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tRNA nucleotidyltransferase is not essential for *Escherichia coli* viability

Liuqin Zhu and Murray P.Deutscher

Department of Biochemistry, University of Connecticut Health Center, Farmington, CT 06032, USA

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The role of tRNA nucleotidyltransferase in *Escherichia coli* has been uncertain because all tRNA genes studied in this organism already encode the -C-C-A sequence. Examination of a *cca* mutant, originally thought to contain 1-2% enzyme activity, indicated that it actually produces an inactive fragment of 40 kd compared to 47 kd for the wild-type enzyme due to a nonsense mutation in its *cca* gene. To confirm that the residual activity in extracts of this strain is due to another enzyme, and that tRNA nucleotidyltransferase is non-essential, we have interrupted the *cca* gene *in vitro*, and transferred this mutant gene to a variety of strains. In all cases mutant strains are viable, although as much as 15% of the tRNA population contains defective 3' termini, and no tRNA nucleotidyltransferase is detectable. Mutant strains grow slowly, but can be restored to more normal growth by a *relA* mutation or by a decrease in RNase T activity. In the latter case the amount of defective tRNA decreases dramatically. These findings indicate that tRNA nucleotidyltransferase is not essential for *E. coli* viability, and therefore, that all essential tRNA genes in this organism encode the -C-C-A sequence.

Key words: tRNA/-C-C-A terminus/RNA processing

Introduction

In vitro tRNA nucleotidyltransferase catalyzes the accurate synthesis of the -C-C-A terminus of tRNA (Deutscher, 1982). However, *in vivo* the situation is less clear. In all eukaryotic cells so far examined the -C-C-A sequence is not encoded in the tRNA genes (Deutscher, 1983), and must be added post-transcriptionally, presumably by this enzyme, although no mutants have yet been isolated to conclusively prove this point. In contrast, in prokaryotic systems no discrete pattern has emerged. For example, in *Bacillus* species (Vold, 1985) and in T-even bacteriophages (Moen *et al.*, 1978) certain tRNA genes encode the -C-C-A sequence and others do not. On the other hand, in *E. coli* all tRNA genes studied to date contain the -C-C-A sequence (Fournier and Ozeki, 1985), so that post-transcriptional addition of these residues may not be needed. This, of course, has raised questions as to what the role of tRNA nucleotidyltransferase might be in this organism.

Previous studies with *E. coli cca* mutants deficient in tRNA nucleotidyltransferase (Deutscher and Hilderman, 1974) indicated that at low levels of the enzyme cells accumulated a population of tRNA molecules with defective 3' termini and also grew more slowly (Deutscher *et al.*, 1974; Deutscher *et al.*, 1977a). As expected from the fact that the -C-C-A sequence is encoded, the defective tRNA molecules arose largely as a consequence of the end-turnover process known to occur *in vivo*; tRNA biosynthesis appeared to be unimpaired (Deutscher *et al.*, 1977a). In phage T4-infected *cca* strains, in contrast, T4-encoded tRNA precursor molecules lacking the complete 3' terminus were not matured (Deutscher *et al.*, 1974; McClain *et al.*, 1978), demonstrating that at least in this system tRNA nucleotidyltransferase is required for tRNA biosynthesis. However, since the most defective *cca* mutant, strain 35-10, apparently still retained 1-2% of tRNA nucleotidyltransferase activity (Deutscher *et al.*, 1977a), it was unclear whether the enzyme was dispensable in uninfected cells.

In this paper we show that the mutant tRNA nucleotidyltransferase made in strain 35-10 is an inactive fragment protein, produced as a consequence of a nonsense mutation in the *cca* gene, and indicating that the residual activity in extracts of this cell is due to another enzyme. We show further that *E. coli* strains containing an interrupted *cca* gene, nevertheless, remain viable. These findings demonstrate that tRNA nucleotidyltransferase is not essential in laboratory strains of *E. coli*, and therefore, that all essential tRNA genes in this organism probably encode the -C-C-A sequence.

