Effect of Bases Contiguous to AUG on Translation Initiation*

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We report that bases 3′ to AUG have a much smaller effect on the formation of initiation complexes than do bases on the 5′ side. This relative lack of effect was shown to hold under conditions which stringently require initiation proteins for complex formation. The following oligonucleotides formed initiation complexes as efficiently as did AUG: AUGA, AUGG, AUGC, AUGAU, AUGGCU, AUGUCC, AUGUUU, AUGCUC, and AUGAGC. In contrast, a more efficient formation of a 30 S initiation complex occurred with the UAAG versus RAUG or AUGN tetraders. This was reflected in an enhanced rate of complex formation. All complexes formed were stable at 0°C. At 24°C, the complexes exhibited slow first order dissociation kinetics in dilute solution. All AUGN-programmed complexes exhibited similar rates of reversibility under these conditions. The preference for 5′ pyrimidines was observed with a series of pentamers and hexamers of varied base composition. This discrimination of context by the 30 S subunit took place in the presence or absence of initiation factors. Since UAAG does not directly bind to fMet-tRNA, but AUGA does, the data suggest that the anticodon of fMet-tRNA is altered during formation of the initiation complex.

The rate and extent of fMet-dipeptides synthesized with UAUGU3 was higher than with AAUGU3, or with hexamers containing AUG followed by A, U, or G, e.g. AUGAUG, AUGGCU, AUGUUU, and AUGUUA, underscoring the importance of the 5′ uridine in stimulating initiation.

The precise mechanism by which initiation codons signal protein synthesis is not completely understood (1–3). Particularly unclear is the selection mechanism whereby the correct codon is recognized as initiator while similar internal codons are ignored. Also, the same AUG can either signal the start of a protein or direct the insertion of an internal methionine (4).

There is evidence that purine-rich sequences 5′ to AUG are important but not sufficient determinants of initiation specificity (1–12). Since the common denominator in the initiation reaction is the RUG codon, recent efforts have been directed at learning, by biochemical or genetic means (7–12), whether base context around the AUG codon modulates initiation.

To study the effect of base sequences on the initiation reaction in its simplest terms, we have used model oligomers of the type (N)AUG(N), (N)AUG, or AUG(N), (where N is any base) and have compared their ability to bind fMet-tRNA and to direct peptide bond synthesis. Using this approach, we have observed that polymers with pyrimidines on the 5′ side of the initiator are more effective in binding fMet-tRNA to 70S ribosomes than those with purines in this position (12).

More recently, it has been proposed that the initiator tRNA of prokaryotes may be unusual in that four bases of the anticodon loop interact with four bases of the mRNA, thus modulating initiation (13). This hypothesis was suggested to account for a differential binding of fMet-tRNA in a set of Q6 RNA mutants having altered bases 3′ to the initiation codon. The idea has received some experimental support in that tetrancleotides of the short AUGR were reported to bind fMet-tRNA slightly better than the AUGY controls (14).

We report that bases immediately adjacent to the 5′ side of AUG stimulate formation of an initiation complex with 30S particles and favor peptide bond formation with 70S ribosomes, whereas bases 3′ to AUG have a much smaller effect in these reactions.

MATERIALS AND METHODS

Materials

Oligonucleotides were purchased from Resolute Lake Limited, Edmonton, Alberta, Canada; methyl(phenyloxazolyl)benzene and 2,5-dimethyl-4-hydroxybenzyl chloride from Aldrich Chemical Company, Milwaukee, WI; uridine nucleosides from CalBiochem, La Jolla, CA; and [3H]methionine (50–100 Ci/mmol) were purchased from The New England Nuclear Corp., Boston, MA. All cells were purchased from the Microbiological Associates, Bethesda, MD.

Chemical Synthesis and Characterization of Oligonucleotides

The oligonucleotides (tetramers and hexamers) used in this work were prepared by the general phosphotriester synthetic method (15–17). Preparative data, purification procedure and characterization by RIA incremental analysis (18.30) of tetramers and hexamers used in this work will be published elsewhere. Data for other oligonucleotides is included in a previous publication (19).

