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Biochemistry

RNase T is responsible for the end-turnover of tRNA in *Escherichia coli*

(CCA terminus)

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ABSTRACT **A mutant strain deficient in RNase T was isolated and used to study the role of this enzyme in *Escherichia coli*. Strains lacking as much as 70% of RNase T activity, alone or in combination with the absence of other RNases, display normal growth properties. However, in *cca* strains, which lack tRNA nucleotidyltransferase, RNase T-deficient derivatives accumulate lower levels of defective tRNA and grow at increased rates compared to their RNase T^+ parents. Slow-growing *cca* strains revert to a faster-growing form that contains less defective tRNA but which is still *cca*. All of these strains have decreased levels of RNase T. These data indicate that RNase T is responsible for nucleotide removal during the tRNA end-turnover process and that the amount of defective tRNA in cells is determined by the relative levels of RNase T and tRNA nucleotidyltransferase.**

The end-turnover of tRNA has been known since the earliest studies of tRNA metabolism (1). The process consists of the removal and readdition of the 3′ terminal AMP and penultimate CMP residues of tRNA. Although the physiological function, if any, of end-turnover is unclear, it is known to involve tRNAs for only some amino acids and to require uncharged tRNA (2). Furthermore, if the readdition of missing terminal residues is prevented, defective tRNA molecules accumulate (3), leading to a considerable slowdown of growth of *Escherichia coli* and the elevation of guanosine 3′-diphosphate 5′-diphosphate (ppGpp) levels (4).

It has been known for a number of years, through the use of *E. coli* mutants, that the readdition phase of the turnover process requires the enzyme tRNA nucleotidyltransferase (2). However, the enzyme responsible for removal of the terminal residues was unknown. Since tRNA nucleotidyltransferase is simply a scavenger of incomplete tRNA molecules and is present in large excess in cells, the repair of defective tRNA appears to be unregulated. Thus, any control of the turnover process would be at the level of nucleotide removal. To understand this process more fully, identification and study of the enzyme required for nucleotide removal is a necessary prerequisite.

We have recently described a previously unidentified *E. coli* exoribonuclease, RNase T, whose *in vitro* specificity suggested that it might be the nuclease involved in end-turnover (5). RNase T has been purified to homogeneity (6). It is an α_2 dimer with a molecular weight of 50,000 (6). The enzyme is highly specific for tRNA-C-C-A, releasing AMP and tRNA-C-C (5). RNase T initiates attack at a free 3′ terminus of tRNA; aminoacyl-tRNA is not a substrate (6). In this paper we describe the isolation of an *E. coli* mutant deficient in RNase T. Studies of the mutant have provided direct evidence that this enzyme is responsible for nucleotide removal during the end-turnover process.

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EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. Wild-type *E. coli* strain CA265, its $RNase^-$ derivative strains CAN, CAN/20, and CAN/20-12, and the multiple RNase-deficient derivative CAN/20-12E have been described (5, 7). The RNase T-deficient strains 20-12E/18 and 20-12E/18-11 were isolated as described below. *cca* derivatives of the various strains, lacking tRNA nucleotidyltransferase, were constructed by bacteriophage P1-mediated transduction of the *cca* locus from strain 35-10, using *tolC* as the selectable marker as described (8). Cells were grown in YT medium containing 0.4% glucose (9). Tetracycline-resistant strains were routinely grown in the presence of tetracycline (10 μg/ml).

Mutagenesis and Screening of Mutants. Strain CAN/20-12E was grown to an OD_{550} of 1 in YT medium containing tetracycline and then was mutagenized with nitrosoguanidine (0.5 mg/ml) for 30 min at 37°C as described (10). Survival after this procedure was about 2%. The mutagenized culture was divided into 30 portions, diluted with fresh YT medium, and grown overnight at 30°C. Cells from each stock tube were plated on YT agar, grown at 30°C overnight, and then transferred to 42°C for 8–16 hr. Small colonies were picked and retested for temperature-sensitive growth. One-hundred twenty-six temperature-sensitive colonies were obtained by this procedure. The colonies displaying temperature-sensitive growth were screened for RNase T activity.

Identification of colonies containing decreased RNase T activity was accomplished by assaying extracts for their ability to hydrolyze tRNA-C-C-[^{14}C]A. Cells were grown in YT medium to an OD_{550} of 1 at 30°C, centrifuged, resuspended in one-fourth volume of 20 mM glycine/NaOH, pH 8.9/0.1 mM EDTA/1 mM dithiothreitol, and sonicated for 30 sec. After centrifugation to remove cell debris, 100-μl aliquots were incubated for 2 min at 37°C and then assayed for RNase T activity for 30 min at 30°C in 200-μl reaction mixtures containing 20 mM glycine/NaOH (pH 8.9), 5 mM $MgCl_2$, and 30 μg of tRNA-C-C-[^{14}C]A.

