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Isolation and Partial Characterization of *Escherichia coli* Mutants with Low Levels of Transfer Ribonucleic Acid Nucleotidyltransferase

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To determine the function of the enzyme transfer ribonucleic acid (tRNA) nucleotidyltransferase in vivo, five mutants of *Escherichia coli* containing low levels of this enzyme were isolated. Since no selection procedure for such mutants existed, these strains were isolated by assay of large numbers of colonies from a heavily mutagenized stock. A procedure employing cells made permeable to tRNA and ATP was used to screen the large number of colonies required for the isolation. All the mutants contained less than 20% of the normal level of the AMP-incorporating activity of tRNA nucleotidyltransferase in extracts prepared by several methods, and the best mutant contained only about 2% of this activity. Three of the mutants also had equally low levels of the cytidine 5'-monophosphate-incorporating activity of the enzyme. Despite these low activities, the mutant strains displayed relatively normal growth characteristics at all temperatures examined. The enzyme in the mutant strains was not temperature sensitive, nor were any other abnormal biochemical properties detected. tRNA isolated from the mutant strains was missing significant amounts of its 3' terminal adenosine 5'-monophosphate residue, amounting to 10 to 15% in the best mutant. However, only small amounts of the terminal cytidine 5'-monophosphate residue were missing. The results indicate that tRNA nucleotidyltransferase is involved in some aspect of synthesis or repair of the 3' terminus of tRNA, and that the enzyme is present in large excess over its requirements for this function.

All transfer ribonucleic acid (tRNA) molecules contain the identical trinucleotide sequence -C-C-A at their 3' terminus, and enzymes are known which incorporate nucleotides into this sequence in vitro (see 8 for references). Despite the fact that these highly purified tRNA nucleotidyltransferases (EC 2.7.7.25) can synthesize a perfect -C-C-A sequence when presented with adenosine 5'-triphosphate (ATP), cytidine 5'-triphosphate (CTP), and tRNA molecules lacking all, or some, of the terminal residues (3, 7, 17), the physiological function of this enzyme is still not known. It has been assumed that tRNA nucleotidyltransferase plays a role in tRNA biosynthesis since labeling of tRNA in the presence of actinomycin (9, 15) and hybridization studies (5) suggested that the -C-C-A sequence is added in a post-transcriptional process. However, the data of Altman and Smith (2), on the sequence of a precursor of $tRNA^{Tyr}$, demonstrated that the -C-C-A sequence is already present in the precursor and followed by three additional nucleotides. From these data it was concluded that the -C-C-A terminus is synthesized during transcription.

tRNA nucleotidyltransferase has also been implicated in the end turnover of tRNA. However, since this process appears to be limited mainly to the terminal adenosine 5'-monophosphate (AMP) residue (12, 18, 19), whereas purified tRNA nucleotidyltransferase incorporates both AMP and cytidine 5'-monophosphate (CMP) residues into tRNA (6), the function of the enzyme probably is not limited solely to the repair of tRNA molecules. In addition, the ubiquitous nature of this enzyme and its extremely high turnover number (8) also would be consistent with another function. This function may be tRNA biosynthesis, but other alternatives are also possible.

To determine the role of tRNA nucleotidyltransferase in vivo, we have attempted to isolate *Escherichia coli* mutants with decreased levels

of this enzyme and to assess the physiological consequences of such a lesion. In this initial paper we describe the isolation and partial characterization of five independent mutants which contain altered levels of tRNA nucleotidyltransferase in extracts. The results suggest that tRNA nucleotidyltransferase is involved in some aspect of the synthesis of the 3′ terminus of tRNA, and that this activity is present in large excess over its functional requirements.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* AB301 (*metB1*) was obtained from the Yale University Genetic Stock Center and A19 (*metB1*, *rnsI*), a derivative of strain AB301 (10), was obtained from J. Speyer. In addition to low levels of ribonuclease I, strain A19 also has an unidentified trace growth requirement. Both strains carry the prophage λ.

