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The gene for a tRNA modifying enzyme, m^{5}U54-methyltransferase, is essential for viability in *Escherichia coli*

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ABSTRACT **One of the most abundant modified nucleosides in tRNA is 5-methyluridine (m^{5}U or rT, ribothymidine). The enzyme tRNA(m^{5}U54)methyltransferase [*S*-adenosyl-L-methionine:tRNA (uracil-5-)-methyltransferase, EC 2.1.1.35] (the *trmA* gene product) catalyzes *S*-adenosylmethionine-dependent methylation of the uracil in position 54 (TΨC loop) in all *Escherichia coli* tRNAs to form m^{5}U. Hitherto no modified nucleoside in tRNA has been shown to be essential for growth, although their importance in fine tuning the function of tRNA is well established. In this paper, we show that the structural gene *trmA* is essential for viability, although the known catalytic activity of the tRNA(m^{5}U54)methyltransferase is not.**

Transfer RNAs of eubacteria, eukaryotes, and archaebacteria have together more than 50 different modified nucleosides, all derived from the four major nucleosides (A, C, G, and U) (1). Many positions in the tRNA can be modified and in *Escherichia coli* some 50 genes (≈1% of the genome) are devoted to tRNA modification (2). Although the modified nucleosides influence the efficiency and fidelity of the tRNA, so far no modified nucleoside has been shown to be essential for viability. One of the most abundant of these modifications, C-5 methylation of uracil-54 (designated m^{5}U54), is present in the TΨC loop of all tRNAs of most eubacteria and in most elongator tRNAs from eukaryotes, which suggests a pivotal role of this modified nucleoside. Even the exceptional Archaebacteria, which lack m^{5}U54, contain the sterically similar 1-methylpseudouridine modification at this position, suggesting convergent evolution (3). Direct tests *in vitro* have shown that the presence of m^{5}U54 in the tRNA increases the fidelity of protein synthesis and the stability of the three-dimensional structure of the tRNA and influences the rate of protein synthesis (4–7). Evidence that m^{5}U54 modification is not essential came from findings that U54 is not modified in the tRNA of *Mycobacterium smegmatis* or of several different *Mycoplasma* species (8–12). A nonconditional mutant (*trmA5*) of *E. coli*, which completely lacks m^{5}U54 in its tRNA, has been isolated (13), and the only detectable phenotype *in vivo* was a 4% reduction of growth rate (ref. 14; G.R.B., unpublished observation). Also, a yeast mutant (*trm2*) lacking m^{5}U54 in the tRNA grows normally (15). Given the growth-stimulating but nonessential nature of m^{5}U54, we expected that *E. coli* strains with insertions in *trmA* would be fully viable. However, here we report that strains with an insertion relatively early in the *trmA* gene are nonviable. This indicates that the *trmA* gene product is indeed essential for growth and suggests that the *trmA* transcript or protein has a second vital function, distinct from that of tRNA m^{5}U54 synthesis.

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MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions. Strains, phage, and plasmids are listed in Table 1. Strain GB1701 is TrmA$^-$ and has Tn*5* inserted at codon 338. The latter was determined by PCR amplification using oligonucleotides located upstream of the *Sal* I site in the *trmA* gene and downstream of the unique *Hin*dIII site in Tn*5*. The *Sal* I/*Hin*dIII fragment was cloned into pBluescript and sequenced. The chromosomal inserts of λ4G11 and plasmids are shown in Fig. 1. Plasmid pCG1502 was constructed by inserting an *Eco*RI/*Pvu* II fragment from pGP200 (22) containing a *trmA*::*cat* (cat, chloramphenicol acetyltransferase) fusion into an *Eco*RI/*Pvu* II-digested pGP1509 (20).

Bacteria were grown in LB broth (23) supplemented with 0.2% maltose. As solid medium, TYS (10 g of trypticase peptone/5 g of yeast extract/5 g of NaCl/15 g of agar per liter) was used. Antibiotics were used in concentrations of 50 μg/ml for carbenicillin and 15 μg/ml for chloramphenicol.

Transductions. Transductions with λ were performed essentially as described (22).

