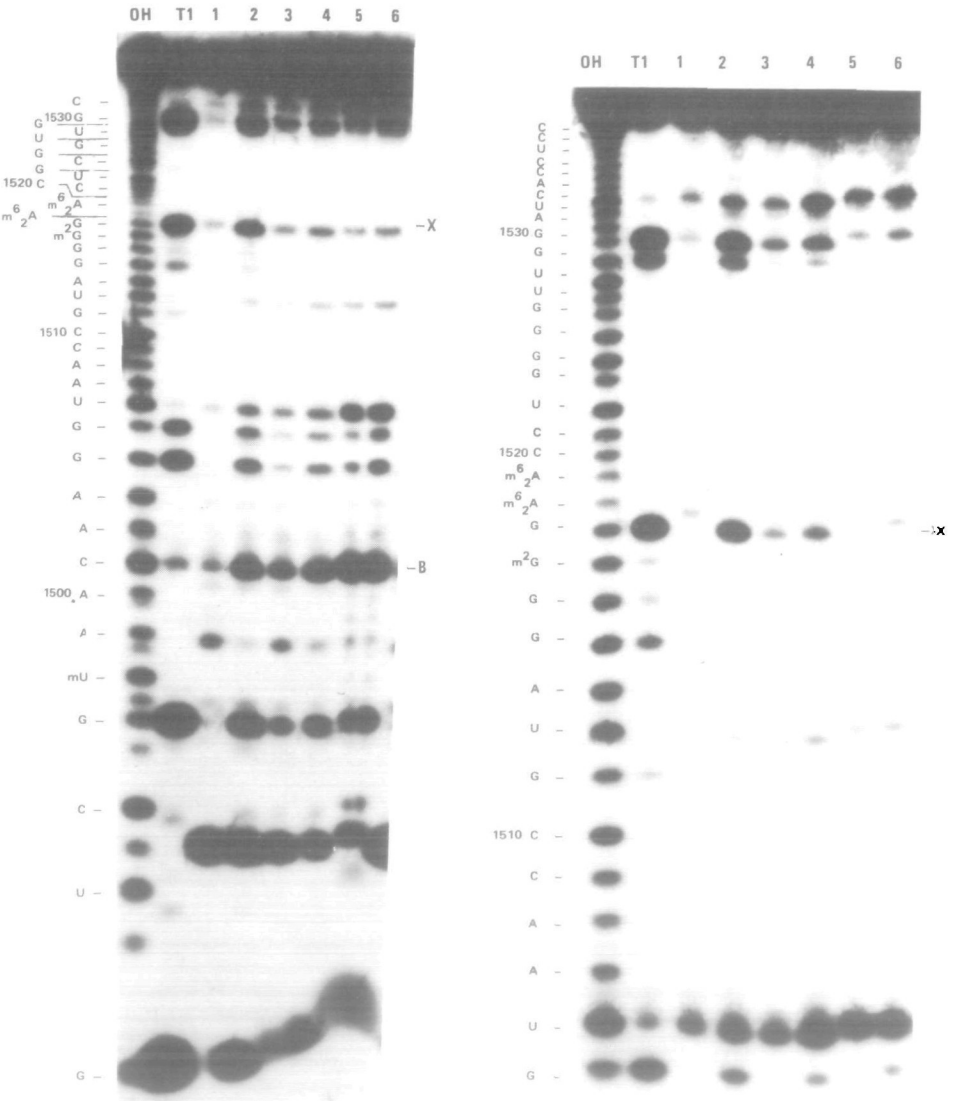


Fig. 4. Equimolar IF3-oligonucleotide reactions. Oligonucleotide concentration was 0.7 μM, except as indicated. The gel was 20% acrylamide, 1.5 mm thick. Lane OH: base hydrolysis ladder; lane T1: RNase T1 hydrolysis; lane 1: nuclease S1 hydrolysis; lane 2: preincubation with 0.7 μM IF3 before nuclease S1 addition; lane 3: as in lane 1, but diluted to 10 μl; lane 4: as in lane 2, but diluted to 10 μl before IF3 addition; lane 5: as in lane 1, but diluted to 50 μl; lane 6: as in lane 2, but diluted to 50 μl

before IF3 addition. Lane 3-6 samples were briefly reduced in volume under vacuum before application to gel. Part A: second loading; part B: first loading.

had not been washed in high salt, and therefore presumably retained their initiation factors, including IF3. Hence, the 3' terminus of 16S RNA in a ribosome with IF3 bound may exist in structure A, although differences in conformation not revealed by the above analysis could exist between the isolated cloacin fragment and the 3' end of intact 16S RNA in a 30S subunit. The nuclear magnetic resonance studies (23) did not reveal the extra base pairs of structures B and C, even though IF3 was not present. This discrepancy is probably due to the fact that the spectra were measured at 25°, near the melting temperature of the extra base pairs (24,25), resulting in the broadening of those particular resonances beyond detectability.