Mutagenesis. Strain A19 was grown in YT medium (8 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) to an absorbancy at 550 nm (A_{550}) of about 1.0 (about 4×10^8 cells/ml). Cells from 4 ml of culture were centrifuged, washed twice with tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (1), and suspended in 1 ml of the buffer. The concentrated cell suspension was added to 3 ml of warmed buffer containing 2 mg of *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine and incubated for 30 min at 37 C with gentle shaking. The cells were centrifuged, washed twice with Tris-maleate buffer, and suspended in 40 ml of YT medium. Portions of 2 ml were distributed to 20 tubes, grown overnight at 25 C to allow segregation of the mutant phenotype, and plated on YT medium. Segregants were grown at 25 C for about 2 days and stored in the cold prior to assay. The extent of mutagenesis was routinely determined by the frequency of appearance of streptomycin-resistant cells.

Screening of mutants. Since there was no obvious selection procedure for mutants deficient in tRNA nucleotidyltransferase, it was necessary to assay every colony for its level of this enzyme. Also, to allow for the possibility that the mutant we sought might be conditionally lethal, cells were grown at 25 C and assayed at 43 C. Inasmuch as large numbers of colonies had to be assayed, a rapid screening procedure was devised. This procedure involved assaying tRNA nucleotidyltransferase in cells made permeable to ATP and tRNA by treatment with toluene (see accompanying paper).

Individual colonies were picked and grown for 16 to 18 h at 25 C in 1 ml of YT broth in small centrifuge tubes. Under these conditions cells were in late log phase. The contents of each tube were scored by eye for reasonable growth and centrifuged at 8,000 × *g* for 10 min in a Sorvall SE-12 rotor. The cell pellets were resuspended in 200 μliters of 50 mM glycine-NaOH buffer, pH 9.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA). Toluene, 3 μliters, was added and the suspension was vortexed and incubated for 5 min at 37 C. To the cells made permeable with toluene was added 200 μliters of a mixture containing: 50 mM glycine buffer, pH 9.0; 10 mM $MgCl_2$; 1 mM [α-^{32}P]ATP or [^{14}C]CTP (about 1,000 counts per min per nmol) and 400 μg of yeast tRNA (about 50% tRNA-C-C) or tRNA-C. Reaction mixtures were incubated for 10 min at 43 C, and terminated by the addition of 3 ml of 10% trichloroacetic acid-0.02 M sodium pyrophosphate. After 10 min in ice the samples were filtered through Whatman GF/C filters, washed six times with 3 ml of 2.5% trichloroacetic acid-0.02 M pyrophosphate, one time with ethanol-ether (1:1), and dried under an infrared lamp, and acid-precipitable radioactivity was determined.

In the presence of ATP and tRNA-C-C the assay measured predominantly tRNA nucleotidyltransferase activity as shown by the fact that AMP incorporation was into the 3′ terminal position of the tRNA (see accompanying paper). In addition, tRNA-C-C-A and tRNA-C were not acceptors of AMP. However, some incorporation of ATP into acid-precipitable material, amounting to about 20% of that with tRNA, also occurred in the absence of tRNA. This reaction, which was probably due to polynucleotide phosphorylase and poly(A) polymerase (M. P. Deutscher, unpublished data), did not interfere with the identification of mutants containing low levels of tRNA nucleotidyltransferase. Nevertheless, the presence of this tRNA-independent reaction did make it more difficult to quantitate exactly the low activities in the mutants by the screening assay (see below). When CMP incorporation into tRNA-C was determined, only a negligible tRNA-independent reaction was observed. Although yeast tRNA's were used in the experiments reported here, identical levels of AMP incorporation were obtained with *E. coli* tRNA-C-C in the screening assay.

