Repair of the 3′-terminal -CCA sequence of tRNA generally requires the action of the enzyme tRNA nucleotidyltransferase. However, in Escherichia coli, in the absence of this enzyme, a decreased level of tRNA end repair continues. To ascertain the enzymes responsible for this residual repair, mutant strains were constructed lacking tRNA nucleotidyltransferase and other enzymes potentially involved in the process, poly(A) polymerase I and polynucleotide phosphorylase (PNPase). Strains lacking tRNA nucleotidyltransferase and either one of the other enzymes displayed decreased growth rates and increased levels of defective tRNA compared with the single cca mutant. Triple mutants lacking all three enzymes grew very slowly, had even more defective tRNA, and were devoid of activity incorporating AMP into tRNA-C-C. Overexpression of poly(A) polymerase I, but not PNPase, partially compensated for the absence of tRNA nucleotidyltransferase. These data show that poly(A) polymerase I and PNPase participate in the end repair process and are required to maintain functional tRNA levels when tRNA nucleotidyltransferase is absent.

tRNA molecules from all organisms contain an identical trinucleotide sequence (-CCA) at their 3′ termini that plays an important role in the biological functions of this nucleic acid (reviewed in Ref. 1). Depending on the organism, the -CCA sequence arises either during transcription or by post-transcriptional addition catalyzed by the enzyme tRNA nucleotidyltransferase, which is capable of utilizing CTP and ATP to incorporate CMP and AMP residues into tRNAs with complete -CCA termini (1). In laboratory strains of Escherichia coli, all tRNA genes encode the -CCA sequence (2), so post-transcriptional addition of these residues is not needed. The role of E. coli tRNA nucleotidyltransferase, encoded by the cca gene, lies not in the biosynthesis of tRNAs, but in its repair. It has been known since the earliest studies of tRNA metabolism that the terminal -CCA sequence normally undergoes an end turn-over process in vivo that consists of the removal and re-addition of the 3′-terminal AMP and penultimate CMP residues of tRNA (3). It is now well established that the exoribonuclease RNase T is responsible for nucleotide removal (4) and that tRNA nucleotidyltransferase is responsible for tRNA repair (5). An E. coli cca strain totally devoid of tRNA nucleotidyltransferase activity is viable, but grows slowly because it accumulates defective tRNAs and has an increased level of ppGpp (5, 6). In such a strain, 10–15% of the total tRNA population contains incomplete 3′ termini because of the decreased ability to carry out repair; however, tRNAs from different amino acid families are affected unequally (7).

Previous studies showed that even in cells devoid of tRNA nucleotidyltransferase, a low level of tRNA end repair continues. Thus, incubation of a cca mutant strain with chloramphenicol (7) or of a cca null mutant in the presence of kanamycin (8), to prevent further end turnover, led to a decrease over time in the amount of defective tRNA originally present. In addition, in an experiment in which the encoded -CCA sequence of the tRNA tyrr su1 gene was changed to -CCC, -CCG, or -CCU and then introduced into a cca mutant strain, it was found that 15% of the suppressor activity found in a wild-type strain was retained despite the fact that removal and repair of the incorrect 3′-terminal residue was required (8). Finally, a low level of activity incorporating AMP into tRNA-C-C can be detected in extracts from cells devoid of tRNA nucleotidyltransferase (5, 8).

All of this evidence suggested that additional activity able to slowly repair the 3′ terminus of tRNA is present in E. coli cca mutant cells. In this paper, we provide genetic and biochemical evidence that this residual tRNA end repair involves the known enzymes poly(A) polymerase I and polynucleotide phosphorylase (PNPase). A model describing the action of these enzymes in the repair process is presented.

EXPERIMENTAL PROCEDURES

Materials—[3H]ATP, [14C]ATP, and a ¹H-labeled amino acid mixture were purchased from DuPont NEN Life Science Products. E. coli tRNA nucleotidyltransferase was purified from a strain that overproduces this enzyme (9). tRNA was prepared by phenol extraction and isopropyl alcohol fractionation as described (10).

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains used in this work are derivatives of the E. coli K12 strain CA244 (lacZ, trp, relA, spoT) (11). P1-mediated transduction was performed to construct a series of strains lacking poly(A) polymerase I (PAP), polynucleotide phosphorylase (PNP), and tRNA nucleotidyltransferase (CCA) in various combinations. The following selectable markers, prepared by the introduction of antibiotic resistance cassettes, were used: penB::kan for PAP (12), pnp::Tn5 for PNP (13), and cca::cam for CCA (5). For construction of the PAP · PNP · double mutant and the CCA · PAP · PNP triple mutant strains, the pnp::Tn5 mutation was introduced into the PAP · CCA · PAP strains using a nearby Tn10 insertion as the selectable marker and assaying tetracycline-resistant transductants for the loss of PNPase activity. The mutations in penB, pnp, and cca are either deletion/substitution or interruption mutations, and all lead to a null phenotype based on direct assay of the relevant enzyme. Plasmids pJL89 (14) and pKAK7 (15), carrying penB and pnp, respectively, were obtained from Dr. Sidney
Kushner. Strains carrying these plasmids overexpress poly(A) polymerase 5–10-fold and PNPase 3–5-fold.

Cells were routinely grown in liquid YT (yeast extract/Tryptone) medium or, for doubling time measurements, in YT medium supplemented with 0.2% glucose. Antibiotics, when present, were at the following concentrations: kanamycin, 25 μg/ml; chloramphenicol, 50 μg/ml; and tetracycline, 12.5 μg/ml.

