AMINOACYLATION OF METHOXYAMINE MODIFIED TYROSINE TRANSFER RNA

A.R.CASHMORE

Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, England

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1. Introduction

Methoxyamine reacts selectively with a small percentage of the cytosine bases in mixed *E. coli* tRNA, producing a uniform loss in acceptor activity [1]. In su^+_{III} tyrosine-tRNA the specific cytosines which react with methoxyamine have been characterised [2] (fig. 1). The effect of the individual modifications on acceptor activity has now been established by examining the chargeable RNA after methoxyamine treatment.

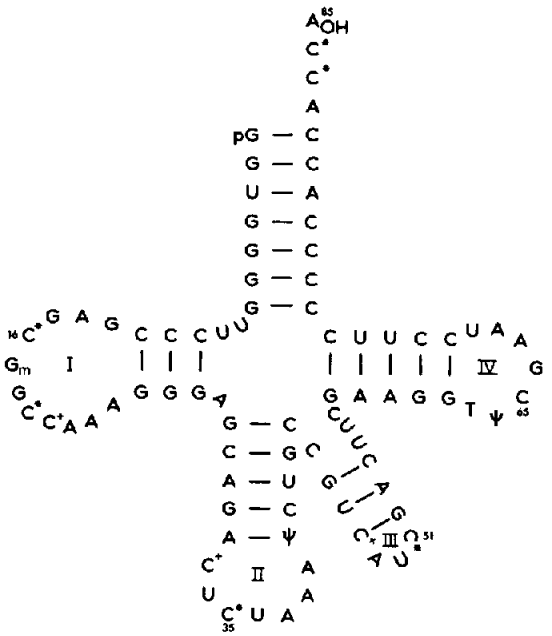

Fig. 1. Reactive cytosines in su^+_{III} tyrosine-tRNA. C* signifies cytosines reactive with methoxyamine and C^+ indicates partial reactivity.

This tRNA differs from the non-chargeable solely with respect to modification of the 3′-terminal cytosines.

2. Materials and methods

^{32}P-labelled transfer RNA, specifically enriched in su^+_{III}, was prepared from *E. coli* CA274 [3]. Tyrosine-tRNA, with A38 unmodified [4], was isolated by fractionation on a BD cellulose column [5]. This tRNA was aminoacylated with ^{3}H-tyrosine using tyrosyl RNA synthetase purified by ammonium sulphate precipitation and DEAE cellulose chromatography [6]. The aminoacylated tRNA was purified by phenoxyacetylation, similar to that described by Gillam et al. [7], and BD cellulose chromatography (fig. 2). The phenoxyacetylated aminoacyl-tRNA was hydrolysed by incubation in 2 M tris (pH 9.1) at 37° for 30 min.

Methoxyamine treatment was with 3 M reagent (pH 5.5), in 10 mM $MgCl_2$, for 8 hr at 37°. The RNA was recovered by ethanol precipitation from 0.2 M acetate (pH 5.0). Nucleotide sequences were determined by the method of Sanger et al. [8].

3. Results and discussion

Fully chargeable tyrosine-tRNA, isolated by the procedure of phenoxyacetylation, was incubated with 3 M methoxyamine for 8 hr. The modified RNA charged with ^{3}H-tyrosine to 26 percent of its original acceptance. After aminoacylation the RNA was phenoxyacetylated and fractionated on a BD cellulose column (fig. 3). The three peaks were examined by T_1

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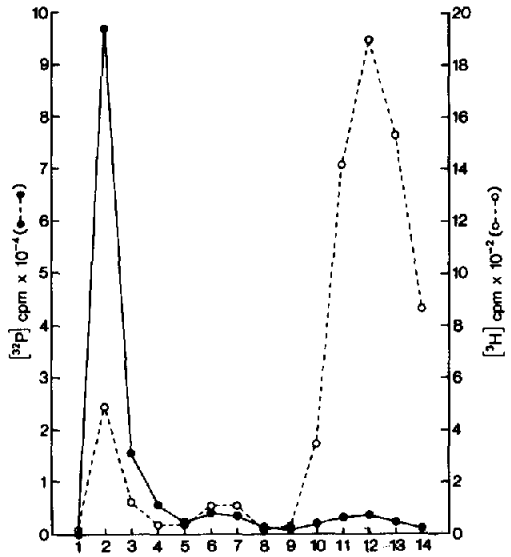

Fig. 2. Purification of tyrosine-tRNA. Transfer RNA, charged with ^{3}H-tyrosine and phenoxyacetylated, was applied to a small BD cellulose column and eluted with an acetate buffer (10 mM, pH 5.0) containing 10 mM $MgCl_2$, 3 mM mercaptoethanol and 1.0 M NaCl (fractions 1–5); 1.0 M NaCl, 5 percent EtOH (fractions 6–8); 1.0 M NaCl, 10 percent EtOH (fractions 9–14).