Results

tRNA nucleotidyltransferase in strains 35-10

In our earlier studies of the *cca* mutation in strain 35-10 we were unable to examine the structure of its tRNA nucleotidyltransferase because the low activity precluded extensive purification. Our recent cloning and over-expression of the *E. coli cca* gene (Cudny *et al.*, 1986), and the preparation of an antibody against tRNA nucleotidyltransferase (Zhu *et al.*, 1986), have now provided us with alternate procedures to examine this question.

Plasmid-directed protein synthesis in maxicells by the cloned 35-10 *cca* gene (Figure 1, lane 2) reveals two protein bands, one of 40 kd and one of 20 kd, that are absent in the vector (lane 1). Neither of these bands corresponds to that of the wild-type tRNA nucleotidyltransferase at 47 kd (lanes 3 and 4). The lower molecular weight protein is not the product of the *cca* gene since it is not made from a 1.9-kb insert containing the wild-type *cca* gene (lane 3), but is synthesized when the larger 4-kb insert is used (lane 4). These results suggest that the 35-10 *cca* gene product is the 40-kd protein.

In order to confirm this assignment, immunoblotting of extracts from various strains was carried out using a rabbit antiserum raised against wild-type tRNA nucleotidyltransferase. As shown in Figure 2, clones containing the wild type *cca* gene (on either the 4-kb or 1.9-kb insert) showed a major reactive band at 47 kd (lanes 4 and 6 respectively), as expected for the wild-type enzyme. In contrast, the product of the 35-10 *cca* gene, cloned in either orientation, is a protein of 40 kd (lanes 3 and 5), supporting the maxicell data (Figure 1). These results indicate that the tRNA nucleotidyltransferase present in strain 35-10 is smaller than the normal protein.

DNA sequencing of the *cca* gene from strain 35-10 revealed only a single nucleotide change that converts the tryptophan

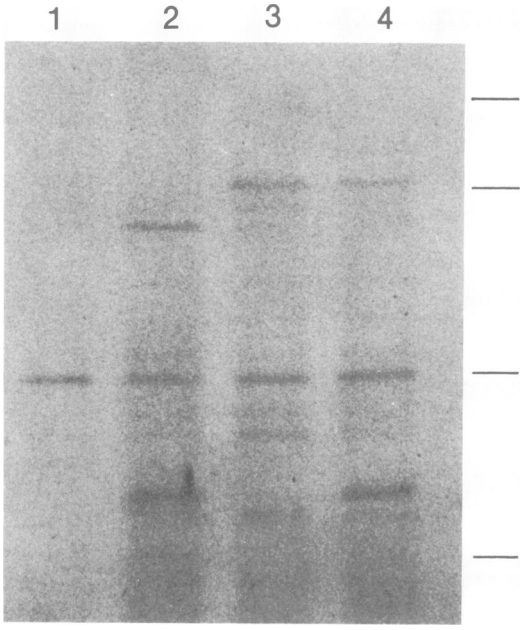

Fig. 1. Maxicell analysis of cloned 35-10 *cca* gene. Maxicell strain CSR603 cells were transformed with either pUC8 (vector alone, **lane 1**), pEC-1M (35-10 *cca* gene on 4-kb insert, **lane 2**), pEC-4 (wild-type *cca* gene on 1.9-kb insert, **lane 3**) or pEC-6 (wild-type *cca* gene on 4-kb insert, **lane 4**), labeled with [^{35}S]methionine and extracts run on SDS-PAGE as described (Cudny and Deutscher, 1986). The migration positions of the molecular weight standards from top to bottom are shown on the right: bovine serum albumin, 68 kd; ovalbumin, 44 kd; α-chymotrypsinogen, 26 kd; β-lactoglobulin, 18 kd. The constant band in all lanes is β-lactamase encoded by the vector.