Construction of λ*trmA*::*cat*. Construction of λ*trmA*::*cat* was done according to the alleleic replacement method of Kulakauskas *et al.* (24). We prepared a derivative of phage λ4G11(*trmA*$^+$) (20) carrying the *trmA*::*cat* fusion of plasmid pCG1502(*trmA*::*cat*), with *cat* inserted 480 nucleotides into the *trmA* coding sequence. The obligate lytic phage λ4G11 from the Kohara library carries the *trmA* gene (Fig. 1). Phage carrying *trmA*::*cat* from plasmid pCG1502 were obtained by growing phage λ4G11 on *E. coli* strain MC1061/pCG1502. This phage stock was used as a donor and strain DB1434, which is lysogenic for λ*plac5*(c*I*857^{ts}), was used as a recipient. Stable chloramphenicol-resistant (Cm^R) transductants were selected at 30°C. These Cm^R colonies were screened for ampicillin sensitivity (Ap^S) to exclude recombinants that had transferred plasmid pCG1502 by a single crossover between the plasmid and the phage. A Cm^R, Ap^S transductant was induced at high temperature for lytic growth of λ phage. Single plaques from the resulting phage stock, which were white on 5-bromo-4-chloro-3-indolyl β-D-galactoside plates (the helper phage λ*plac5* is blue), were isolated, amplified, and tested for their ability to transduce strain DB1434 to Cm^R at high frequency. A phage (denoted λ*trmA*::*cat*) with this ability was used in the transductions described in this paper.

Southern Blot Analysis. Chromosomal DNA was prepared according to Silhavy *et al.* (25), digested with *Pvu* II, and separated on a 0.5% agarose gel. Southern blotting and hybridization were carried out using Hybond-N filters (Amersham) and with solutions and conditions as recommended by the manufacturer. As a probe plasmid pGP100, ^{32}P-labeled by the oligolabeling method as described by Feinberg and

Abbreviation: Cm^R, chloramphenicol resistant.

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Table 1. Strains and plasmids

Strain/plasmid	Phenotype	Source
Bacteria		
CAG12185	*argE86*::Tn*10*	(16)
MC1061	Δ(*ara-leu*), *araD139*, Δ*lacX74*, *galU*, *galK*, *strA*, *hsr*	(17)
MW100	Wild type	(18)
GB1-5-39	*trmA5*, *argH*, *lac*	(19)
GB1701	*trmA21*::Tn*5*	This work
Phage		
λ4G11	Chromosomal insert covering *trmA* and *argE* (see Fig. 1)	(20)
λ*trmA*::*cat*	λ4G11 with a transcriptional *cat* fusion in *trmA*	This work
Plasmids		
pCG1502	*trmA*::*cat*	(21)
pGP100	*trmA*$^+$	(22)
pUST106	*trmA*$^+$	(21)

Vogelstein (26, 27), was used. Autoradiography was with Amersham-MP x-ray film.

Western Analysis. Strain GB1-5-39 (*trmA5*) and strain MW100 were grown in 500 ml of LB broth to OD_{600} = 1.0, harvested, and washed with 0.9% NaCl. The cells were sonicated and cell debris was removed by centrifugation. The lysate was made 0.6 M $(NH_4)_2SO_4$; ribosomes were removed by centrifugation at 310,000 × *g* in an SW40 rotor (Beckman). One hundred micrograms of protein from the supernatant was applied on an SDS/12% polyacrylamide gel. The separated protein was blotted onto a Hybond-N filter (Amersham) and Western analysis was performed essentially as described by Sambrook *et al.* (28). The primary antibodies, specific for the tRNA(m^5U54)methyltransferase [*S*-adenosyl-L-methionine: tRNA (uracil-5-)-methyltransferase, EC 2.1.1.35], were a gift from D. V. Santi (San Francisco). The secondary antibodies were goat anti-rabbit IgG coupled to horseradish peroxidase and were purchased from Bethesda Research Laboratories.

RESULTS

Construction of a λ Phage with a Transcriptional *cat* Fusion in *trmA*. To study transcriptional regulation of the *trmA* gene, we tried to replace the chromosomal *trmA*$^+$ allele with a *trmA*::*cat* transcriptional fusion relatively early in the gene (at codon 161) by a linear transformation method (29). This strategy failed, despite prior evidence that a Tn*5* (*trmA21*) insertion close to the 3′ end of *trmA* [at the 338th codon, 14 codons downstream of the cysteine involved in methyl group transfer (30)] is not lethal, although it eliminates methylation at U54 (data not shown). We therefore decided to use another allelic replacement method that would give the desired *trmA*::*cat* insertion on the chromosome or prove this insertion to be lethal. This method entails the transfer of plasmid-borne genes with inserted resistance markers, to the corresponding λ phage from the Kohara library (20) and from there onto the chromosome by homologous recombination (24).