Preparation of extracts and assay. Extracts of strain A19 and the putative mutants were also prepared in a variety of other ways in order to confirm the low tRNA nucleotidyltransferase activity observed in the assay of toluene-treated cells. The first method of preparing extracts was by centrifugation of toluene-treated cells. Cells grown to 1.0 A_{550} unit were concentrated fivefold by centrifugation and treated as in the screening procedure (except that EDTA was omitted) with 1.5% toluene for 15 min at 37 C. The cell suspension was then centrifuged for 10 min at 15,000 × *g*, and the supernatant fluid was withdrawn. This procedure served to extract a large proportion of the tRNA nucleotidyltransferase, probably owing to its relatively small size, whereas the tRNA-independent activity remained associated with the cell pellet. For this reason, this method of preparing extracts was the most useful for obtaining an accurate measure of the low level of tRNA nucleotidyltransferase in the mutants.

The second method of preparing extracts was by treatment with lysozyme-EDTA. Cells at 1.0 A_{550} were concentrated and suspended as in the screening procedure, followed by addition of 20 μg of egg white lysozyme and incubation for 10 min at 37 C with gentle shaking. The viscous cell suspensions clarified during this procedure were used directly.

Finally, extracts also were prepared by sonic dis-

ruption. Cells at 1.0 A_{550} were concentrated fivefold in 50 mM glycine-NaOH, pH 9.0, and subjected to sonic oscillation until clear (usually 45 s, with cooling after 30 s). Sonically treated preparations were usually used directly since centrifugation appeared to have no effect. The tRNA-independent activity in these extracts was appreciable and made exact quantitation of the mutant activities more difficult. In all cases, extracts were assayed as in the screening procedure. Thus, 200 μliters of the assay mixture was added to 200 μliters of the extract.

Isolation of tRNA. tRNA was isolated from the mutants and A19 by phenol extraction and isopropanol fractionation. Approximately 8 g (wet weight) of log-phase frozen cells was partially thawed, suspended in 14 ml of 0.9% NaCl, and shaken for 30 min at room temperature with 19 ml of 90% liquefied phenol (Fisher). Chloroform, 1.9 ml, was added and the mixture was shaken for an additional 15 min. The sample was then centrifuged at 27,000 × *g* for 10 min. To the upper aqueous layer was added 1.4 ml of 20% potassium acetate and 35 ml of ethanol. After 2 h at −15 C, the precipitate was collected by centrifugation, washed once with 75% ethanol, and dissolved in 10 ml of 0.3 M sodium acetate, pH 7.0. Isopropanol, 5.2 ml, was added with stirring at room temperature, and the precipitate was removed by centrifugation. An additional 4.8 ml of isopropanol was added to the supernatant solution; as before, the precipitate was collected, and the pellet was dried in a stream of air and dissolved in sterile water. Approximately 20 mg of purified tRNA was obtained by this procedure. The tRNA preparation was devoid of any high molecular weight material, but was contaminated with about 10% 5*S* RNA as shown by chromatography on Sephadex G-100.

Other methods. One-step growth of phage T4 was performed essentially as described by Stent (20) except that the basal level of infective centers was determined by subtracting the level of free phage after chloroform treatment, rather than by the use of anti-T4 serum. Protein was determined by the method of Lowry et al. (13). tRNA concentration was calculated from the absorbance at 260 nm assuming that a solution of 1 mg/ml would have an A_{260} of 22.0.

RESULTS

Isolation of mutants. Since no selection procedure existed for isolation of tRNA nucleotidyltransferase mutants, our approach involved the assay of large numbers of colonies from a heavily mutagenized stock (3 to 5% survival). Approximately 3,500 individual colonies, obtained from three separate mutageneses, were assayed, and 5 clones with low levels (<20%) of tRNA nucleotidyltransferase were isolated. In addition, several other strains with intermediate levels (20 to 60%) of enzyme were obtained, but were not characterized further. The frequency of mutation for this particular gene was between 0.1% and 0.3% of the cells examined, which is in the range observed for other markers (16). All of the mutant strains retained the phenotype of the A19 parent in requiring methionine and a trace nutrient for growth, and in containing low levels of ribonuclease I.