Initiation Complex Formation

Preparation of Ribosomes. Seventy-five ribosomes were isolated from Escherichia coli, Ifd or from midlog M900 cells as described (19,20). Cells were washed 3 times in 0.1 M Tris-Cl, pH 7.4, 0.1 M KCl, 50 mM MgCl2, 0.5 mM EDTA, and 0.05 M sucrose. The lysates were centrifuged at 35,000 × g for 1 hr. The supernatant was added to 10 ml of 30,000 × g ribosomes and 10 ml of 30,000 × g ribosomes. After the first centrifugation, the pellets were resuspended in 0.05 M Tris-Cl, pH 7.4, 0.1 M KCl, 0.1 M MgCl2 and 0.05 M EDTA and then centrifuged again at 30,000 × g. The ribosomal pellets were added to 10 ml of 100 µM methionine and stored at 0°C. The ribosomes in M900 cells were prepared as above, with the following modifications. After the first centrifugation, the pellets were resuspended in 0.1 M KCl, pH 7.4, 0.1 M KCl, 0.1 M MgCl2, and 0.05 M EDTA and then centrifuged again at 30,000 × g. The ribosomal pellets were added to 10 ml of 100 µM methionine and stored at 0°C. The ribosomal pellets were resuspended in 0.1 M KCl, pH 7.4, 0.1 M KCl, 0.1 M MgCl2, and 0.05 M EDTA and then centrifuged again at 30,000 × g.

The abbreviations used are: AUG, trinucleotide diophosphate ApUpG; fMet-tRNA, N-formylmethionyl-tRNA; IP, initiation factor; EF, elongation factor; DTT, dithiothreitol; POP, POPPO, 1,4-bis[2-phenyloxazolyl]benzene. Abbreviations for translation factors follow Caskey et al. (60). Other abbreviations follow recommendations of IUPAC-IUB Commission (51).
Effect of Bases 5' and 3' to AUG on Translation Initiation

The primary sequence near the start point clearly is also important. Several lines of evidence indicate that the Shine-Dalgarno region pairs with 16 S rRNA, thus defining part of the ribosome binding site during initiation and presumably narrowing the search for the correct signal (5-7, 37). However, this is not an inevitable requirement for initiation, as the λ ci short transcript contains no bases on the 5' side of the starter AUG codon and is translated in vivo, albeit with low efficiency (10).

Furthermore, at least two other Escherichia coli mRNA restart regions contain no bases that can pair the 18 S rRNA, and these are also translated in vivo (38). Likewise, in vitro initiation can proceed in the absence of precistronic sequences (39). The Shine-Dalgarno region is also not sufficient to define an initiation signal, as a number of internal methionine and valine codons follow sequences indistinguishable from functional Shine-Dalgarno regions. Some of these sites may, however, be legitimate initiators of undiscovered gene products (1, 4). Nevertheless, the number of such locations within well defined cistrons suggests that most of them cannot be functional start signals.

Are there other primary structural identifiers of correct initiation signals? A rather large and statistically significant proportion of known precistronic regions contain non-codons within a short distance of the initiation codon (1, 3, 8). Their significance remains to be determined, although it is tempting to suggest that they may indicate initiation sites. In vitro formation of an initiation complex with 70 S ribosomes is more efficient with YAG as message rather than RAUG (12). Tentative evidence suggests that 3' bases may also affect initiation. Methionine, alanine, and serine are heavily favored over other amino acids at the NH2 termini of E. coli proteins (41). Taniguchi and Weissmann (13) observed that ternary complexes formed with Q8 mRNA carrying an initiation site mutated from AUG to AUGG bound more tightly to ribosomes. Schmitt et al. (14) reported that, in the presence of IF-2, AUGG bound more tightly to ribosomes.

It is known that the initiation rRNA is unusual in that the bases 3' to the anticodon are not modified and hence are potentially available to base pair with base 3' to the mRNA. Also, two bases 3' to the anticodon could potentially discriminate down from internal RUG codons.

Synthetic oligoribonucleotides of defined sequence up to about 12 residues allow us to study the effect of primary structure on initiation systematically and independently of higher order structure. In previous studies, we observed that tetrans of composition YAG formed initiation complexes 5-fold more efficiently than those programmed by RAUG. Similar kinetic and dose-response curves were observed with 70 S ribosomes programmed with the pentamer AUAGG, which is complementary to the CAUAU anticodon of fMet-tRNA and UUAUG. Hexamers of different sequence varied in their ability to form initiation complexes, but in general, pyrimidine in the next position favored formation of initiation complexes. The specificity of this interaction assessed with hexanucleotides was not affected by the initiation proteins IF-1 and IF-2.

The following compounds formed initiation complexes with 70 S ribosomes approximately 2-fold less efficiently than the AUG reference: AUGUAA, AUGUUA, AUGUUG, AUG(A)n, AUG(A1)n, and AUG(U1)n, where n = 12 (18).

Since it is generally accepted that the initiation reaction of translation is the business of the 30 S particle, it seems necessary to learn if 30 S ribosomes maintain the specificity of formation of the initiation complex (34-36).
for purines and pyrimidines 5' to AUG and whether bases 3' to AUG modulate initiation.