The RNase T mutant strain 20-12E/18 was converted to one with temperature-insensitive growth by P1 transduction, followed by selection for cells able to grow at 44°C. The resulting derivative strain, which retained its RNase T deficiency, was termed 20-12E/18-11.

Other Materials. tRNA was isolated from *E. coli* by phenol extraction and isopropanol fractionation as described (3). The RNase T substrate, tRNA-C-C-[^{14}C]A was prepared by [^{14}C]AMP incorporation into tRNA-C-C by means of tRNA nucleotidyltransferase (5). [^{14}C]ATP was obtained from Schwarz/Mann. Ultrogel AcA 44 was from LKB. All salts were reagent grade.

Other Methods. The amount of terminally defective tRNA present in various strains was determined by isolating tRNA and measuring the level of [^{14}C]AMP incorporation in the presence of purified rabbit liver tRNA nucleotidyltransferase

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Table 1. RNase T activity of strains CAN/20-12E and RNase T-deficient mutants

Strain	Relevant phenotype	RNase T activity: [^{14}C]AMP released, nmol/10 min	RNase T activity: %
CAN/20-12E	RNase I$^-$,II$^-$,D$^-$,BN$^-$	0.140	100
20-12E/18	RNase I$^-$,II$^-$,D$^-$,BN$^-$, T$^-$; ts	0.042	30
20-12E/18-11	RNase I$^-$,II$^-$,D$^-$,BN$^-$,T$^-$	0.037	26

Cells were grown in YT medium to an OD_{550} of ≈1.6. Extracts were prepared by sonication as described in *Experimental Procedures*, and 50-μl aliquots were assayed for RNase T activity for 10 min at 37°C. Preparation of extracts by disruption with a French press showed that the relative level of activity in 20-12E/18-11 was decreased to the same level as in extracts prepared by sonication. ts, Temperature-sensitive.

(5). Standard RNase T assays were as described (5). Protein was determined colorimetrically by the method of Bradford (11).

RESULTS

Isolation of a RNase T-Deficient Mutant Strain. By using a "brute-force" isolation procedure to assay colonies from a heavily mutagenized stock of CAN/20-12E, we identified one RNase T-deficient strain among 126 independent, temperature-sensitive colonies tested. Extracts of this strain, 20-12E/18, contained about 30% the normal level of RNase T activity (Table 1). Partial purification of RNase T from mutant and parental strains by Ultrogel AcA 44 and DEAE-Sephadex chromatography revealed that both showed similar chromatographic properties; however, the enzyme from the mutant strain was more temperature-sensitive and had a slightly higher K_m for the tRNA substrate (data not shown), suggesting that the mutation is in the structural gene for RNase T. Since RNase T is the only nuclease in extracts of strain CAN/20-12E that is active on tRNA-C-C-A, the 70% decrease in activity in the mutant strain must be due to an effect on this enzyme.

Table 2. State of 3′ termini in tRNAs from RNase$^-$/*cca* double mutants

Strain	AMP incorporation, nmol/mg of tRNA: *cca*$^+$ derivative	AMP incorporation, nmol/mg of tRNA: *cca* derivative
CAN	<0.1	3.3
CAN/20-12E	0.2	3.6
20-12E/18	0.2	1.0
20-12E/18-11	0.1	0.8

tRNA was isolated from each of the indicated strains, and [^{14}C]AMP incorporation into 100 μg was determined with purified tRNA nucleotidyltransferase as described (5). CAN data were taken from ref. 5.

To determine whether the temperature-sensitive growth of 20-12E/18 was due to its deficiency of RNase T, revertants and transductants of this strain able to grow at 44°C were isolated. Twenty-four of 24 spontaneous revertants and 15 of

