THE 3′-TERMINAL HEXANUCLEOTIDE, CAACCA, IS NOT ESSENTIAL FOR FRAGMENT RECOMBINATION OR CROSS-LINKING IN *E. COLI* $tRNA^{fMet}$

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1. Introduction

We have recently shown that the light-induced cross-linking of 4-thiouracil at position 8 and cytosine at position 13 [1, 2] in *E. coli* $tRNA^{Val}$ [3] and $tRNA^{fMet}$ [4] is completely dependent on the presence of parts of the tRNA molecule which are not closely connected in primary sequence to the two residues involved in this reaction. Thus, no cross-linking took place after irradiation of the 5′-half molecule (residues 1–34) of $tRNA^{Val}$ unless the complementary 3′-half molecule was added and annealed to the 5′-half by preliminary incubation treatment. Similarly, the 5′-1/4 molecule (residues 1–20) of $tRNA^{fMet}$ (fig. 1) was not able to become cross-linked unless the complementary 3′-fragment (residues 21–77) was added. These studies, and those of others [6, 7], show that the correct juxtaposition in space of the 4-thiouracil and cytosine residues that will allow this photochemical reaction to take place [8] requires the participation of non-adjacent regions of the tRNA molecule. Consequently, this reaction can be used as a probe for those sequences which determine the proper folding of tRNAs.

Initially, we chose to test the ability of the fragment (21–71) from $tRNA^{fMet}$ to substitute for (21–77) in the cross-linking assay because of an earlier report [9] that the fragment (21–71) in which the 3′-terminal hexanucleotide had been removed was unable to compete with the intact fragment (21–77) for complex formation with the complementary fragment (1–20). We wished to determine whether this hexanucleotide was also essential for induction of cross-linking. In this report, we present evidence that removal of the 3′-terminal CAACCA has no effect on the induction of cross-linking, demonstrating that neither the 3′ single-stranded region nor the first base pair are involved in this aspect of tRNA tertiary structure. In addition, we have found by competition studies that (21–71) makes as strong a complex with (1–20) as does (21–77). Consequently, it is not necessary, at least in this tRNA, to assign any major structural role to these six nucleotides.

2. Experimental

Fragments (1–20), (21–77) and (21–71) were prepared by partial digestion of *E. coli* $tRNA^{fMet}$ (purified as in [4], specific activity 1244 pmoles of methionine bound per A_{260} unit) with T_1-RNase followed by chromatographic purification [10]. Single symmetrical peaks were obtained after a second DEAE-cellulose-urea column at pH 7.6 [9]. The fragments were desalted and stored at −170°. The purified fragments were identified by their column elution positions [9], by the ratio $A_{337}:A_{260}$ which is a measure of the chain length of the 4-thiouracil-containing oligonucleotide, and by the ability of (1–20) to be complemented by (21–77) but not by (21–71) for reconstitution of methionine acceptor activity. Titration of purified (1–20) with (21–77) gave maximal acceptance (87% of intact $tRNA^{fMet}$) when the ratio between the 5′- and 3′-fragments in terms of A_{260} was 1:2.75. The theoretical ratio based on the respective chain lengths is 1:2.86.

Irradiation of fragments was done as before [4] in 20 mM Hepes buffer, pH 8.0, 2 mM magnesium acetate and 2 mM KCl after a 20 min prior incubation at 37°. The concentration of (1–20) was 0.66 A_{260} unit