In the present study, reactions were therefore carefully optimized such that 90–96% of the 30 S particles were active both in formation of the initiation complex as well as in translation of the MS2 coat protein. We find that this condition is met if subunits are prepared by the method of Kirillov et al. (19) as modified (see "Materials and Methods"), and if sufficient IF-2 is included in the reactions (data not shown). The reconstruction of initiation efficiency depended principally on addition of purified IF-2.

Since Mg" can substitute for the initiation proteins (42, 43), reactions were studied between 2 and 4 mM Mg centroid IF-1 and IF-2 stimulate complex formation 20- and 10-fold, respectively. At 15 mM Mg" centroid, where the relative efficiency of AUGA and AUGU were previously compared (14), the initiation factors stimulated the reaction 20 to 30% (data not shown).

Under conditions which strictly require the initiation proteins, 30 S ribosomes bind fMet-tRNA more efficiently in response to UAUG than to AUG (Fig. 1A). A slightly lower level of binding was also consistently found with CAUG compared to UAUG (Fig. 1A). In contrast, tetranucleotides of the type AUGN were similar in their ability to complex fMet-tRNA to 30 S subunits (Fig. 1B). Fig. 2A shows that there is an approximately 2-fold difference in the rate of the initiation complex formed with UAUG relative to AUG. In contrast, no consistent differences in the rates of complex formation could be observed with any of the AUGN tetramers (see Fig. 2, B and C). Varying the components of the ternary complex or carrying out the experiments at 2.5 mM Mg") did not alter these results. UAUG remained preferred over AUG, GAUG, or CAUG, and no consistent differences in binding efficiency could be observed among any of the AUGN compounds tested.

The association constant \( K_a \) for most ribosome-polymer complexes is in the range of \( 10^7 \) (M") (37). With a 500-fold excess of oligonucleotide to 30 S ribosomes, the \( K_a \) for fMet-tRNA is in the order of \( 2 \times 10^7 \) (M") for AUG and UAUG, and about \( 5 \times 10^6 \) (M") for AUG or AUGN. These data further suggest that the affinity of the 30 S-UAUG complex is preferentially enhanced by fMet-tRNA.

All complexes were stable at 0 and at 24 °C. Decay of 30 S complexes after a 100-fold dilution at 24 °C approximated slow first order kinetics for 5 min, followed by a complex approach to equilibrium. Interestingly, a slightly more stable complex formed with the tetramer UAUG than with AUG or other tetranucleotides tested (Fig. 3). Control experiments indicated that the inability to observe differences in stability or rates of formation complex formation was not the result of degradation of the tetranucleotides or of the fMet-tRNA.

Furthermore, NMR incremental analysis was used to check the purity and sequence integrity of each oligoribonucleotide. This is superior to classical enzymatic digestion analysis which cannot identify partial internucleotidic diphosphate isomerization or other trace impurities in the compounds (32).

NMR incremental analysis was also examined with 70 S ribosomes in the presence and absence of initiation factors. In the absence of initiation factors, the reference codon AUG induced about 2-fold more binding than any of the AUGN tetramers tested (see Ref. 12). Initiation factors equalized the binding of AUG relative to AUGN, while UAUG remained slightly superior to AUG. This result agrees with recent reports by Schmitt et al. (14) and Eckhardt and Lührmann (44).

After completion of this work, Eckhardt and Lührmann (44) also observed that UAUG codes more efficiently for fMet-

\* M. C. Ganoza, unpublished observations.

![Fig. 1. Extent of initiation complex formation with 30 S subunits, UAUG, AUG, or AUGN. Initiation complex formation was carried out as described under "Materials and Methods" in 0.025-m1 incubations, except that 2.3 pmol of 30 S subunits, 11.8 pmol of fMet-tRNA, 3.3 μg of IF-1, 5.7 μg of IF-2, and the indicated levels of tetramers were used. The final MgCl₂ concentration was 43 mM.](image1)

![Fig. 2. Time course of initiation complex formation with UAUG, AUG, or AUGN. Reaction mixtures (0.175 ml) were as described under "Materials and Methods" and in Fig. 1 and contained 580 pmol of 30 S subunits, 700 pmol of tetratmer, or no tetratmer (C—Δ), 23.4 μg of IF-1, 18.9 μg of IF-2, and 18.9 pmol of N-acetyl[2H₅]Met-tRNA. 25-μl aliquots were withdrawn at the indicated time intervals prior to measuring formation of the initiation complex.](image2)
to AUG were examined for their ability to bind fMet-tRNA. Fig. 4 shows, for example, that complex formation with fMet-tRNA and 30 S ribosomes is identical if the oligonucleotides are extended at the 3’ side. Thus, AUGAUG, AUGUUA, and AUGUUU bind fMet-tRNA with about the same efficiency as the AUG codon.