If IF3 indeed binds to the 5' end of the cloacin fragment in an intact 30S subunit, it is quite interesting that ribosomal protein S1 has been reported to bind to the 3' end of the same fragment (36). Since both of these proteins have been crosslinked to the 3' terminus of 16S RNA (11,37), it is helpful to be able to distinguish their precise binding sites. Since structures B and C involve base pairing between the 5' and 3' legs of the cloacin fragment, binding of either IF3 or ribosomal S1 should allow only structure A. However, the two proteins differ in the nature of their binding to the fragment. The association constant range reported here for IF3, 1.4-7.0 x 10^7 M^{-1}, based on the equimolar dilution experiments of Fig. 4, is close to that for IF3 binding to 30S subunits, 5.8 x 10^7 M^{-1}, under similar conditions, but at 25° (38). On the other hand, the association constant for ribosomal S1 binding to the fragment, 5 x 10^6 M^{-1} (24), is 40-fold lower than that for S1 binding to 30S subunits, 2 x 10^8 M^{-1} (39). While S1 binding to the cloacin fragment is only one of its interactions with 30S subunits, and may be dispensed with (40), it appears from the results reported here that IF3 binding to the cloacin fragment may be its predominant interaction with 30S subunits. The existence of some other interactions was indicated in sedimentation studies of IF3 binding to 30S subunits lacking the colicin fragment (41).

The modest reduction in protection by IF3 of certain residues as the ionic strength increased, seen in Fig. 5, implies that the interactions which lead to protection involve significant non-ionic interactions. Hence, IF3 probably interacts directly with the bases of the protected nucleotides. On the other hand, for those nucleotides more readily attacked by nucleases in the presence of IF3, such potentiation decreased with increasing ionic strength. This phenomenon could be due to the stabilizing of secondary structure in the cloacin fragment, probably structure B, as increasing ionic strength reduces phosphate-phosphate repulsion. The potentiation of nuclease attack by IF3 is most likely a long range destabilization of secondary structure, resulting from IF3 binding to the protected nucleotides. As the ionic strength increases above physiological levels, direct binding by IF3 to its site on the fragment may be slightly reduced, but the long range effects seem to be sharply reduced by the increased stability of the fragment's secondary structure. It is even possible that partial double strand formation between fragment molecules may be favored over hairpin loops as the ionic strength increases, as seen previously for synthetic hairpins of the form A_nUGU_m (42).

In the loop of the central hairpin of the cloacin fragment (Fig. 1), both adenosines are dimethylated on N6. In methylation deficient, kasugamycin resistant mutants, the loop adenosines display less stacking, and the hairpin is more stable as a result (25,26). Such mutants also require greater concentrations of IF3 for full initiation activity (43). This observation now may be explained by the destabilization of the hairpin resulting from IF3 binding. The more stable mutant hairpin is more difficult for IF3 to disrupt, so higher IF3 concentration is required for the physiologically necessary degree of destabilization.

The 5' end of the cloacin fragment, where IF3 appears to protect some residues and potentiate nuclease attack on others, has the sequence GUCGmUAACAAG. Interestingly, a similar sequence, GCUGCAACAAG, occurs in the 5' untranslated region of the probable IF3 mRNA sequence at positions 216-226 of the *infC* gene (5). A further similarity is that the sequence GAA occurs just 5' of both the former sequence in 16S RNA (32), and the

Figure 5a.