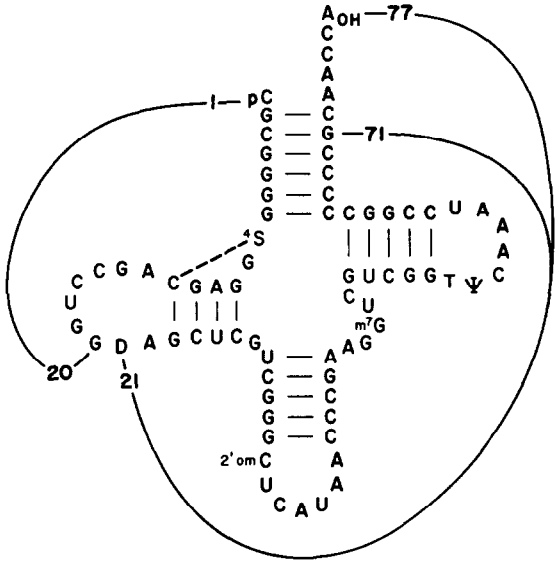

Fig. 1. Cloverleaf model of $tRNA^{fMet}$ from the known primary sequence [5]. The fragments (1–20), (21–71) and (21–77) are as indicated. The dashed line connects the two nucleotides (C, cytosine; S, 4-thiouracil) involved in the cross-linking reaction [1, 2].

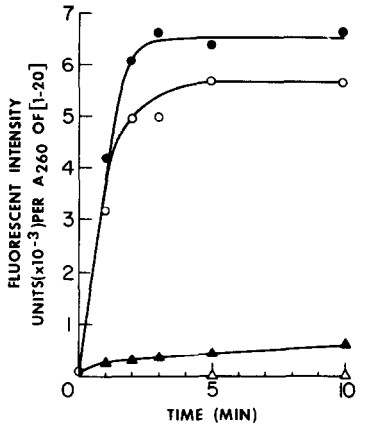

Fig. 2. Kinetics of cross-link formation. 0.86 A_{260} unit of (1–20) alone, combined with 2.47 A_{260} units of (21–77), or with 2.45 A_{260} units of (21–71) were irradiated at 2–4° in a final vol of 1.3 ml as described in Experimental. 0.77 A_{260} unit of (21–77) and 0.74 A_{260} unit of (21–71) in 0.9 ml were irradiated separately under the same conditions. Aliquots were removed after various times of irradiation for assay of cross-link formation by fluorescence. 0.0264 A_{260} unit of 5′-fragment were taken from tubes containing the 5′-fragment or mixtures of 5′ and 3′-fragments, and 0.1 A_{260} unit of (21–77) or (21–71) alone were assayed. Fluorescence intensity was recorded as arbitrary units per A_{260} unit of the 5′-fragment. The value for intact $tRNA^{fMet}$, previously irradiated for 30 min but assayed simultaneously with the fragments, was 6900 units per A_{260} of (1–20). ▲–▲–▲: (1–20); Δ–Δ–Δ: (21–71) or (21–77); ○–○–○: (1–20) plus (21–77); ●–●–●: (1–20) plus (21–71).

per ml in each case, and approximately stoichiometric amounts of the complementary 3′-fragments, (21–77) and (21–71), were added as indicated such that the final A_{260} ratio (21–77) or (21–71):(1–20) was 2.87 and 2.85, respectively. The stoichiometry for (21–71) was calculated from its chain length relative to (21–77), assuming the same degree of purity as (21–77).

Formation of the cross-linked product after irradiation was assayed by the appearance of a fluorescent compound following $NaBH_4$ reduction of the irradiated samples as described previously [4, 11] except that the final concentration of $NaBH_4$ was doubled in order to ensure complete reduction. Intact $tRNA^{fMet}$, irradiated previously for 30 min, was assayed simultaneously as a standard.

The conversion of 4-thiouracil into the covalently linked 4-thiouracil–cytosine dimer was also followed by recording the characteristic variation in the 4-thiouracil absorption spectrum [1, 6, 11]. The irradiated samples for spectral analysis were the same as those assayed for cross-linking by fluorescence. Absorption was measured in a Cary Model 15 spectrophotometer. The reference solution was the same buffer irradiated along with the samples.

For competition experiments, varying amounts of (21–71) were added to a mixture of (1–20) and (21–77) in a final volume of 230 μl containing 110 mM Hepes, pH 8.0, 11 mM magnesium acetate, and 11 mM potassium chloride. The A_{260} ratio of the functionally active fragments, (21–77):(1–20) was 2.93, so that the 3′-fragment was present in 1.2-fold excess according to the methionine acceptance titration curve for these particular preparations (equivalence point A_{260} ratio of 2.41). After a 20 min preliminary incubation at 37°, the mixture was assayed for methionine acceptance as before [10] in a 30 min incubation at 37°.