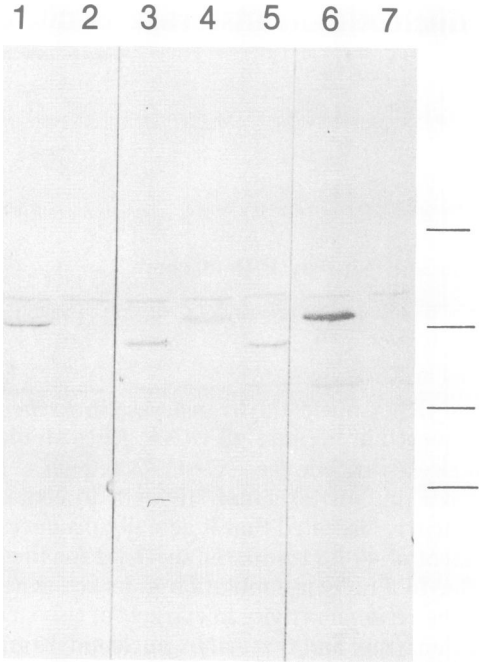

Fig. 2. Immunoblotting of extracts from strain UT481 harboring various plasmids. Cells were grown to an A_{550} of ~1. Cells from 10 ml of culture were spun down, resuspended and sonicated in 0.4 ml of the extraction buffer described in Table I. Aliquots of 10 μl were run on SDS–PAGE, transferred to nitrocellulose and treated with rabbit antiserum raised against tRNA nucleotidyltransferase and with ProtoBlot. **Lane 1**, pEC-CAT-7; **lane 2**, pEC-CAT-10; **lane 3**, pEC-1M; **lane 4**, pEC6; **lane 5**, pEC-2M; **lane 6**, pEC-4; **lane 7**, pUC8. Under these conditions the low level of tRNA nucleotidyltransferase in strain UT481, itself, is not visible (e.g. lanes 2 and 7). The migration positions of the molecular weight standards, as in Figure 1, are shown on the right.

codon, UGG, at position 362 to the termination codon, UGA (Cudny *et al.*, 1986). The molecular mass of a protein terminating at this position was calculated to be 40 540, in excellent agreement with the results of the maxicell and immunoblotting experiments. These data demonstrate that the presence of a tRNA nucleotidyltransferase fragment protein in strain 35-10 is a consequence of a nonsense mutation in the *cca* gene of this cell.

Our earlier studies of strain 35-10 indicated that a low level of AMP- and CMP-incorporating activity remained in extracts, and this activity was attributed to residual tRNA nucleotidyltransferase (Deutscher *et al.*, 1977a). In light of these new findings that the 35-10 enzyme is a fragment, and other information that a deletion mutant lacking 21 C-terminal amino acids is devoid of tRNA nucleotidyltransferase activity (L.Zhu and M.P.Deutscher, unpublished results), we have examined whether the residual activity in strain 35-10 is in fact the product of the *cca* gene. The cloned 35-10 *cca* gene in pUC8 (pEC-1M) was used to transform strains UT481 and 35-10. Since the presence of the wild-type *cca* gene in pUC8 normally leads to over a 100-fold elevation of tRNA nucleotidyltransferase activity (Cudny and Deutscher, 1986), we anticipated that even a 1% level of activity for the 35-10 *cca* gene product should lead to a doubling of activity in strain UT481, and a 100-fold increase in strain 35-10 itself. However, the data in Table I indicate that the presence of a cloned 35-10 *cca* gene does not lead to any elevation of AMP- or CMP-incorporating activity in extracts of either strain. These results demonstrate that the product of the 35-10 *cca* gene cannot catalyze nucleotide incorporation *in vitro*, and that the residual activity found in extracts of this strain must, therefore, be due to another enzyme. However, the 35-10 tRNA nucleotidyltransferase does appear to retain a very small amount of activity *in vivo* since in the presence of plasmid pEC-1M, which overexpresses the mutant enzyme, strain 35-10 contains only half as much defective tRNA as it does in the absence of a plasmid, or when the vector, pUC8, is present.