Growth characteristics. The mutant strains grew almost as well as the parent in YT liquid culture at 25, 37, and 43 C and attained almost identical maximum densities. Under conditions in which strain A19 had a doubling time of about 35 min, that of the mutants varied from 35 to 45 min. At the present time it is not known whether this small difference in growth rate is due to the mutation in tRNA nucleotidyltransferase or other mutations introduced during the heavy mutagenesis. The mutants also gave rise to colonies with the same efficiency as the parent at 25, 37, and 43 C on both rich and minimal media, although some of the strains had somewhat smaller colonies. These data suggested that none of the mutants would contain a temperature-sensitive tRNA nucleotidyltransferase activity (see below). The mutant strains appeared to have no additional nutritional requirements over those of the parent, suggesting that auxotrophic mutations were probably not introduced in these strains during the mutagenesis. We have not yet determined what effect, if any, the absence of ribonuclease I has on the growth of the mutant strains.

tRNA nucleotidyltransferase levels in mutants. Under conditions of the screening assay, in which cells were made permeable with toluene, all the mutants contained low or undetectable levels of the AMP-incorporating activity (Table 1). The relative activity in the mutants in this assay should only be regarded as a close approximation since the tRNA-independent re-

TABLE 1. *tRNA nucleotidyltransferase levels in toluene-treated cells (screening assay)*[a]

Strain	Relative sp act	
	AMP	CMP
A19	(100)	(100)
5C15	5	122
15A16	0	5
15A39	0	25
21-51	0	3
35-10	0	2

[a] Cells were grown, treated with toluene, and assayed for AMP incorporation into yeast tRNA-C-C or CMP incorporation into yeast tRNA-C. Incorporation in the absence of tRNA has been subtracted from each value. Specific activites for strain A19 were 5.8 nmol per 10 min per mg of protein for AMP incorporation and 2.4 for CMP incorporation.

action interfered to some extent (see Materials and Methods). The tRNA-independent reaction amounted to about 20% of that in the presence of tRNA with strain A19, and its specific activity in the mutants was almost identical to that in the parent strain. However, since this reaction was inhibited to a small extent by tRNA, some of the mutants actually had lower activity in the presence of tRNA than in its absence. On the other hand, the tRNA-independent reaction did not interfere in the measurement of CMP incorporation into tRNA-C, so that the relative percentage in this case (Table 1) was an accurate measure of the level of activity in the mutant. With this assay, two of the mutants, 5C15 and 15A39, appeared to contain considerably higher amounts of CMP-incorporating activity than would have been expected from their level of AMP incorporation.

To substantiate the relative activities of AMP and CMP incorporation observed in the screening assay, extracts were also prepared from strain A19 and the mutant strains by several other procedures. These included preparation of toluene supernatant fractions, treatment with lysozyme-EDTA or sonic oscillation. The first method had the advantage that the tRNA-independent reaction was absent, so that more accurate levels for the AMP activity could be ascertained. The latter two methods led to complete rupture of cells so that any possible difficulties in making the mutants permeable with toluene would be overcome. However, since sonic treatment led to extremely high values for the tRNA-independent reaction, these latter values are somewhat less accurate. As can be seen in Table 2, all the methods of preparing extracts gave essentially the same relative activities for the mutants compared to the parental strain. Thus, all the mutants had low levels of activity for AMP incorporation, with strain 35-10 containing as little as 2% of the normal activity. In addition, strains 15A16, 21-51, and 35-10 contained equally low amounts of the CMP-incorporating activity. In contrast, as seen earlier in the screening assay, mutants 5C15 and 15A39 had anomalously high levels of the latter activity. In fact, CMP incorporation catalyzed by extracts of strain 5C15 repeatedly was more active than that of strain A19. Since it has previously been shown that AMP and CMP incorporation are catalyzed by a single protein (3, 6), the mutations in strains 5C15 and 15A39 must have differentially affected the two activities. The observation that most of the mutants had low levels of both activities lends further support to the conclusion that only one protein is involved. On the other hand, the isolation of a mutant (5C15) in which AMP incorporation is greatly reduced, but in which CMP incorporation is normal should prove useful for studies of the active site of this enzyme.

