ACTIVE COMPLEXES DERIVED FROM *ESCHERICHIA COLI* FORMYLMETHIONINE tRNA WHICH LACKS THE DIHYDROURIDINE-CONTAINING LOOP

T.SENO, I.KOBAYASHI, M.FUKUHARA and S.NISHIMURA
Biology Division, National Cancer Center Research Institute, Tsukiji 5-chome, Chuo-ku, Tokyo, Japan

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We have previously demonstrated that a cleavage of $tRNA^{fMet}$ from *Escherichia coli* into two halves at the dihydrouridine-containing loop (D-loop) (see fig. 1) did not destroy the methionine-acceptor activity as well as transformylation ability, indicating that the intactness of the sequence in the D-loop is not necessarily required for either methionyl tRNA synthetase or transformylase [1, 2]. Since the sequence in this region is sufficiently different in tRNAs to serve as recognition sites and, in fact, Yaniv and Gros have shown an evidence that this region is a promising candidate for the specificity sites [3], it should be interesting to examine whether the sequence in this region, though it no longer forms a loop in the case of the recombined halves, specifies the recognition sites. In this respect, we have attempted to carry out the stepwise removal of nucleotides from the D-loop region to a various extent by the use of snake venom phosphodiesterase and examined its effects on the recovery of the activities. The results showed that 40% of the initial methionine-acceptor activity were still maintained after elimination of the looped-out sequence, but further removal of one nucleotide from the stem region diminished the activity to 10%; the removal of two or more nucleotides from the stem abolished the activity. Practically the same results were obtained for the transformylation ability. It is suggested that the stem part of the D-loop region may play an important role in constructing the active form of the reconstituted molecules.

Splitting of $tRNA^{fMet}$ at the D-loop was carried out as reported previously [1, 2]. Fragment N is the mixture of two kinds of pC-half molecules consisting of 19 or 20 nucleotides with the 3′-terminal sequence –CUG or –CUGG, respectively. Fragment L is the CCA-half complementary to Fragment N and consists of 57 nucleotides, in which dihydrouridine terminates at the 5′-end [2] (see fig. 1). In order to carry out the stepwise removal of nucleotides from the 3′-end of Fragment N, 10 $A_{260\,nm}$ units of Fragment N were dissolved in 5 ml of 0.04 M triethylammonium bicarbonate (pH 7.9) and at first incubated at 37° for 2 hr

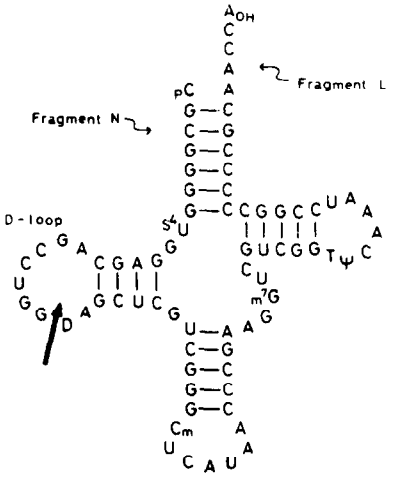

Fig. 1. Nucleotide sequence of Fragment L and Fragment N obtained by a single cleavage at the D-loop in *E. coli* $tRNA^{fMet}$. The arrow indicates the site of the cleavage by ribonuclease T_1. The primary sequence and the arrangement in the cloverleaf structure of the tRNA was taken from Dube et al. [7, 8]. The U derivative in the original sequence was identified as 4-thiouridine [9].

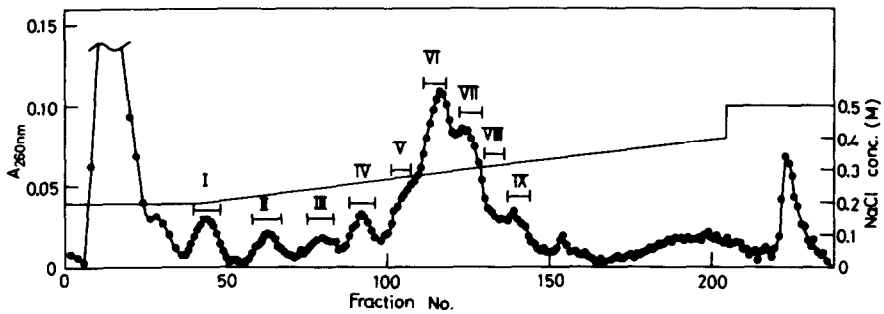

Fig. 2. Column chromatographic separation of partially digested pC-half of $tRNA^{fMet}$ by snake venom phosphodiesterase. 10 A_{260nm} units of Fragment N were digested by snake venom phosphodiesterase as described in the text and chromatographed on a column of DEAE-cellulose (Whatman DE 23) (0.3 cm × 100 cm). A linear gradient elution was performed at 20° using 100 ml of 0.4 M NaCl, 7 M urea and 0.02 M tris-HCl (pH 7.5) in the reservoir and 100 ml of 0.1 M NaCl, 7 M urea and 0.02 M tris-HCl (pH 7.5) in the mixing vessel. The flow rate was 3 ml/hr. Fractions of 1 ml were collected. The materials were recovered from each peak as indicated according to Rushizky and Sober [10]. The digests before the chromatography were found to maintain the acceptor activity corresponding to 40% of the original one when assayed by recombining with complementary Fragment L.

Table 1

Methionine- and formate-acceptor activities recovered from each Fraction shown in fig. 2.