Table I. tRNA nucleotidyltransferase activity in strain containing the 35-10 *cca* gene or the interrupted *cca* gene

Strain (plasmid)	Relevant properties	Nucleotide incorporation nmol/mg protein	
		AMP	CMP
35-10	CCA^-	4.2	1.9
35-10 (pUC8)	CCA^-, vector	3.3	<1.0
35-10 (pEC-1M)	CCA^-, 35-10 *cca* gene on plasmid	2.9	1.2
UT481	CCA^+	36.2	26.2
UT481 (pEC-1M)	CCA^+, 35-10 *cca* gene on plasmid	37.5	26.9
CF881	CCA^+	83.6	36.4
CF101	CCA^-, interrupted *cca* gene	2.8	2.1

Cells were grown to an A_{550} of ~1, concentrated 10- to 20-fold in 10 mM Tris–HCl, pH 8.0, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and sonicated in two 20-s pulses with cooling between. Aliquots were assayed for AMP or CMP incorporation into yeast tRNA for 15 min at 37°C as described (Cudny and Deutscher, 1986).

Isolation of a strain with an interrupted cca gene

The implications of the results presented above are that tRNA nucleotidyltransferase is not essential for the viability of *E. coli*.

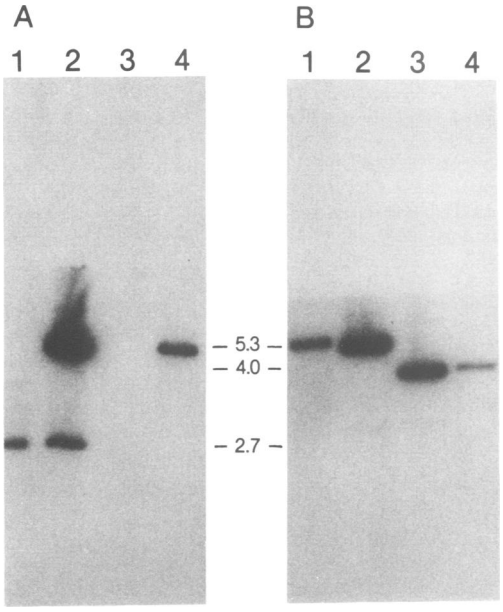

Fig. 3. Hybridization analysis of the interrupted *cca* gene. Chromosomal DNA from strains CF881 (wild-type *cca* gene) and CF101 (interrupted *cca* gene), and plasmid DNA from pEC-6 (wild type *cca* gene) and pEC-CAT-10 (interrupted *cca* gene) were digested with *Bam*HI, electrophoresed on 0.8% agarose, transferred to nitrocellulose and hybridized to ^{32}P-labeled probes specific for the *cat* gene or the *cca* gene. **Panel A**, *cat* gene probe. **Lane 1**, pEC-6; **lane 2**, pEC-CAT-10; **lane 3**, CF881; **lane 4**, CF101. **Panel B**, *cca* gene probe. **Lane 1**, CF101; **lane 2**, pEC-CAT-10; **lane 3**, pEC-6; **lane 4**, CF881. The sizes of bands are based on standards run on the same gel.

Table II. State of tRNA termini in strains containing an interrupted *cca* gene

Strain	Relevant phenotype	Nucleotide incorporation nmol/mg protein	
		AMP	CMP
CA265	RNase T$^+$	0.4	0.1
CA265 *cca*$^-$	RNase T$^+$	5.0	0.9
20-12E/18-11	RNase T$^-$	0.4	0.2
20-12E/18-11 *cca*$^-$	RNase T$^-$	1.8	0.2
CP78	Stringent	0.3	0.1
CP78 *cca*$^-$	Stringent	5.3	0.8
CP79	Relaxed	0.2	0.2
CP79 *cca*$^-$	Relaxed	5.6	0.8

tRNA was isolated from each of the indicated strains, and the extent of nucleotide incorporation into ~100 μg was determined with purified tRNA nucleotidyltransferase as described (Deutscher *et al.*, 1985).