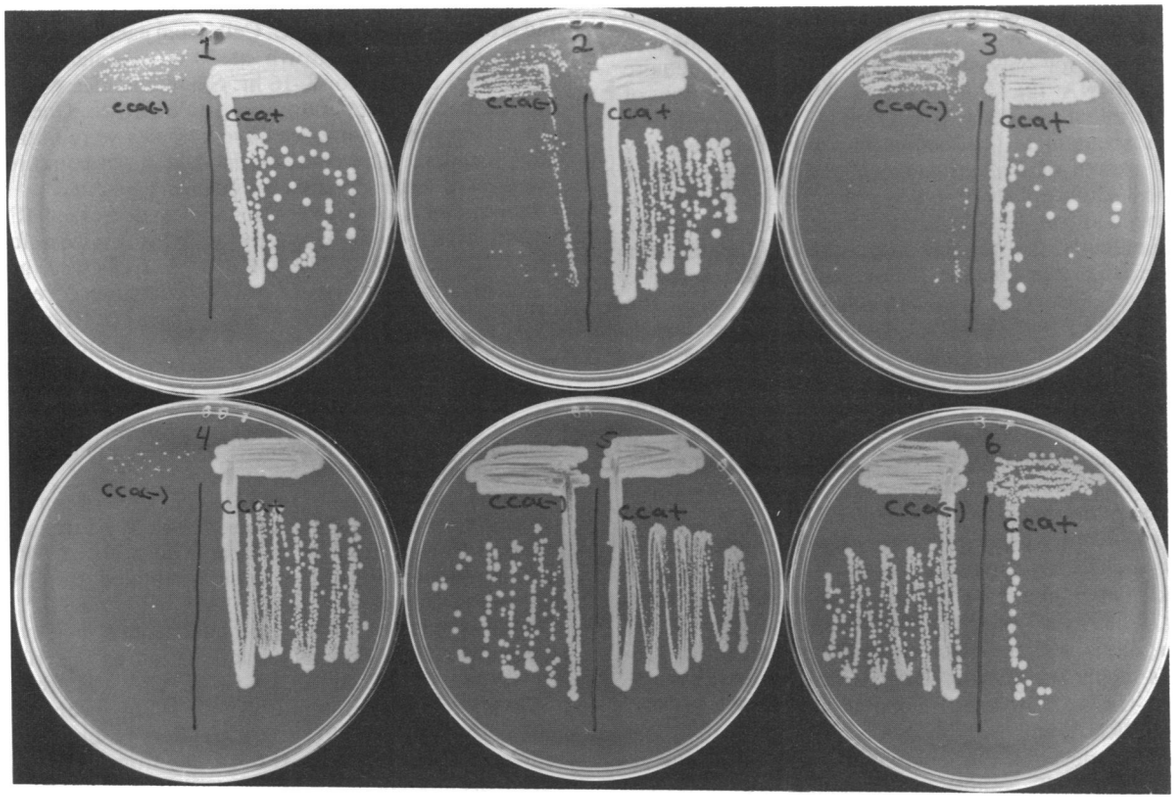

FIG. 1. Growth of *cca*$^+$ and *cca* derivatives (on right-hand and left-hand sides of each plate, respectively) of various RNase-deficient strains. Cells were streaked on YT or YT/tetracycline agar plates and grown overnight at 37°C (30°C for strain 20-12E/18). Plates: 1, CA265 (wild type); 2, CAN (RNase BN$^-$); 3, CAN/20-12 (RNase BN$^-$,II$^-$,D$^-$); 4, CAN/20-12E (RNase I$^-$,BN$^-$,II$^-$,D$^-$); 5, 20-12E/18 (RNase I$^-$,BN$^-$,II$^-$,D$^-$,T$^-$;ts); 6, 20-12E/18-11 (RNase I$^-$,BN$^-$,II$^-$,D$^-$,T$^-$). In liquid culture, all *cca*$^+$ strains grow with a doubling time of 28–30 min at 37°C. *cca* derivatives of the RNase T$^+$ strains grow with a doubling time of >120 min, whereas the RNase T$^-$/*cca* strain 20-12E/18-11 doubles in 40 min at 37°C. It was not possible to measure the growth of 20-12E/18 at 37°C because of its ts phenotype.

Table 3. RNase T activity of fast-growing *cca* revertants

Revertant strain	No. of revertants RNase T^-/no. tested	Relative RNase T specific activity	No. of revertants TNT^-/no. tested
CA265 *cca*	18/18	0.26–0.73	17/17
CAN/20-12E *cca*	24/24	0.52–0.76	8/8

Fast-growing revertants of strains CA265 *cca* and CAN/20-12E *cca* were isolated, and extracts were assayed for both RNase T and tRNA nucleotidyltransferase (TNT) activity. Enzyme activity is expressed relative to the cca^+ parental strains and has been corrected for the amount of protein in the extracts.

15 transductants retained the decreased level of RNase T activity yet grew at 44°C. These findings indicated that the temperature-sensitive growth of 20-12E/18 was unrelated to its RNase T defect. One of the transductants, 20-12E/18-11, was retained for further study. Despite its decreased RNase T activity (Table 1), this strain grew with the same doubling time as 20-12E in YT medium at 37°C and at 44°C.