Polyacrylamide disc gel electrophoresis was performed in a vertical electrophoresis apparatus

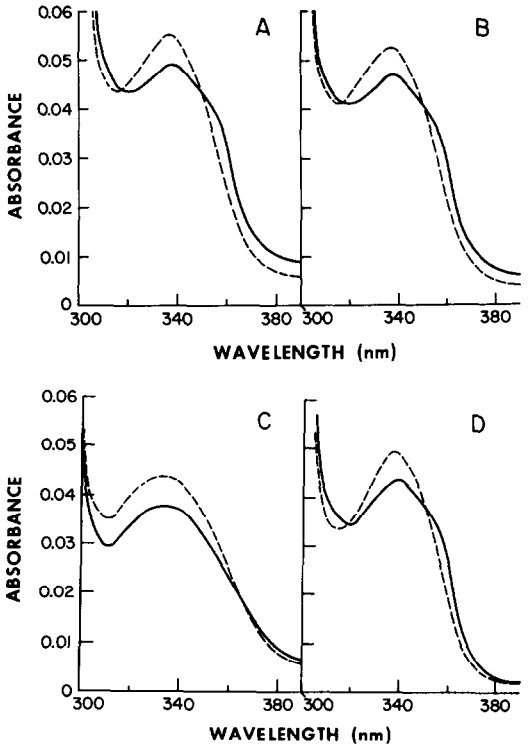

Fig. 3. Ultraviolet absorption spectra of irradiated and control $tRNA^{fMet}$ and fragments. Absorption spectra of the same samples used in fig. 1 which contained 0.66 A_{260} unit per ml of (1–20) alone or in combination with (21–77) or (21–71) were recorded at 0 time (-----) and after 5 min irradiation (——) as described in Experimental. The spectrum of intact $tRNA^{fMet}$ was recorded in 10 mM cacodylate buffer, 10 mM $MgCl_2$, pH 6.9. A: (1–20) + (21–77); B: (1–20) + (21–71); C: (1–20); D: $tRNA^{fMet}$.

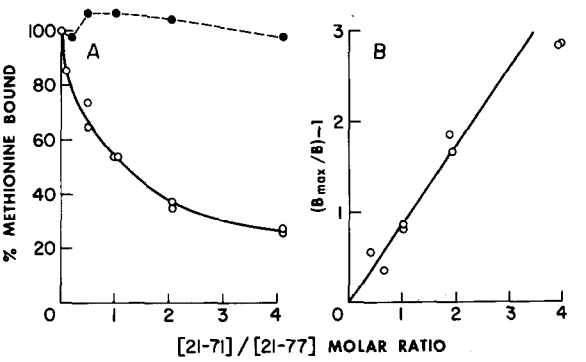

Fig. 4. Competition of (21–71) with (21–77) for complex formation with (1–20). A: Increasing amounts of (21–71) were added to a mixture containing 0.0086 A_{260} unit of (1–20) and 0.0252 A_{260} unit of (21–77) and assayed for methionine acceptance after 20 min preliminary incubation in Hepes buffer, pH 8.0 as described in Experimental. As a control, 0.0243 A_{260} unit of intact $tRNA^{fMet}$ was used in place of (1–20) + (21–71). All points represent the net activity after subtracting the sum of activities due to fragments alone. The (21–71)/(21–77) molar ratio was computed on the basis of the amount of A_{260} units added, assuming equal purity for both fragments. The solid circles were plotted on the abscissa to correspond with the same amount of (21–71) used for the open circle experiment. 100% Methionine bound was 38.1 pmoles (4580 cpm). o–o–o: (1–20) + (21–77) + (21–71); •–•–• $tRNA^{fMet}$ + (21–71). B: Analysis of the data of part A. B_{max} and B are the amounts of methionine bound in the absence and presence of inhibitor, respectively and (21–71)/(21–77) molar ratio refers to the uncomplexed molar concentrations at equilibrium calculated as in [13]. Analysis as described in the text. Slope = 0.87.