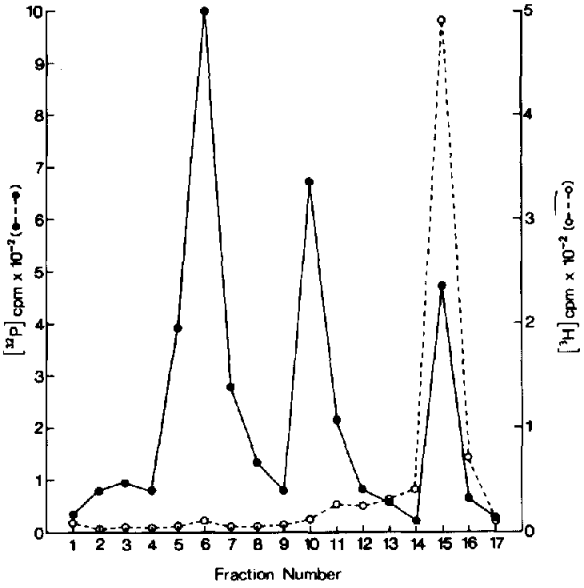

Fig. 3. Fractionation of chargeable and non-chargeable methoxyamine modified tRNA. Phenoxyacetylated RNA was chromatographed on a BD cellulose column according to the procedure of fig. 1. The elution buffer contained 1.0 M NaCl (fractions 1–4); 1.0 M NaCl, 5 percent EtOH (fractions 5–9); 1.0 M NaCl, 10 percent EtOH (fractions 10–13) and 2.0 M NaCl, 20 percent EtOH (fractions 14–17).

ribonuclease digestion followed by two dimensional electrophoresis (fig. 4). The two non-chargeable RNA peaks presumably reflect a small quantitative difference in modification, as they yielded essentially identical fingerprints. In contrast, the fingerprint of the chargeable RNA differed markedly from the fingerprints of the non-chargeable in the relative amounts of the modified 3′-terminal sequences. The 3′-end with both cytosines modified was a minor component (16 percent) of the 3′-terminal sequences in the chargeable RNA fraction whereas it was the major species (66 percent) in the non-chargeable fraction (table 1). The relative amounts of the other modified sequences were similar for chargeable and non-chargeable RNA. It is concluded that methoxyamine modification of the 3′-terminal cytosines inhibits aminoacylation, in contrast to the modification of the other reactive cytosines.

Some aspects of the experimental procedure warrant further discussion. In a control experiment with the aminoacylation step omitted, 4 percent of the RNA eluted in the "chargeable" fraction compared with 17 percent of chargeable RNA found in fig. 3. This 4 percent "spil-over" provides a possible explanation for the small fraction of RNA containing the doubly modified 3′-end eluting in the "chargeable" fraction. Alternatively this RNA may charge at a greatly diminished rate, although no acceptor activity was found for fractions 1–9 (fig. 3). A second point to be considered is loss in acceptor activity which is not due to the effect of the reagent. Treatment of mixed tRNA, from *E. coli* CA265, with 3 M methoxyamine for 20 hr results in 74 percent loss in acceptor activity [1]. Under identical reaction conditions, but in the absence of the reagent, 9 percent loss in amino acid acceptance is observed. This loss in acceptor activity, which is not due to the reagent, would be expected to be considerably greater for the overall experimental procedure. Non-chargeable RNA, with one or neither of the 3′-terminal cytosines modified could be accounted for by this factor.