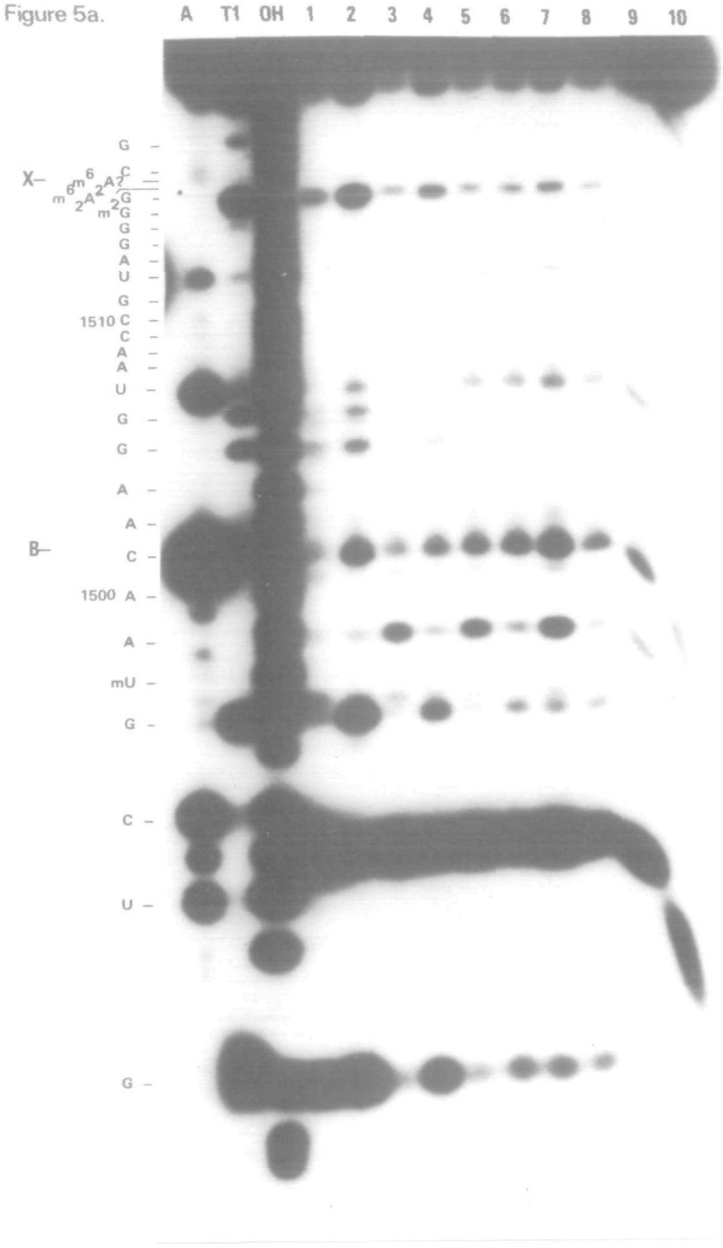
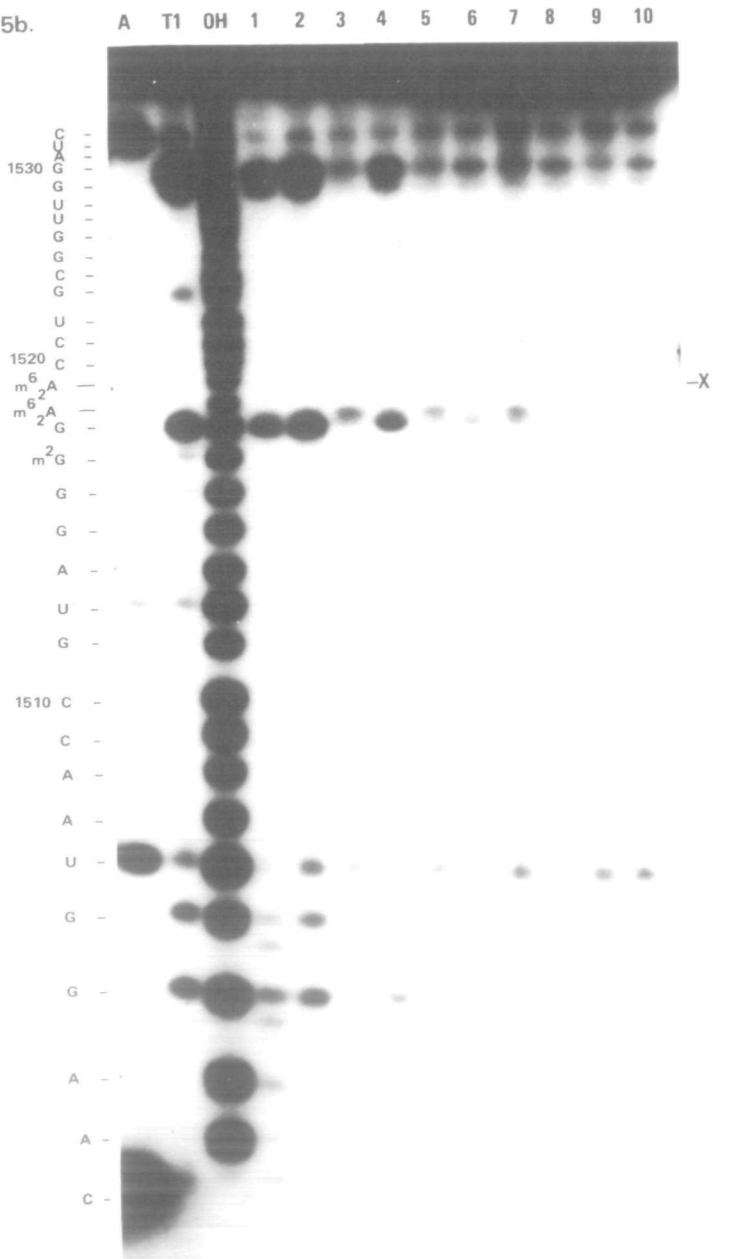