Both 30 S and 70 S ribosomes responded similarly to the various oligonucleotides, and no significant differences were observed when the rates were measured under a variety of conditions in which the components of the ternary complex were varied.

Binding studies alone do not adequately characterize the effect of signal context on the overall process of initiation. There are several reasons for this. First, such experiments provide no information about the phase of the oligonucleotide bound to the ribosome. For instance, AUG may bind with AUG in the entry site, and AUGU with UGU in the entry site, under otherwise identical conditions. Second, aminoacyl-tRNA insertion is not equally proficient for all aminoacyl-tRNAs (45–47). Thus, if fMet-tRNA bound weakly due to context, the reaction could nevertheless be stabilized by a strongly and rapidly bound aminoacyl-tRNA. Likewise, tightly interacting fMet-tRNA may score poorly in overall initiation if the second aminoacyl-tRNA were inefficiently bound. Thus, binding information must be supplemented with data such as those from dipeptide synthesis.

To avoid these problems, conditions were chosen such that dipeptide synthesis was linearly dependent on the hexanucleotide concentration. Of particular interest is the finding that UAUGUUU promotes the rate of fMet-Phe-tRNA synthesis 2-fold better than AUGUUU or AUGUU. The same results were observed when the reactions were measured at 5, 10, 15, 20, or 30 min. On the other hand, we find that AUGUUU and AUGUUCU (both coding for Met-Ser) trigger AUGAUG and AUGUUCU stimulate essentially the same rate and extent of dipeptide synthesis (data not shown).

We find no evidence that fMet-tRNA recognizes a 3’-extended four-base codon, as originally suggested by Taniguchi and Weisssmann (13). This model assumes 5’-stacking of the anti- codon loop, which is the hypothesized configuration during tRNA binding to the ribosome-mRNA complex (48). However, the anti- codon loop is 3’-stacked in crystalline tRNA and is thought to assume this form after binding of the tRNA (25, 48). Therefore, it could be equally well argued that fMet-tRNA could recognize a 5’-extended codon. The 3’-neighboring base of the fMet-tRNA anti- codon is A. A prediction of this model is that UAUG should stimulate initiator tRNA binding better than any other NAUG. We indeed consistently find such a difference between UAUG and NAUG (Fig. 1 and Ref. 12). We suggest that fMet-tRNA can recognize the extended codon UAUG better than it can AUGA. This implies a ribosomal effect, as UAUG cannot interact directly with fMet-tRNA while AUGUUCU (49). In light of this data, we suggest that the coding properties of fMet-tRNA are altered during formation of the initiation complex.

Although sequences 3’ to the start codon may, in certain cases, influence initiation in native mRNA (e.g. Ref. 13), these may well result from alterations in the secondary structure and tertiary folding of the molecule.

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REFERENCES


Fig. 3. Stability of initiation complexes formed with 30 S subunits, UAUG or AUGN, and fMet-tRNA. Reaction mixtures were carried out as described in Fig. 1 and under “Materials and Methods.” Incubations (0.075 ml) contained 7.2 nmoles of N-acetyl[3H]Met-tRNA, 10 µg of IF-1, 5 µg of IF-2, 249 pmol of 30 S subunits, and 300 pmol of each tetranucleotide. After a 15-min incubation at 24°C, each 0.075-ml incubation was diluted 10-fold with a buffer containing 0.05 M Tris, pH 7.8, 0.005 M NH4Cl, 0.001 M GTP, 0.015 M Dithiotreitol, 4.3 mm MgCl2, and 3 mM of the corresponding tetranucleotide. Reactions were then incubated at 24°C, and 0.1-ml aliquots were withdrawn at the indicated times. 100% activity refers to the binding observed immediately after dilution of each initiation complex. AUG was identical in stability to AUGN.

Fig. 4. AUGN2N2N stimulation of [f3H]Met-tRNA binding to 30 S ribosomes. Initiation complex formation was measured for 15 min at 24°C using the indicated concentrations of AUG or each hexamer. Preparation and activation of 30 S subunits and of [f3S]Met-tRNA, purification of initiation factors, and assay procedures are described under “Materials and Methods.” Each reaction mixture (final volume, 0.06 ml) contained 100 pmol of activated 30 S subunits, 93 pmol (158,700 dpm) of [f3S]Met-tRNA, 1.8 µg of IF-2, 0.68 µg of IF-1, and 8.5 mM MgCl2 buffer. The final concentration of MgCl2 was 11.1 mM. A and B are separate experiments. Increasing the level of IF-2 increased the fraction of active ribosomes without altering their coding pattern or their kinetic behaviour.
Effect of Bases 5’ and 3’ to AUG on Translation Initiation

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