However, in order to conclusively prove this point, and to eliminate the possibility that even the very low level of enzyme in strain 35-10 might have some effect *in vivo*, we have interrupted the *cca* gene *in vitro* with a DNA fragment containing the *cat* gene. This construct was used to replace the endogenous *cca* gene in strain CF881 (see Materials and methods for details) generating strain CF101. As shown in Figure 3, strain CF101 contains a single 5.3-kb *Bam*HI fragment that hybridizes with both a *cat* gene probe and a *cca* gene probe, whereas with the parental strain, CF881, there is no hybridization to the *cat* probe, and the *cca* probe hybridizes only to the usual 4-kb *Bam*HI fragment (Cudny *et al.*, 1986). Likewise, plasmid pEC-CAT-10, used to replace the chromosomal *cca* gene, contains the same 5.3-kb fragment hybridizing with both probes, whereas pEC-6, which contains the wild-type *cca* gene, is missing this fragment and contains a 4-kb fragment hybridizing only with the *cca* gene probe.

Additional evidence for interruption of the *cca* gene in strain CF101 comes from the immunoblotting experiment shown in Figure 2. The presence of plasmid pEC-CAT-10, harboring the interrupted *cca* gene, does not lead to the synthesis of any protein cross-reacting with antiserum to tRNA nucleotidyltransferase (lane 2). In contrast, the presence of plasmid pEC-CAT-7 containing another *cat* insertion, nearby, but outside of the *cca* gene, leads to a cross-reacting band at the expected position of 47 kd (lane 1). Furthermore, extracts of strain CF101 show a very low level of AMP- and CMP-incorporating activity, amounting to only a few percent of that in the parental strain, CF881 (Table I). Taken together, these data indicate that the *cca* gene in strain CF101 is interrupted with a *cat* fragment, and therefore, that an intact gene is not required for *E. coli* viability. Apparently, the low level of activity seen in extracts of this strain is due to another enzyme (see below).

Properties of strains with an interrupted cca gene

Although strain CF101 is viable, its growth rate (~70 min doubling time) is considerably slower than its *cca*$^+$ parent, strain CF881 (~30 min doubling time), in agreement with previous studies of the *cca* mutation in strain 35-10 (Deutscher *et al.*, 1974). The earlier studies also showed that the growth rate of 35-10 *cca* mutants could be increased by the presence of either a *relA* mutation (Deutscher *et al.*, 1977b) or by a mutation decreasing the activity of RNase T (Deutscher *et al.*, 1985). Similar results have been obtained in the present work. The interrupted *cca* gene from strain CF101 was transferred by P1 transduction to strains CP78 and CP79, an isogenic *relA*$^+$–*relA* pair respectively, and to strains CA265 (RNase T$^+$) and 18-11 (RNase T$^-$). Most importantly, in all cases strains containing the interrupted *cca* gene were viable, and as before, the RNase T and *relA* mutations increased the growth rate of the *cca* strains. In fact, the RNase T mutation in strain 18-11 dramatically increased the growth rate of its *cca* derivative compared to that of CA265 (~30 min versus ~85 min doubling time), to a value which is close to that of the *cca*$^+$ parent (~25 min doubling time).

Another property of *cca* mutants is that they accumulate tRNA molecules with defective 3′ termini (Deutscher and Hilderman, 1974; Deutscher *et al.*, 1977a). Strains containing the interrupted *cca* gene exhibit the same phenotype (Table II). The maximum amount of defective termini accumulated is ~5 nmol/mg, or ~15% of the total tRNA, despite the fact that the cell is devoid of tRNA nucleotidyltransferase. Furthermore, the defect largely affects only the terminal AMP residue since only about one-fifth as many CMP residues are absent. As observed earlier, the presence of a mutation in RNase T substantially reduces the amount of defective tRNA (Deutscher *et al.*, 1985), whereas the *relA* mutation has no such effect (Deutscher *et al.*, 1977b). These findings indicate that the phenotypic properties of cells with an interrupted *cca* gene are similar to those of cells containing the 35-10 *cca* mutation, although slightly more extreme.