Figure 5b.



latter sequence in the IF3 gene. It may be noteworthy that the similar sequences cover 14 nucleotides, which is the binding site size of IF3 (20-22). In the probable IF3 mRNA, this sequence lies in a region of dyad symmetry, capable of forming a stable hairpin loop, which may have some role in mRNA nuclease resistance or regulation. One might speculate that, in vivo, excess IF3 might bind to and unwind such a sequence, as shown in Fig. 6, although such binding could only have a modest regulatory effect, since IF3 production in overproducing cells is gene dosage dependent (45). The 14 nucleotide sequence from 16S RNA discussed above is almost perfectly conserved in cytoplasmic and mitochondrial small ribosomal subunit RNAs of all organsims studied, including humans (46,47). It is tempting to speculate that a polypeptide homologous in function to IF3 has also been conserved, in one of the eucaryotic initiation factors, in all forms of life.

The oligopurine region found 5-12 nucleotides upstream from the initiation codon in procaryotic mRNAs forms base pairs with the 3' terminus of 16S RNA (7). Examination of structure A in Fig. 1 suggests that, in such a base paired mRNA:rRNA complex, the AUG initiation codon would probably be located just below the central hairpin. Since the data reported above indicate that IF3 binds to the 5' leg of structure A, any interaction which may occur between IF3 and mRNA ribosome binding sites (6) would probably be limited to the initiation codon and the earliest structural codons.

These studies show that IF3 may participate in destabilizing secondary structure in the 3' terminus of 16S RNA in 30S subunits, thus changing the 30S subunit conformation, in accord with some models for IF3 function (9,10). On the other hand,

Fig. 5. NaCl dependence of IF3 interactions. Oligonucleotide concentration was 0.7 μM; IF3 concentration was 0.9 μM, where present. The gel was 20% acrylamide, 1.5 mm thick. Lane A: RNase A hydrolysis; lane T1: RNase T1 hydrolysis; lane OH: base hydrolysis; lane 1: nuclease S1 hydrolysis in buffer lacking NaCl; lane 2: as in lane 1, but preincubated with IF3; lanes 3,4: as in 1,2, but with 0.1 M NaCl (standard buffer); lanes 5,6: as in 1,2, but with 0.2 M NaCl; lanes 7,8: as in 1,2, but with 0.3 M NaCl; lanes 9,10: as in 1,2, but with 0.4 M NaCl. Part A: second loading; part B: first loading.

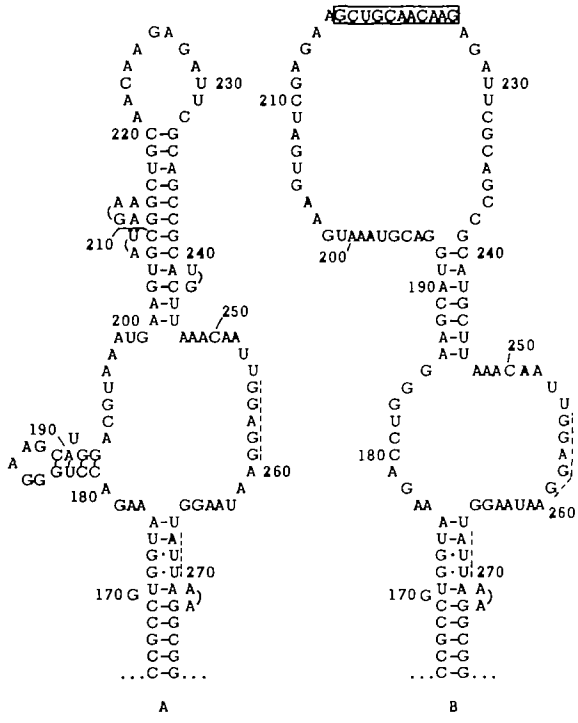


Fig. 6. A,B: alternative structures for the 5' untranslated region of the probable IF3 mRNA deduced from the sequence of the infC gene (5), using the secondary structure prediction program of Zuker and Stiegler (44). A potential IF3 binding site is enclosed by a box in B. The GAA to the left of the box also occurs in that position in 16S RNA (32).

these results do not rule out IF3 (6,8) or ribosomal S1 (8,29) interaction with mRNA, since both of these proteins may have two RNA binding sites (21,48). Hence, it will be of interest to look for any similarly strong interactions of IF3 with the same or other regions of 16S RNA in whole 30S ribosomal subunits, where the 3' or 5' termini have been radiolabeled in situ. It will also be of interest to look for comparable interactions of IF3 with mRNA ribosome binding sites.

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