(Buchler Instruments) essentially as described by Philippsen et al. [12]. Running gels consisted of 12% acrylamide cross-linked with 0.6% bis-acrylamide and the spacer gels were polymerized from 5% acrylamide and 0.25% bis-acrylamide. Both gels contained 62.5 mM imidazole-HCl pH 6.4, 1 mM magnesium acetate, 0.08% (v/v) *N, N, N', N'*-tetramethylethylenediamine and 3 mM ammonium persulfate. The reservoir buffer was 30 mM cacodylic acid, 2.5 mM imidazole, and 1 mM $Mg(OAc)_2$, final pH 5.5. Samples were applied to the gels as described in the legend to fig. 5 with bromphenol blue added as a running marker. Electrophoresis was run at 350 V (ca. 6 mA/tube) for 110 min at 0–4°. Gels were stained with 0.2% methylene blue in 0.4 M KOAc, pH 5.0 for 1 hr and scanned in a Gilford Model 2400 spectrophotometer at 600 nm after destaining against water for 40 hr.

^{14}C-Methionine was purchased from New England Nuclear, acrylamide and bisacrylamide from Eastman Kodak, and sucrose (RNase-free) from Schwarz Bio-Research.

3. Results

3.1. *Cross-linking ability*

The ability of (21–77) and (21–71) to induce cross-linking in (1–20) after irradiation is illustrated in fig. 2. In this experiment, cross-linking was measured by the specific fluorescence properties of the $NaBH_4$ reduction product of the cross-linked tRNA

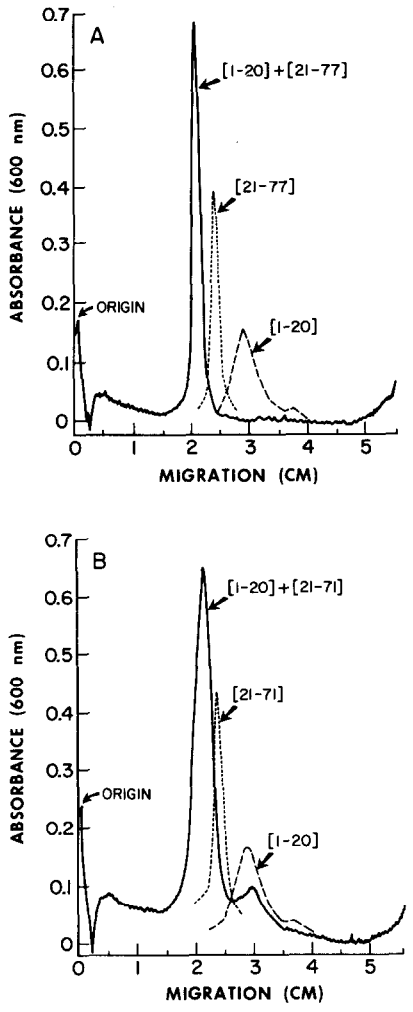

Fig. 5. Disc-gel electrophoresis of $tRNA^{fMet}$ fragments. 0.086 A_{260} unit of (1–20) mixed with 0.25 A_{260} unit of (21–77) or with 0.23 A_{260} unit of (21–71) was incubated for 15 min at 37° in a final vol of 100 μl containing 5 mM Tris, pH 7.1, 5 mM magnesium acetate, and 5 mM potassium chloride in 30% RNase-free sucrose, and then placed on the gels for electrophoresis. The same amounts of uncombined fragments were treated identically. The details of the electrophoretic run are described in Experimental.

fragments. As previously reported [4], (1–20) alone did not form a cross-link, but required the participation of (21–77). The same stimulation was also observed for (21–71) even though the 3′-hexanucleotide was absent in this fragment. Note the similar rate and extent of reaction in both cases. The slight difference in plateau values noted in this figure has not been consistently observed. With other preparations of (21–77), no difference was detected. The final yield of cross-link was about the same as that for intact $tRNA^{fMet}$ similarly treated. The small amount of cross-link formation observed in (1–20) alone may be due to contamination with a complementary fragment [4] or to a slow rate of reaction similar to that observed for the free nucleosides [8].