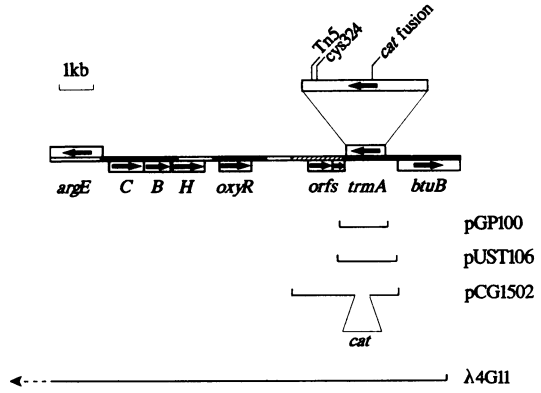

FIG. 1. The chromosomal region around 89.5 min on the *E. coli* chromosome. Arrows indicate direction of transcription of the indicated genes. The *trmA* gene consists of 366 codons. In the enlarged section of the *trmA* gene the positions of the *cat* gene fusion (codon 161), the cysteine residue responsible for methyltransferase activity (codon 324), and the position of the Tn*5* insertion in *trmA21* (codon 338) are shown. Chromosomal inserts in plasmids pGP100, pCG1502, and pUST106, and phage λ4G11 are indicated. Solid bars, sequenced areas (EMBL data library); open bars, unsequenced areas; hatched bar, unpublished sequence determined in this laboratory. Plasmid pUST106 contains chromosomal DNA from *S. typhimurium*; all other chromosomal inserts are derived from *E. coli*.

Phage λ4G11 carrying the *trmA* gene (Fig. 1) was allowed to recombine with plasmid pCG1502, which carries a *trmA*::*cat* transcriptional fusion with the fusion point 480 nucleotides into the *trmA* coding sequence. The desired recombinant phage, denoted λ*trmA*::*cat*, was purified and amplified (see *Materials and Methods*) and used in the transductions described in this paper.

Recombination of *trmA*::*cat* from the Phage onto the Chromosome Requires the Presence of a Complementing Plasmid. To exchange *trmA*::*cat* with the chromosomal *trmA*$^+$ allele, strain MC1061 and a derivative harboring plasmid pGP100(*trmA*$^+$) were used as recipients and λ*trmA*::*cat* was used as a donor (Table 2). No Cm^R transductants were obtained with strain MC1061, whereas 127 Cm^R transductants were obtained when strain MC1061/pGP100(*trmA*$^+$) was the recipient. This suggests that the *trmA* gene is essential.

This result might, however, also be achieved if the chromosomal *trmA* locus were, for some reason, not accessible for recombination. To test this possibility, we repeated the transduction in an *argE* strain (CAG12185). The phage λ*trmA*::*cat* (Fig. 1) also carries the wild-type *argE* gene, and we investigated whether both *trmA*::*cat* and *argE*$^+$ could be transferred to the chromosome in a single event. Cm^R and Arg$^+$ colonies were selected separately using strain CAG12185 or strain CAG12185/pGP100(*trmA*$^+$) as recipients. The same frequency of Arg$^+$ transductants was found in both strains (Table 2). However, stable Cm^R transductants were only found with strain CAG12185/pGP100(*trmA*$^+$) as the recipient. One Cm^R transductant was found in transduction of strain CAG12185. This rare transductant (frequency, $<10^{-10}$) was very unstable and rapidly lost its *trmA*::*cat* copy when streaked on nonselective (chloramphenicol free) medium. This instability of the Cm^R transductant suggests that it contains a duplication (*trmA*$^+$-*trmA*::*cat* stabilized) over the *trmA* gene, which was also verified by Southern blot analysis (data not shown). This result is consistent with the fact that in every bacterial population a small fraction of the bacteria will have partial chromosomal duplications (31). About 10% of the stable Cm^R transductants when strain

Table 2. Transductions using phage λ*trmA*::*cat* as donor

Recipient strain	Selection	Frequency	Screened marker
MC1061	Cm	–	NA
MC1061/pGP100	Cm	2.3×10^{-7}	NA
CAG12185	Cm	$\approx 10^{-10}$	NA
CAG12185/pGP100	Cm	7.8×10^{-8}	10% Arg$^+$
CAG12185	Arg$^+$	1.8×10^{-3}	No Cm^R
CAG12185/pGP100	Arg$^+$	1.8×10^{-3}	0.005% Cm^R

Frequency was calculated as number of indicated transductants obtained per added phage. No Cm^R transductants were found when strain MC1061 was used as recipient ($<10^{-10}$). Also, no stable Cm^R transductants were found when strain CAG12185 was used as recipient. Frequency includes one unstable Cm^R transductant recovered in this cross (see text). NA, not applicable.