The time course of AMP incorporation catalyzed by toluene-supernatant extracts from strain A19 and the two best mutants, 21-51 and 35-10, is shown in Fig. 1A. The relative activity in the mutants remained essentially the same over a period of 20 min, and was only slightly greater than the non-tRNA control. Similarly, the relative activity in the mutants was unchanged over an eightfold range in the amount of protein added to the assay (Fig. 1B).

To eliminate the possibility that the mutants contained an inhibitor of tRNA nucleotidyltransferase, mixing experiments were performed with extracts of strain A19 and the mutants. We found that AMP incorporation in the presence of both strain A19 and a mutant extract was exactly that expected from additivity, indicating that no free inhibitor was present. Likewise, mixing extracts of mutants, in pairs, suggested that in vitro complementation could not take place.

Other biochemical properties of tRNA nucleotidyltransferase mutants. Enzyme activity was originally determined at 43 C in cells grown at 25 C in order that heat-sensitive enzyme in conditional lethal mutants might be detected. Once the mutants had been identified we were interested in determining whether the tRNA nucleotidyltransferase in these cells was, in fact, thermolabile. These studies were carried out both with the toluene supernatant extracts in order to eliminate interference from the tRNA-independent reaction and with sonically treated extracts. We found that the relative

TABLE 2. *tRNA nucleotidyltransferase levels in extracts prepared by various methods*[a]

Strain	Relative sp act					
	Toluene supernatant		Lysozyme-EDTA		Sonic oscillation	
	AMP	CMP	AMP	CMP	AMP	CMP
A19	(100)	(100)	(100)	(100)	(100)	(100)
5C15	17	119	23	130	21	108
15A16	6	4	1	7	11	9
15A39	6	18	4	40	0	27
21-51	3	2	2	8	7	9
35-10	2	3	0	7	0	6

[a] Cells were grown and extracts prepared either by toluene treatment, lysozyme-EDTA, or sonic oscillation. Extracts, 200 μliters, were assayed with 200 μliters of the assay mix for 5 min at 43 C exactly as described for the screening assay.