An additional means of assaying for cross-link formation makes use of characteristic changes in that portion of the ultraviolet absorption spectrum of tRNA which is due to the presence of the 4-thiouracil residue [1, 6, 11]. The effect is illustrated for intact $tRNA^{fMet}$ in fig. 3D. After 5 min of irradiation, there were no further changes in the spectrum in agreement with the kinetic measurements by fluorescence in fig. 2 and previously [4]. Clearly, both (21–77) (fig. 3A) and (21–71) (fig. 3B) in combination with (1–20) show spectral changes after irradiation indicative of cross-link formation while (1–20) alone (fig. 3C) does not. The 13% decrease in (1–20) absorption after 5 min irradiation depends on the extent of irradiation and appears to be due to some photochemical decomposition reaction unrelated to cross-link formation. The main point to note is the absence of the characteristic shoulder at 335 nm in the (1–20) spectrum.

3.2. *Complex forming ability*

The ability of (21–71) to induce cross-linking despite the absence of the 3′-terminal hexanucleotide prompted us to re-examine the previously reported requirement for this hexanucleotide for complex formation with (1–20) [9]. An incubation was prepared such that both (21–77) and (21–71) were competing with each other for complex formation with limiting amounts of (1–20). After 20 min at 37°, the mixture was assayed for the amount of (1–20)–(21–77) formed by its ability to accept methionine after a second 30 min incubation with excess enzyme. (1–20)–(21–71) complexes cannot accept amino acid. Although the time required to reach equilibrium was not directly examined, it is known from other experiments that complex formation occurs very rapidly even at 0° since the rate of cross-linking at 0° is not increased by prior incubation of fragments [4]. It is clear from fig. 4A that

(21–71) readily inhibited complex formation by (21–77) and that when equal amounts of the two fragments were added, the inhibition was approximately 50%. As a control, intact $tRNA^{fMet}$ was used in place of the functionally active fragments (1–20) plus (21–77) in order to show that the added fragment (21–71) was not contaminated with some nonspecific inhibitor. The inability of the latter fragment to affect the acceptor activity of $tRNA^{fMet}$ also demonstrates that the fragment was not competing for the binding site on the synthetase under these experimental conditions showing that saturating levels of enzyme were indeed present in the assay mixture. The data of fig. 4A may be analyzed to give a more quantitative measure of the binding of (21–71) to (1–20) on the assumption that equilibrium did exist among the added fragments and that acylation of the (1–20)–(21–77) complex did not disturb this equilibrium. So long as (1–20) is limiting, the relationship at equilibrium can be readily shown to be $(B_{max}/B) - 1 = K_{21-71}/K_{21-77}$ times (21–71)/(21–77) where B_{max}, B, and (21–71)/(21–77) are defined in the legend to fig. 4B, and K is the association constant for complexing of the 3′-fragment with (1–20). This analysis has been previously applied in another context [13]. The slope of the line plotted in fig. 4B is a measure of the association constant for (21–71) relative to that for (21–77). This value was 0.87 showing that the affinity of (21–71) for (1–20) is virtually the same as (21–77).

In order to verify the existence of this complex by direct means, polyacrylamide gel electrophoresis was used since the fragments and their complexes are easily distinguished by this method. Electrophoresis was run in the presence of Mg^{2+} to stabilize the complexes. Fig. 5 shows the results of combining approximately stoichiometric amounts of the complementary fragments. The positions of the individual fragments run separately are also indicated. A definite complex was formed between (1–20) and (21–71) as well as between (1–20) and (21–77). The approx. 20% of (1–20) which did not form a complex with (21–71) in this experiment was not observed in a second electrophoresis run.

4. Discussion

These experiments were initiated in order to determine the minimum complementary 3′-fragment needed for induction of cross-linking in (1–20). Because of the stereochemical features of this reaction, the 3′-fragment requirement should show which sequences are involved in the folding of this part of the tRNA molecule. It seems clear that the 3′-terminal hexanucleotide including one base pair of the amino acid acceptor stem is not necessary. We previously showed, on the other hand, that the 13 residues from the 3′-end were not enough at least in the case of $tRNA^{Val}$ [3]. Further studies will be necessary to delineate the size and sequence of the smallest active fragment and to show whether it is the same for both initiator and for internal tRNAs.