In order to assess the source of the residual nucleotide-incorporating activity in strain CF101, high-speed supernatant fractions from this strain and its parent, CF881, were compared by gel filtration on Ultrogel AcA44 (data not shown). Two peaks of AMP- and CMP-incorporating activity were observed for the parental strain, one at a position corresponding to a molecular mass of ~50 000, and one in the void volume (M_r >130 000). In contrast, only a single peak of nucleotide-incorporating activity was found when the *cca* gene was interrupted, correspon-

ding to the enzyme eluting in the void volume. These data confirm the absence of tRNA nucleotidyltransferase in strain CF101 and demonstrate the presence of an additional activity capable of nucleotide incorporation.

Discussion

The data presented here, using two distinct *cca* mutants, indicate that tRNA nucleotidyltransferase is not essential for *E. coli* viability. Our initial analysis of strain 35-10 indicated that a low level of nucleotide-incorporating activity remained in extracts of the mutant (Deutscher *et al.*, 1977a), so that it was uncertain whether the enzyme is dispensable. It is now clear that the tRNA nucleotidyltransferase made in this strain is a fragment of 40 kd that has no activity *in vitro*. Since the possibility remained that this large fragment protein might have some residual effect *in vivo*, even when present at very low levels due to a single mutant chromosome *cca* gene, additional studies with strains containing an interupted *cca* gene were performed. These confirmed that cells are viable even in the absence of any tRNA nucleotidyltransferase protein.

The ability of *E. coli* to survive in the absence of tRNA nucleotidyltransferase strongly suggests that all essential tRNA genes in this organism must encode the −C−C−A sequence. This conclusion agrees with and expands what has been found to date from the sequencing of *E. coli* tRNA genes (Fournier and Ozeki, 1985). In this respect *E. coli* differs from all other organisms studied (Deutscher, 1983; Vold, 1985). It is not clear whether this is a consequence of many years of culture under laboratory conditions, or is a distinct property of this bacterium. Analysis of tRNA genes from natural *E. coli* populations would be of interest in this regard. Nevertheless, at present, the only known function of tRNA nucleotidyltransferase in *E. coli* is the repair of the 3′ terminus of defective tRNA molecules that arise by the occasional action of RNase T (Deutscher *et al.*, 1985). Inasmuch as the level of this population does not exceed 15% of the total tRNA, and is largely lacking only the terminal AMP residue, the role of tRNA nucleotidyltransferase appears minimal, and very different from its presumed function in other organisms. Likewise, the role of RNase T *in vivo* remains to be explained.

It is still unclear what enzyme(s) might account for the residual nucleotide-incorporating activity present in extracts, and whether it might act on tRNA *in vivo*. The high molecular weight of the second activity suggests that polynucleotide phosphorylase might be involved, but further work is necessary to substantiate this idea. Previous studies with strain 35-10 indicated that defective tRNA could be repaired very slowly *in vivo* in the presence of antibiotics which prevented further end-turnover or new tRNA synthesis (Deutscher *et al.*, 1977a). If this is so, then the very low level of mutant tRNA nucleotidyltransferase or the unidentified activity may function *in vivo*. Alternatively, the defective tRNA could also be degraded slowly since the enzyme, RNase D, which is known to act on these molecules *in vitro* (Ghosh and Deutscher, 1978), is present in these cells.

Materials and methods

Materials

Ribonucleoside triphosphates and antibiotics were obtained from Sigma Chemical Co. Yeast tRNA, [^{14}C]ribonucleoside triphosphates and [α-^{32}P]ATP were from Schwarz/Mann. Restriction endonucleases, phage T4 DNA ligase and the Klenow fragment of DNA polymerase I were products of New England Biolabs. Proto Blot anti-rabbit IgG alkaline phosphatase conjugate was obtained from Promega Biotec. All chemicals used were reagent grade.