Fractions	Net incorporation of ^{14}C-methionine (cpm/0.002 A_{260nm} units equivalent to Fragment N*)	%	Net incorporation of ^{14}C-formate (cpm/0.01 A_{260nm} units equivalent to Fragment N*)	%
I	40	2	20	2
II	10	0	0	0
III	70	3	40	5
IV	100	4	50	6
V	250	11	100	12
VI	920	41	250	30
VII	1240	55	280	34
VIII	2100	93	830	100
IX	2200	98	820	100
PMase-Fragment N**	2200	98	–	–
Intact Fragment N	2250	100	820	100

For methionine-acceptor assay, 0.002–0.006 A_{260nm} units of pC-half fragments as specified were combined with 0.09 A_{260nm} units of Fragment L. For the formate-acceptor assay, 0.006–0.02 A_{260nm} units of pC-half fragments as specified were combined with 0.08 A_{260nm} units of the complementary CCA-half which had already been charged with non-labeled methionine. The assay procedures were followed as reported previously [2]. ^{14}C-methionine specific activity, 187 mCi/mmole, a product of New England Nuclear Corp.; ^{14}C-formate specific activity, 33 mCi/mmole, prepared as described previously [2].

* Actual absorbancy in each Fraction was normalized to the absorbancy equivalent to Fragment N with the aid of molar extinction coefficient and the chain length of oligonucleotide (see fig. 3).

** Phosphates were removed from both termini of Fragment N by alkaline phosphomonoesterase.

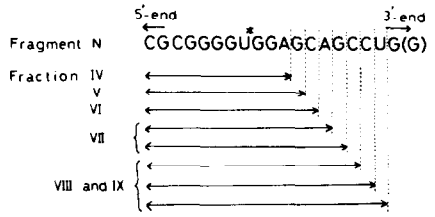

Fig. 3. Nucleotide sequence of partially deleted Fragment N. The arrows indicate the nucleotide sequence of the oligonucleotides recovered from each Fraction (fig. 2). The sequence was determined as described in the text. Fraction VII was the mixture of tetradeca- and pentadeca-nucleotides. As for Fractions VIII and IX, exact sequence at the 3'-end could not be determined, however, both Fractions did not contaminate the original Fragment N. As for Fractions I, II and III, the sequence was not determined.

with *E. coli* alkaline phosphomonoesterase (kindly donated by Dr. K.Tanaka of Shionogi Research Lab.) to remove phosphates from the terminal nucleotides and then incubated for additional 17 min in the presence of snake venom phosphodiesterase (a product of Worthington Biochemical Corp.) at the concentration of 20 μg per ml. After heating at 100° for 2 min, the digests were chromatographed on a DEAE-cellulose column as shown in fig. 2. The oligonucleotides were recovered from each fraction as specified in fig. 2, and were subjected to finger-print analysis of the ribonuclease T_1 digest by two-dimensional thin-layer chromatography as reported previously [4]; 0.3–0.5 A_{260nm} units from each digest were developed on a cellulose plate (10 cm × 10 cm) and the chromatogram was examined by an ultraviolet lamp at 253.7 nm or at 365.0 nm. All products which are expected from the ribonuclease T_1 digestion were clearly separated from each other on the plate. The sequence of the oligonucleotides present in Fractions IV–IX was determined by such T_1-fingerprint analysis and is shown in fig. 3.

Table 1 shows the methionine-acceptor and the transformylation activities restored from each Fraction by combining with the complementary Fragment L. In order to test the formate-acceptor activity, aminoacylated Fragment L prepared with non-labeled methionine as reported previously [2] was used. The results in table 1 should be emphasized as follows: (a) Fractions VIII and IX, in which 1 to 3 nucleotides have been eliminated from the 3'-terminus of Fragment N, maintained almost 100% of the initial activity and (b) 40% of the methionine-acceptor activity was still maintained after elimination of the heptanucleotide, AGCCUGG (Fraction VI) which covers the whole looped-out sequence of the pC-half fragment (see figs. 1 and 3). This further extends the results obtained with yeast $tRNA_1^{Val}$ that the removal of dinucleotide from the D-loop diminished only 20% or so of the initial acceptor activity [5]. It is evident that this looped-out region is not necessarily required for the recognition of the synthetase. (c) The removal of the octanucleotide, CAGCCUGG, which results in the loss of one base-pairing in the stem region in addition to the looped-out sequence (see fig. 1) decreased the activity to 10% (Fraction V). This drop of activity suggests the importance of the stem region in the reconstituted molecules. Practically similar results were obtained for the formate-acceptor activity, indicating that the transformylase requires the same order of structure in the tRNA molecule as does the methionyl tRNA synthetase. The data shown in table 1 also revealed that the removal of phosphates from both termini of Fragment N by alkaline phosphomonoesterase did not destroy the recovery of the activities. The conversion of s^4U (4-thiouridine) to U in the pC-half (fig. 3) had similarly no effect. The latter was confirmed by a separate experiment in which s^4U in Fragment N was converted to U by the BrCN-treatment [6].

Our present data have strongly suggested that it is the stem of the D-loop region that plays an important role in recognizing the aminoacyl tRNA synthetase. However, it is unlikely that any particular nucleotide or sequence of nucleotides within the stem region serves as the specific recognition site, since the recovery of the activity did not drop abruptly to zero in a particular Fraction; the gradual decrease of activity was reproducibly observed in Fractions VI–III. The nucleotides in the looped-out region may help in stabilizing the base-pairs in the stem region. Our results further imply that the specificity for the synthetase locates in regions other than the D-loop and the sequence in the D-loop stem is only physically important in maintaining the stable complex with the CCA-half. The removal of nucleotides from the 5'-terminus of Fragment N or Fragment L, or enzymic re-addition of extra oligonucleotides starting with those shortened pC-half should be possible and give more information about this problem.

Acknowledgements

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