3′ Termini in tRNAs from RNase T^-/*cca* Double Mutants. tRNAs isolated from *cca* cells contain incomplete 3′ termini because of their inability to repair the defective ends generated during the end-turnover process (2). If RNase T were the exonuclease responsible for removal of the 3′ terminus, we might expect that double mutants lacking both tRNA nucleotidyltransferase and RNase T would have less defective tRNA than *cca*, RNase T^+ cells. Analysis of tRNA from strains containing RNase T or lacking this enzyme confirmed this prediction: the RNase T^- mutation reduced the level of defective tRNA about 4-fold (Table 2). We showed previously that *cca* strains with RNase II^-, RNase D^-, RNase BN^-, or RNase R^- mutations contained the same level of defective tRNA as their $RNase^+$ parents (5), indicating that these RNases do not play a role in end-turnover. Furthermore, as shown in Table 2, a *cca* derivative of the multiple RNase-deficient strain, CAN-20/12E (RNase I^-,II^-,D^-, BN^-), has the normal amount of defective tRNA. These data indicate that of the known exoribonucleases, only RNase T participates in the end-turnover of tRNA *in vivo*.

Growth of RNase T^-/*cca* Double Mutants. Strains containing a *cca* mutation grow slowly (12); likewise, *cca* double mutants lacking either RNase BN; RNases II, D, and BN; or RNases I, II, D, and BN also grow slowly (Fig. 1). In contrast, a RNase T^-/*cca* double mutant is able to grow much more rapidly (Fig. 1), demonstrating that the slow-growth phenotype is a consequence of the presence of RNase T. It has been shown previously that the growth rate of *cca* strains is inversely dependent on their level of defective tRNA (4, 12). Thus, the more rapid growth of RNase T^- strains, which contain less defective tRNA (Table 2), is entirely consistent with this earlier finding.

Fast-Growing *cca* Strains Are RNase T^-. Slow-growing *cca* strains often revert to a faster-growing form that remains *cca* but which contains only about half as much defective tRNA as the original strain (4, 12). The data presented here suggest that these revertant strains may have become RNase T^-. If this idea is correct, the reversion of *cca* strains to faster growth might provide a way to select for RNase T^- mutants. To test this possibility, *cca* derivatives of the wild type strain CA265 and the multiple RNase-deficient strain, CAN/20-12E were grown to stationary phase to allow enrichment for faster-growing cells. Individual cultures were diluted, regrown, and plated, and the largest colonies were tested for RNase T and tRNA nucleotidyltransferase activity (Table 3). In the case of strain CA265 *cca*, all large colonies tested (18 of 18) displayed decreased levels of RNase T that varied from 26 to 73% of the activity in strain CA265. In addition, all the strains tested remained *cca*. Likewise, of 24 large CAN/20-12E *cca* colonies tested, all showed decreased RNase T activity, amounting to 52 to 76% of the activity of CAN/20-12E (Table 3). Taken together, these findings indicate that the slow growth of *cca* cells is due to the accumulation of defective tRNA and that the defect can be eliminated to a large extent by decreasing the activity of RNase T.

Although the RNase T^-, *cca* revertants grow much more rapidly than RNase T^+/*cca* cells (≈40 min versus ≈150 min doubling time), these cells still grow somewhat more slowly than their cca^+ counterparts (doubling time ≈30 min). The large difference in growth rates between RNase T^-/*cca* and RNase T^+/*cca* cells, however, makes it extremely difficult to maintain liquid cultures of the slow-growing cells since fast-growing revertants rapidly take over.

DISCUSSION

The data presented here provide evidence that RNase T is the ribonuclease responsible for the end-turnover of tRNA. However, this role for RNase T only becomes evident in *cca* genetic backgrounds. In the absence of tRNA nucleotidyltransferase, RNase T-deficient strains accumulate less defective tRNA and grow at increased rates. In cca^+ strains the absence of as much as 70% the usual amount of RNase T has no effect on the growth properties of a wild-type strain or of one lacking RNases I, II, D, and BN. The apparent dispensability of these RNases remains to be explained. It strongly suggests that additional exoribonucleases must be present in *E. coli* that compensate for the ones that are missing, but the reason for the high degree of redundancy among the exoribonucleases is not clear.

The amount of defective tRNA in cells is a consequence of the relative levels of RNase T and tRNA nucleotidyltransferase. Under normal circumstances, the amount of tRNA nucleotidyltransferase in cells is sufficient to maintain all tRNAs in an intact form. However, in *cca* mutants the effect of RNase T predominates. It is known that end-turnover only affects uncharged tRNAs *in vivo* and leads to removal primarily of the 3′ terminal AMP residue. The properties of RNase T *in vitro* conform exactly to these restraints: we have recently shown that aminoacyl-tRNA and tRNA-C-C are relatively inactive as substrates for this enzyme (5, 6).

The physiological significance of end-turnover and the role of RNase T are not understood. At first glance the process appears to be a futile cycle. However, the existence of a highly specific ribonuclease that removes only the 3′ terminal AMP of tRNA raises questions about whether the turnover process does have some importance. The presence of a similar activity in *Xenopus* oocytes (13) and in rat liver (unpublished observation) supports this possibility. Further studies of RNase T mutants should help to resolve this issue.

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