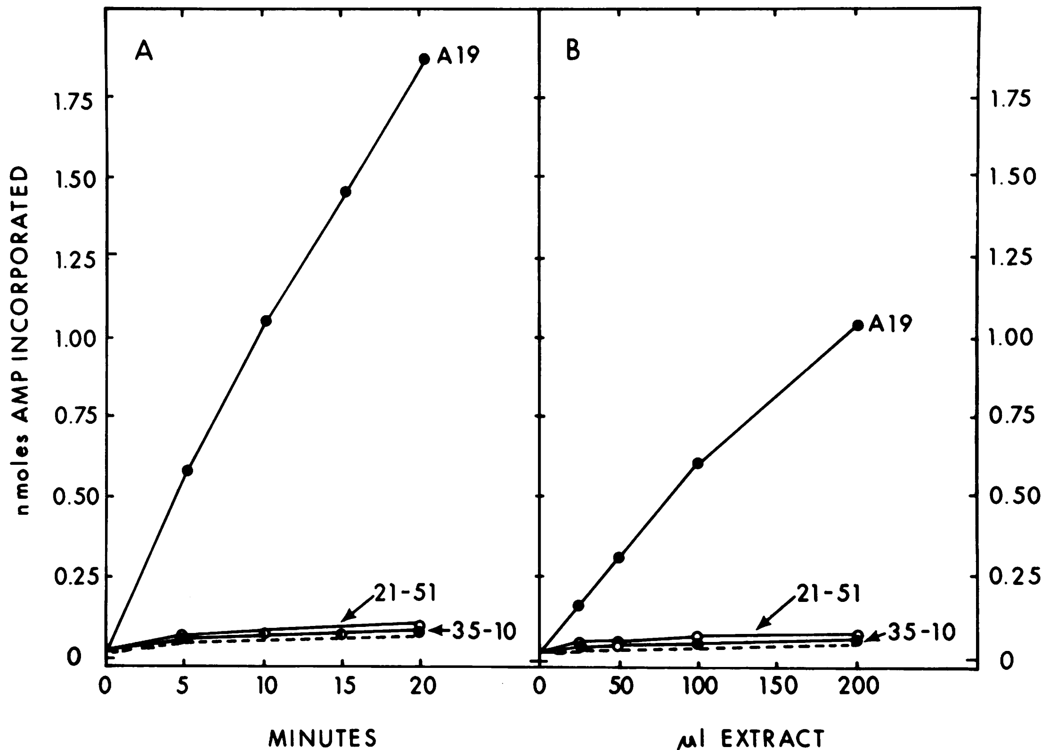

FIG. 1. *Effect of time and extract concentration of tRNA nucleotidyltransferase activity from wild-type and mutant cells. Cells were grown to 1.0* A_{550}*, and the toluene supernatant fraction was prepared. (A) The toluene supernatant extracts (200 µliters) from strains A19, 21-51, and 35-10 were assayed for AMP incorporation for the indicated times at 43 C. (B) Indicated amounts of extract were assayed for AMP incorporation for 10 min at 43 C. The dashed line represents AMP incorporation in the absence of added tRNA, and was identical for all three strains.*

activity in the mutants compared to strain A19 for both AMP and CMP incorporation remained essentially constant at temperatures from 25 to 50C. In addition, since it was possible that enzyme would be inactivated during the toluene treatment at 37 C, cells were extracted at both 25 and 30 C and assayed at low temperatures. Under these conditions, also, the relative AMP-incorporating activity of the mutant extracts was as low as under the standard assay conditions. These data indicated that the tRNA nucleotidyltransferase in the mutants was not temperature sensitive. The results are consistent with the finding that the mutant strains are not temperature sensitive for growth.

Since the mutants did not contain heat-labile enzyme, preliminary studies were carried out to ascertain what was the biochemical defect in tRNA nucleotidyltransferase in the mutants that led to low activity. The relative AMP-incorporating activity in all the mutants remained essentially the same in the pH range of 7.0 to 9.5. In addition, in more detailed studies with mutants 21-51 and 35-10, this relative activity was not affected by changes in ATP concentration from 0.075 to 1 mM, and in tRNA concentration from 0.12 to 1.5 mg/ml. Thus, at the present time we have not been able to determine the biochemical defect in tRNA nucleotidyltransferase which leads to low activity.

Biological properties of tRNA nucleotidyltransferase mutants. Since tRNA nucleotidyltransferase has been thought to be involved in some aspect of the synthesis of the -C-C-A terminus of tRNA, we isolated tRNA from strain A19 and from each of the mutant strains and the state of the 3′ terminal residues was examined. As shown in Table 3, tRNA from each of the mutants was missing a fraction of its terminal AMP residue, and the mutants with lower activity contained a higher amount of defective tRNA. In the mutant, 35-10, which only had about 2% of the AMP-incorporating activity of the parent, about 10 to 15% of the tRNA population was missing its 3′ terminal AMP residue. Identical results were obtained

TABLE 3. *State of 3' termini in tRNA from wild type and tRNA nucleotidyltransferase mutants*[a]

Strain	Nucleotide incorporation (nmol/mg)		Termini missing (%)	
	AMP	CMP	AMP	CMP[b]
A19	<0.1	0.1	0	0.3
5C15	0.2	0.1	0.7	0.3
15A16	0.9	0.6	2.6	1.7
15A39	1.1	0.4	3.1	1.1
21-51	2.3	0.6	6.6	1.7
35-10	3.9	0.7	11.2	2.0

[a] tRNA was isolated from the various strains and the state of its termini was determined by the extent of AMP and CMP incorporation with purified liver tRNA nucleotidyltranferase. Reaction mixtures contained 100 μg of the appropriate tRNA, and excess enzyme, and were incubated for 30 min at 37 C. Incorporation has been corrected for the small amount of non-tRNA material in the preparations. Identical values for AMP incorporation were also found in the presence of CTP.