Bacterial strains, plasmids and growth conditions

Wild-type *E. coli* strain CA265, its RNase$^-$ derivative, 20-12E/18-11 and CCA$^-$ strain, 35-10, have been described (Deutscher and Hilderman, 1974; Deutscher *et al.*, 1985). The isogenic stringent-relaxed pair, CP78-CP79 (Fiil and Friesen, 1968), and strain CSR603, used to prepare Maxicells (Sancar *et al.*, 1979), were obtained from the Coli Genetic Stock Center, Yale University. Strain UT481 [Δ(lac-pro),r$^-$,m$^-$, lacI^q,lacZ], for plasmid growth, was obtained from Dr Gordon Carmichael. Strain CF881 (*recB, xthA, rna1*), for linear DNA transformation, was obtained from Dr Asis Das.

Plasmid vectors, pUC8 and pUC13 (Vieira and Messing, 1982) and pBR325 (Bolivar, 1978) have been described. The construction of plasmid pEC4, containing a 1.9-kb *cca*$^-$ insert in pUC8, has been reported earlier (Cudny and Deutscher, 1986); plasmid pEC6, containing a 4-kb *cca*$^+$ insert in the *Bam*H1 site of pUC13 was a gift from Dr Henryk Cudny. Plasmids pEC-1M and pEC-2M contain a 4-kb insert of the 35-10 mutant *cca* gene in either orientation in the *Bam*H1 site of pUC8. The orientations were verified by sequence analysis; the *cca* promoter in pEC-2M has the same orientation as the *lacZ* promoter in pUC8.

Cells were routinely grown in YT medium ± 0.4% glucose (Miller, 1972). Antibiotics, when present, were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 30 μg/ml.

Other materials

Chromosomal DNA (Hiuga and Miura, 1963) and large-scale (Clewell and Helinski, 1969) and small-scale (Birnbaum and Doly, 1979) plasmid DNA preparations were made by standard procedures. *E. coli* tRNA preparations were made as described (Deutscher and Hilderman, 1974), and their purity was checked by gel electrophoresis in the presence of 8 M urea. *E. coli* tRNA nucleotidyltransferase was prepared as reported previously (Cudny and Deutscher, 1986).

Interruption of the wild-type cca gene

Plasmid pEC6, containing the wild-type *cca* gene was partially digested with restriction endonuclease, *Mlu*I. This enzyme cuts at two sites in the 4-kb insert, one located within the *cca* gene at amino acid 135 and one located downstream of the gene. The 6.7-kb fragments generated by the digestion were filled in with DNA polymerase to generate blunt ends, and ligated to a 1.3-kb *Fnu*DII fragment from pBR325 that contains the *cat* gene encoding chloramphenicol acetyltransferase. The resulting plasmids were used to transform strain UT481, selecting for Amp^r, Cam^r transformants. Assays for tRNA nucleotidyltransferase identified one transformant, harboring plasmid pEC-CAT-7, that had elevated activity, and a second transformant, harboring plasmid, pEC-CAT-10, that had the basal level of activity. The latter plasmid was a candidate for containing an interrupted *cca* gene. Restriction analysis and maxicell analysis confirmed this assignment (see Results).

DNA from plasmid pEC-CAT-10 was digested with endonuclease, *Bam*HI, and the linear fragments were used to transform strain CF881. One Cam^r, CCA$^-$ transformant, termed CF101, was used for further study. Digestion and hybridization of chromosomal DNA from strain CF101, using probes for the *cat* gene and the *cca* gene, indicated that the *cca* gene in this strain was interrupted (see Results). An identical experiment carried out with DNA from plasmid pEC-CAT-7 gave only *cca*$^+$, Cam^r transformants.

Other methods

Maxicells were prepared and labeled as described (Sancar *et al.*, 1979). Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) was carried out by the procedure of Dreyfuss *et al.* (1984) using 10% gels. Immunoblotting was performed according to the instructions with the ProtoBlot kit using a rabbit antiserum raised against purified *E. coli* tRNA nucleotidyltransferase (Zhu *et al.*, 1986). tRNA nucleotidyltransferase was assayed as described (Cudny *et al.*, 1986). Protein was determined by the method of Bradford (1976). DNA sequencing was by the dideoxy chain termination method using chemically-synthesized, 17-base oligonucleotide primers as reported (Zhu *et al.*, 1986).

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