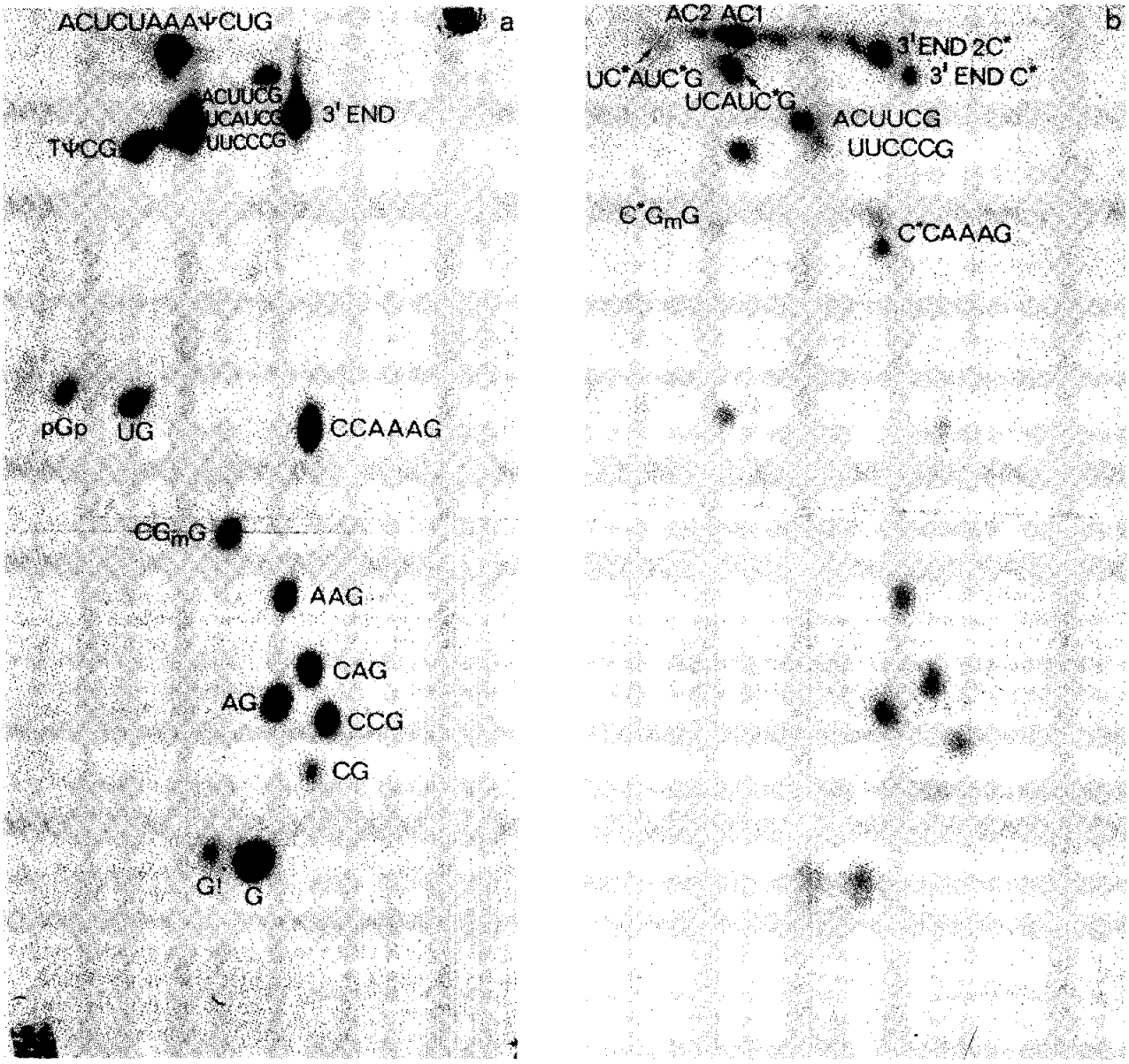

Fig. 4a–c. T_1 ribonuclease fingerprints of methoxyamine tyrosine tRNA. Separation is by electrophoresis on cellulose acetate at pH 3.5 in 7 M urea (right to left) followed by electrophoresis on DEAE paper in 7 percent formic acid 3′ END refers to the sequence AAUCCUUCCCCCACCACCA–OH; 3′ END C* refers to the same sequence with one of the two 3′-terminal cytosines modified; 3′ END 2C* refers to the same sequence with both of the terminal cytosines modified; ACl refers to the sequence ACUC* UAAAΨ CUG and AC2 refers to the sequence AC*UC*UAAAΨCUG. (a) is the T_1 fingerprint of unmodified su^+_{III} tRNA. Fractions 5–7 (b), and 15 (c) are from the column described in fig. 3.