The cross-linking assay does not directly measure the strength or type of complex that is formed other than with relation to orientation of the position 8 in the 4-thiouracil and position 13 in the cytosine. Competition with unmodified fragments, on the other hand, allows a quantitative comparison of affinity relative to the natural fragment. Applying this assay, we found that removal of the 3′-hexanucleotide had almost no effect on the affinity of the 3′-fragment for its complementary 5′-piece and this strong affinity was directly verified by electrophoretic separation of the fragments from the complexes.

It seems clear from these results that at least in this tRNA the 3′-terminus of the molecule does not play any major structural role. It is not yet clear whether this is a particular feature of initiator tRNAs, of this particular initiator tRNA, or is a general property of all tRNAs. Chemical modification [14] and oligonucleotide binding experiments [15] in $tRNA^{Phe}$ of yeast have been interpreted to show protection of the equivalent of residues 74, 75, and 76 of $tRNA^{fMet}$, and some tRNA models have incorporated this feature [14, 16]. Our results do not contradict these findings if applicable to initiator tRNA but do show that the interaction cannot be a very important one structurally. Residues 75 and 76 of $tRNA^{fMet}$ have been implicated in synthetase recognition as a result of photochemical modification experiments [17]. Our results are not directly comparable since we have removed the nucleotides rather than modifying them

and have examined complex formation rather than acceptor activity.

It should be noted at this point that although several assays exist for complex formation between fragments, they do not necessarily measure the same parameters. Cross-linking, the competition assay described above, electrophoresis in polyacrylamide gels, and enzymatic acylation all require complex formation to occur but not necessarily in precisely the same ways. For example, cyanoethylated (21–77) induces cross-linking and complexes with (1–20) about as well as untreated (21–77) as judged by the competition assay, yet it cannot be enzymatically acylated (unpublished experiments). In this sense, the various assays complement each other in detecting different aspects of the complex formed from tRNA fragments and, used together, may help to unravel the three-dimensional structure of tRNA.

References

[1] A. Favre, M. Yaniv and A.M. Michelson, Biochem. Biophys. Res. Commun. 37 (1969) 266.
[2] M. Yaniv, A. Favre and B.G. Barrell, Nature 223 (1969) 1331.
[3] M. Krauskopf and J. Ofengand, FEBS Letters 15 (1971) 111.
[4] M.A.Q. Siddiqui and J. Ofengand, FEBS Letters 15 (1971) 105.
[5] S.K. Dube, K.A. Marcker, B.F.C. Clark and S. Cory, Nature 218 (1968) 232.
[6] A. Favre, A.M. Michelson and M. Yaniv, J. Mol. Biol. 58 (1971) 367.
[7] F. Ponchon, C. Balny, K.H. Scheit and A.M. Michelson, Biochim. Biophys. Acta 228 (1971) 49.
[8] D.E. Bergstrom and N.G. Leonard, Biochemistry 11 (1972) 1.
[9] T. Seno, M. Kobayashi and S. Nishimura, Biochim. Biophys. Acta 190 (1969) 285.
[10] M.A.Q. Siddiqui and J. Ofengand, J. Biol. Chem. 245 (1970) 4409.
[11] M. Krauskopf, C.-M. Chen and J. Ofengand, J. Biol. Chem. 247 (1972) 842.
[12] P. Philippsen, R. Thiebe, W. Wintermeyer and H.G. Zachau, Biochem. Biophys. Res. Commun. 33 (1968) 922.
[13] J. Ofengand and C. Henes, J. Biol. Chem. 244 (1969) 6241.
[14] F. Cramer, H. Doepner, F. van der Haar, E. Schlimme and H. Seidel, Proc. Natl. Acad. Sci. U.S. 61 (1968) 1384.
[15] A. Danchin and M. Grunberg-Manago, FEBS Letters 9 (1970) 327.
[16] A. Danchin, FEBS Letters 13 (1971) 152.
[17] L.H. Schulman, Proc. Natl. Acad. Sci. U.S. 66 (1970) 507.