CAG12185/pGP100(*trmA*$^+$) was the recipient were also Arg$^+$, and ≈5 per 100,000 Arg$^+$ transductants were Cm^R, indicating that the chromosomal *trmA* locus is accessible for recombination (Table 2). The difference in cotransduction frequencies, depending on whether the first selected phenotype was Arg$^+$ or Cm^R, was also reflected in an 8000-fold higher transduction frequency for Arg$^+$ than for Cm^R (when the *trmA*$^+$ plasmid was present) (Table 1). The *trmA*::*cat* insertion is 2.2 kilobases (kb) from the end of the cloned *E. coli* DNA in phage λ4G11, whereas *argE* is near the middle of the cloned segment and 8.3 kb from the end. Thus, the difference in transduction frequency might reflect the different lengths of homology available for *trmA* and *argE* recombination. It might also be explained by the presence of a recombinational hot spot between *trmA* and *argE* (32).

The Lethal Effect of the *trmA*::*cat* Insertion Is not Due to Polarity Effects on Downstream Genes. Hypothetically, the lethal effect of the *cat* insertion in the *trmA* gene might be due to a polar effect on the expression of a downstream gene; therefore, we repeated the transduction with phage λ*trmA*::*cat* using strain MC1061/pUST106 as recipient. Plasmid pUST106 (Fig. 1) harbors the *trmA* gene from *Salmonella typhimurium*. Since recombination between *Salmonella* and *E. coli* DNA is very rare (33), and this plasmid contains only the *trmA* gene, we would obtain Cm^R transductants only if the lethal effect of the *cat* insertion was due to the essentiality of the *trmA* gene and not if it was caused by polarity on downstream genes. We did obtain Cm^R transductants with strain MC1061/pUST106. Analysis of the transductants showed that they still harbored plasmid pUST106 (*trmA*$^+$) and Southern blot hybridization showed that the *trmA*::*cat* allele had been recombined onto the chromosome in the correct position (Fig. 2). This unequivocally demonstrates that the *trmA* gene is essential for growth.

A TrmA Polypeptide of Normal Size Is Synthesized in a *trmA5* Mutant Strain. As mentioned above, the *trmA21*::Tn*5* mutation renders the cell TrmA$^-$ i.e., protein extract from the mutant has no enzymatic activity *in vitro* and its tRNA made *in vivo* is also deficient in m^{5}U54. This suggests that the region downstream of codon 338 is essential for tRNA methylating activity but not for cell survival. More important is the phenotype of the *trmA5* mutant, which has been shown to completely lack m^{5}U in its tRNA (34). The essentiality of the *trmA* gene suggests that a TrmA polypeptide is made in this mutant. Using antibodies toward purified tRNA(m^{5}U54)-methyltransferase, a polypeptide of the same size (42 kDa) as in the wild-type strain was found (Fig. 3). The 54- and 66-kDa bands represent forms of the tRNA(m^{5}U54)methyltransferase that are stably bound to RNA (C.G. and G.R.B., unpublished observation). Thus, although the *trmA5* mutant completely lacks m^{5}U54 in its tRNA, a TrmA peptide of normal size is made and it is still associated with RNA in the same way as the wild-type TrmA protein. We conclude that the essential part of the *trmA* gene is upstream of codon 338. Our results, taken together, show that although the *trmA* gene is essential, the tRNA (m^{5}U54)-methyltransferase activity is not.

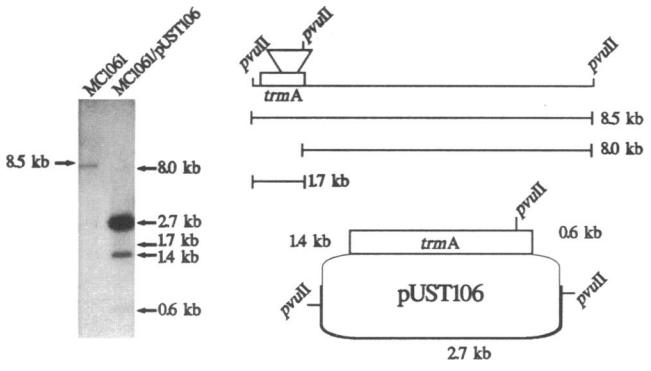

FIG. 2. Southern hybridization of DNA from strain MC1061 and *trmA*::*cat* derivative of strain MC1061/pUST106 (*trmA*$^+$). DNA was degraded by *Pvu* II and the different fragments were identified by using as probe oligolabeled plasmid pGP100. Sizes of the *Pvu* II fragments shown are for the *trmA*$^+$ chromosome (8.5 kb), the *trmA*::*cat* insertion on the chromosome (8.0 and 1.7 kb), and the plasmid pUST106 (*trmA*$^+$) (2.7, 1.4, and 0.6 kb).