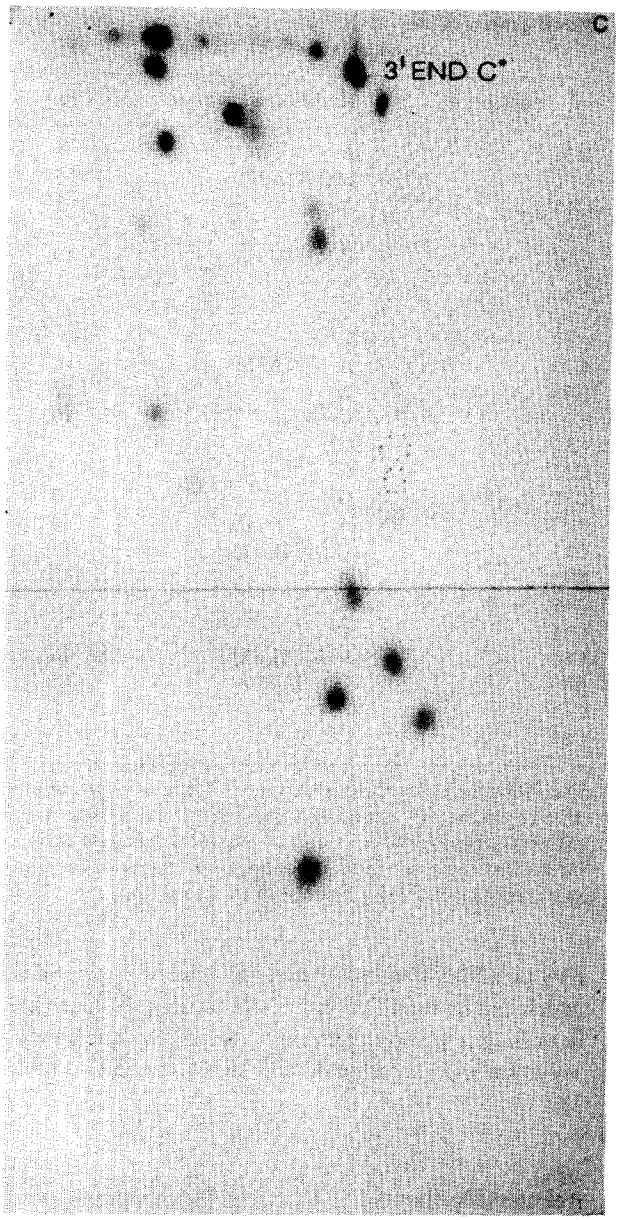

Fig. 4c.

That RNA with one 3′-terminal cytosine modified can be charged is quite clear from fig. 4 and table 1. This RNA may be subdivided according to whether the modification is at C83 or C84 and these two classes differ in the AC content of their 3′-terminal T_1 fragments. The T_1 fragment modified at C83 contains one AC dinucleotide whereas the T_1 fragment modified at C84 contains two AC dinucleotides. The AC content has been estimated after digestion with pancreatic ribonuclease (table 2). In evaluating the data in table 2 it should be noted that methoxyamine modification at C84 may inhibit the pancreatic cleavage of the C83–C84 phosphate bond [2] and thus the apparent AC content of the singly modified 3′-terminal sequences may be anomalously low. From the evidence in table 2 it is therefore concluded that methoxyamine modification of C84 does not prevent

Table 1
Quantitation of T_1 ribonuclease derived oligonucleotides from chargeable and non-chargeable, methoxyamine modified, tyrosine tRNA.

Sequence	Molar yield	
	Non-chargeable	Chargeable
CCAAAG	0.15	0.07
C*CAAAG	0.61	0.64
C*G_mG	0.31	0.36
UC*AUC*G	0.17	0.16
UCAUC*G	0.91	0.87
ACUC*UAAAΨCUG	0.83	0.67
AC*UC*UAAAΨCUG	0.11	0.13
3′END	0.04	0.14
3′END C*	0.17	0.43
3′END 2C*	0.40	0.11

Non-chargeable tRNA refers to fractions 5–7 (fig. 3) and chageable tRNA refers to fraction 15 (fig. 3). Oligonucleotides were counted in a scintillation counter and are related to TΨCG on a molar basis. The low yield of the 3′-terminal fragment is a characteristic feature of this tRNA.