[b] The value for percent CMP residues missing was calculated assuming only one CMP residue was absent per tRNA chain.

with tRNA isolated from stationary phase A19 and 35-10, and from cells grown at 42 C. In contrast to the results for AMP, only a small fraction of CMP residues were missing from any of the mutants (Table 3), although the two mutants with higher CMP-incorporating activities, 5C15 and 15A39, did contain the smallest amount of tRNA lacking CMP residues. These results are consistent with an involvement of tRNA nucleotidyltransferase in the synthesis of at least part of the -C-C-A terminus of tRNA, although it appears that the mutants may still contain too much activity for observation of any major physiological defect (see Discussion).

Nevertheless, during the course of examination of the biological properties of these strains, we observed that phage T4 grew very poorly on all of the mutants. In one-step growth experiments, the burst size of T4 in the tRNA nucleotidyltransferase mutants was considerably lower than that in the parental strain (Table 4). In addition, the latent period was altered in several of the mutant strains. At the present time we do not know the significance of these findings. However, since these strains have been heavily mutagenized the possibility still exists that the poor growth of T4 is due to a secondary mutation. On the other hand, we have shown recentiy (11) that no new tRNA nucleotidyltransferase is made after T4 infection. Thus, since T4 directs the synthesis of new tRNA molecules upon infection (4, 22), a defect in the host enzyme could have some effect on proper T4 maturation, although it should be pointed out that deletions of T4 lacking the tRNA genes are still viable in some *E. coli* strains (23).

DISCUSSION

In this paper we described the isolation and initial characterization of five mutants with low levels of tRNA nucleotidyltransferase. Some of these mutants lacked greater than 95% of tRNA nucleotidyltransferase activity in extracts, yet the cells appeared to be relatively normal. However, the activity measured in extracts may not be an accurate reflection of the situation in vivo. On the other hand, the enzyme may normally be present in such large excess that the presence of only 2% of the wild-type activity may be sufficient to keep most of the tRNA in an active state. That this latter explanation may be correct is suggested from the levels of tRNA and tRNA nucleotidyltransferase in *E. coli* cells. Maaløe and Kjeldgaard (14) reported that 2×10^5 to 6×10^5 molecules of tRNA are present per cell, which is equivalent to 0.3 to 0.9 nmol per 10^9 cells. If it is assumed that tRNA nucleotidyltransferase is involved in tRNA biosynthesis it would have to synthesize this amount of termini in every generation of about 40 min. We have determined the total tRNA nucleotidyltransferase activity in cells opened under a variety of conditions and found that there is sufficient enzyme to incorporate 18 nmol of AMP per 10^9 cells per 40 min. Therefore, this enzyme appears to be present in a 20- to 60-fold excess over its presumed requirements, which could explain why as much as 95% of the enzyme can be absent from mutant cells while normal growth characteristics are retained.

Although we undertook the isolation of tRNA nucleotidyltransferase mutants in order to determine the role of this enzyme in vivo, we are not yet in a position to answer this question completely because the mutants appear to have too much residual activity. Nevertheless, the fact that there is a correlation between the enzyme level and the fraction of tRNA molecules with defective termini indicates for the

TABLE 4. *Burst size of T4 in wild type and tRNA nucleotidyltransferase mutants*

Strain	T4 burst size
A19	59
5C15	4
15A16	3
15A39	4
21-51	10
35-10	7

first time that tRNA nucleotidyltransferase is involved in some way in the synthesis of the terminus of tRNA. However, since there appears to be a much greater effect on the terminal AMP than on the CMP residues, this enzyme may only participate in repair of the end turnover of tRNA rather than in tRNA biosynthesis. At the present time, we also do not know the effect of the presence of the *rnsI* mutation in the mutant strains. These questions can only be answered with the isolation of mutants with lower activity, of temperature-sensitive mutants, or of deletions. As a first step toward these goals we have mapped the position of the mutation in the tRNA nucleotidyltransferase-deficient strains (J. Foulds, R. Hilderman, M. Deutscher, accompanying paper), and found that it is located at about 59 min on the *E. coli* map (21).

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