DISCUSSION

We have used a λ phage from the Kohara library (λ4G11; Fig. 1), which carries the *trmA* gene, and recombined a *trmA*::*cat* insertion onto this phage. This phage was subsequently used to transfer the *trmA*::*cat* insertion into various *E. coli* strains. The data presented show that viable cells that contain the *trmA*::*cat* insertion can be recovered only if that cell has another intact copy of the *trmA* gene, either on a plasmid or as a result of a duplication covering *trmA*. This shows that the *trmA* gene is essential.

A hypothetical explanation for the lethal effect of the *cat* insertion in the *trmA* gene could be polarity on the expression of a downstream gene. If so, we must postulate that sufficient recombination could occur between plasmid and chromosome to allow expression of this putative downstream gene from an uninterrupted transcription unit when a *trmA*$^+$ plasmid is present in the cell. This explanation seems very unlikely, since *trmA* has been shown to be a monocistronic operon (21) and the downstream genes are transcribed in the opposite direction with a terminator shared by the *trmA* operon (cf. Fig. 1). The coding sequence of the downstream gene ends only 6 nucleotides downstream from the termination site of the *trmA* transcript. Furthermore, this possibility was ruled out by a direct experiment (Fig. 2). Since the complementing plasmid pUST106 used in this experiment only carries the *trmA* gene (Fig. 1), this gene must be essential.

The *trmA5* mutation results in a complete lack of m^{5}U in tRNA [detection limit, 0.2% of wild-type level (34)]. If such a low level (<0.2%) were still significant for growth, it would imply that <400 of the 200,000 tRNA molecules present in the cell (36) require m^{5}U54 for their function. If so, the mutant TrmA5 polypeptide must need to recognize these 400 tRNA molecules but none of the remaining >99.8%. Note that the only identity element required for the tRNA(m^{5}U54)-methyltransferase is 4 nucleotides all in the highly conserved TΨC loop (37), which are present in all *E. coli* tRNAs hitherto sequenced and in all 79 tRNA genes present in *E. coli* (38). Thus, all tRNA chains present in *E. coli* contain the identity element for the tRNA(m^{5}U54)methyltransferase. This fact strongly argues against the presence of a unique subpopulation of tRNA with respect to recognition by the tRNA-(m ^{5}U54)methyltransferase. Furthermore, the fact that all tRNA species in *Mycoplasma capricolum* are devoid of

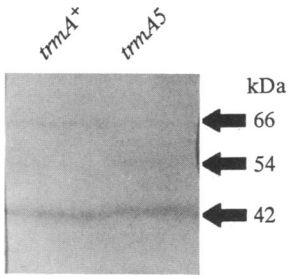

FIG. 3. Western blot analysis of protein extracts from strains MW100 (*trmA*$^+$) and GB1-5-39 (*trmA5*).

m^5U54 (10) shows that the presence of m^5U54 in tRNA is not essential for growth.

We propose that functionally the TrmA protein has two domains and that the domain near the C terminus, shown by biochemical (30) and genetic (*trmA21*::Tn*5*; see *Results*) studies to participate in m^5U54 modification, is distinct from that needed for viability. Note that although the *trmA5* mutation completely destroys the catalytic activity, a normal-sized peptide is still synthesized (Fig. 3).

We speculate that the essential function of TrmA protein is involved in the translation process since the tRNA(m^5U54)-methyltransferase activity is indirectly connected to this process, and it would seem reasonable if both functions were related. One possibility is suggested by the finding that another RNA-modifying enzyme (the *trmC* gene product) has two modification activities (35). Based on this precedent, the second function of TrmA protein might be synthesis of another modified ribonucleoside. If so, this RNA modification, in contrast to m^5U54, would be essential for viability. A second suggestion stems from our recent finding that the tRNA(m^5U54)methyltransferase protein binds covalently to a fragment from the 3′ end of 16S rRNA *in vivo* (35). The biological function of TrmA 16S rRNA covalent interaction is not yet known. The bound 16S rRNA does not influence the m^5U54 modification. Perhaps the observed 16S rRNA TrmA covalent interaction is part of modification of a ribosomal component, assembly of the ribosome, or RNA maturation—e.g., as an RNA chaperone. Since the *cat* insertion used here is not suitable for further studies to unravel the unknown essential function of the *trmA* gene, we need to isolate a conditionally lethal *trmA* mutation.

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