Table 2
AC content of 3′-terminal fragments.

	AC (molar yield)
3′END C* chargeable	1.47
3′END C* non-chargeable	1.50
3′END 2C*	0.83
3′END	1.75

Pancreatic ribonuclease digestion products were separated by electrophoresis on DEAE paper at pH 3.5. The AC content was determined relative to AAU. The abbreviations used are explained in fig. 4.

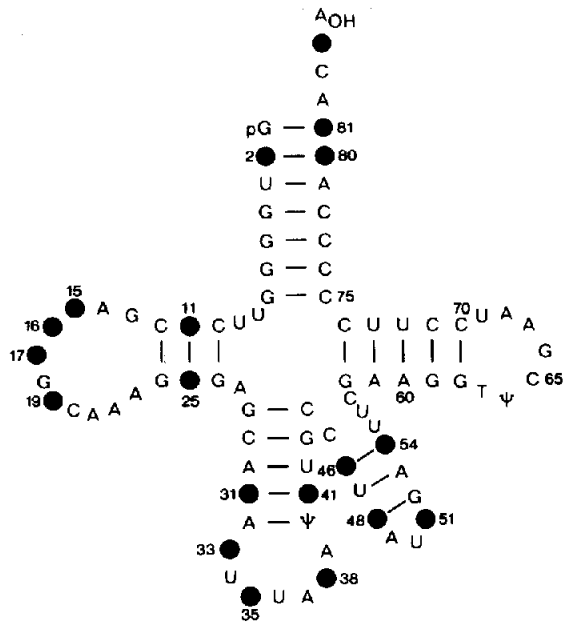

Fig. 5. Modifications of su^{+}_{III} tRNA which do not prevent enzymatic aminoacylation with tyrosine. The modified bases are represented by •. It should be noted that the kinetics of aminoacylation of the modified tRNAs are not necessarily the same as for the su^{+}_{III} tRNA. The modifications are explained in table 3.

Table 3
Modifications of su^{+}_{III} tRNA which do not prevent enzymatic aminoacylation with tyrosine.

su^{+}_{III} nucleotide	Modified nucleotide	Ref.
G2	A	5
C11	U (U11A25)	5
G15	A	3
C16	C*	
C16	U (U16U31)	10
G_m17	A	3, 11
G_m17	G	12
C19	C*	
G25	A (U11A25)	5
G31	A	3
G31	U	10, 13
C33	C*	
C35	C*	
C35	G*	3
A*38	A	4
C41	U (A31U41)	13
G46	A	13
C48	C*	
C51	C*	
C54	U (A46U51)	13
C80	U (A2U80)	5
C81	A	5
C84	C*	

The following abbreviations are used: C*, methoxyamine modified cytosine; G*, a modified G and A*, 2-methylthio-*N*-(6)-isopentenyl-A. Where a double mutant is specified this means that the single mutant has not been examined for tyrosine acceptance, unless given elsewhere in the table.

the aminoacylation of tyrosine-tRNA. The evidence does not allow a decision regarding the effect of modification of C83.

Methoxyamine modification of both 3′-terminal cytosines of su^{+}_{III} tyrosine-tRNA inhibits aminoacylation, in contrast to the modification of the other reactive cytosines. For the experimental conditions described in this report, modification of both 3′-terminal cytosines accounts for approximately 70 percent of the non-chargeable species. Similar results regarding the inactivation of formylmethionine-tRNA by ultraviolet light induced modification of the 3′-terminal cytosines have recently been reported by Schulman [9]. The universal nature of these terminal cytosines precludes their involvement in a specific binding reaction with the synthetase and the inhibitory effect of modification is more likely to involve the catalytic stage in acylation.

The nucleotides C-16, 19, 33, 35, 48 and 51 can now be added to a rather extensive list of nucleotides which can be modified either chemically, mutagenically or *in vivo* without preventing the enzymatic aminoacylation with tyrosine of su_{III} tRNA (fig. 5 and table 3).

Acknowledgements

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