Preparation of Extracts—Cells were grown to A$_{600}$ ≈ 1.0, concentrated 10-fold in 20 mM glycine (pH 9.4) and 1 mM diethiothreitol, and sonicated with two 15-s pulses with a 30-s cooling period while submerged in an ice bath (Heat System/Ultrasonics, Plainview, NY). The sonicate was then centrifuged at 12,000 × g for 10 min, and the supernatant fraction was retained for assays.

Assays—Assays of extracts for activity incorporating AMP into tRNA-C-C were carried out under conditions optimal for tRNA nucleotidyltransferase (16). Reaction mixtures contained the following (in a volume of 50 μl): 50 mM glycine (pH 9.4), 5 mM MgCl$_2$, 1 mg/ml yeast tRNA (predominantly tRNA-C-C), 1 mM [γ-32P]ATP (~ 10$^9$ cpm/nmol), and enzyme fraction. Incubation was at 37 °C for 15 min. Reactions were stopped with cold 10% trichloroacetic acid and 2 mM sodium pyrophosphate, and left on ice for 10 min. Precipitates were collected on Whatman glass-fiber GF/C filters and washed five times with 3 ml of 2.5% cold trichloroacetic acid and once with 5 ml of ethanol/ether (1:1). Filters were dried and counted in a scintillation counter.

Incubation of AMP incorporation was carried out in reaction mixtures of 100 μl containing 50 mM glycine (pH 9.4), 5 mM MgCl$_2$, 1 mM [γ-32P]ATP, 100 μg of tRNA from the indicated strain, and sufficient purified E. coli tRNA nucleotidyltransferase for complete incorporation. Incubation was for 30 min at 37 °C. Precipitation, washing, and counting were as described above.

Assay of tRNA for aminocacylation was carried out in reaction mixtures of 100 μl containing 100 mM Tris acetate (pH 7.4), 10 mM MgCl$_2$, 10 mM KC1, 1 mM ATP, 0.1 mM [γ-32P]adenosine acid mixture, 100 μg of the indicated tRNA, 0.2 mg/ml bovine serum albumin (nuclease-free), and sufficient purified E. coli aminocyl-tRNA synthetase mixture for complete incorporation. Incubation was for 10 min at 37 °C. Precipitation, washing, and counting were as described above except that the 10% trichloroacetic acid contained 2.5% casamino acids. The tRNA used for aminocacylation was first stripped of endogenous amino acids by incubation in 1.7 M Tris-CI (pH 8.0) for 1 h at 37 °C, followed by ethanol precipitation (7). Protein was determined by the method of Bradford (17).

Northern Blot Analysis—tRNA samples of ~ 9 μg were run on 6% acrylamide gels containing 8.3 M urea. Sample buffer contained 95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF. Gels were run at 1250 V (~32 V/cm) until the xylene cyanol dye had migrated 28 cm. The tRNA was transferred to a GeneScreen Plus membrane using a Genie Blotter. After transfer, the membrane was rinsed in 2 × SSC, dried, and baked for 2 h at 80 °C. Prehybridization and hybridization were carried out as described (18) with the indicated $^{32}$P-labeled oligonucleotide probe specific for a single tRNA species. Membranes were stripped for reuse by boiling for 20 min in 0.1 × SSC containing 1% SDS.

RESULTS

Growth Rates of Cells Lacking tRNA Nucleotidyltransferase, Poly(A) Polymerase I, and/or PNPase—Earlier studies in this laboratory (8)$^2$ focused attention on PNPase and poly(A) polymerase I as possibly contributing to the residual AMP-incorporating activity observed in extracts of E. coli CCA$^+$ cells. To investigate the role of these enzymes in detail, strains were constructed that contained null mutations in the cca, pcnB, and pnp genes encoding tRNA nucleotidyltransferase, poly(A) polymerase I, and PNPase, respectively, alone or in combination (see “Experimental Procedures”).

Growth of these strains on YT plates at 37 °C showed that single mutant strains each produced colonies slightly smaller than the wild type. Each of the double mutant cells grew even more slowly than the single mutants; triple mutant cells, depending on the method of construction, either did not grow or produced extremely small colonies. Thus, when the triple mutant strain was prepared by introducing cca::xam into the PAP·PNP$^-$ double mutant and selecting for chloramphenicol-resistant transductants, essentially no colonies could be recovered. Alternatively, when pnp::Tn5 was introduced into the CCA$^+$·PAP strain using a closely linked Tn10 insertion and selecting for tetracycline-resistant transductants, viable triple mutant colonies could be recovered, although they grew considerably more slowly than any of the double mutants. The differential effects of the antibiotics on the growth of the triple mutant were not investigated further. The identity of the triple mutant was confirmed by direct assay of the relevant activities.

A more quantitative measure of the growth rates of the various strains was obtained from determination of their doubling times in liquid YT/glucose cultures (Table I), and these growth rates agreed well with the relative growth observed on plates. The double mutant strains grew more slowly than the single mutant strains, and the triple mutant strain grew even slower. For many of the CCA$^-$ strains, measurement of accurate doubling times was difficult because of the tendency of these cells to revert to faster growing forms (4, 10). To minimize this problem, the values reported here are for the slowest reproducible growth rates observed in many growth experiments. The mutations resulting in faster growth are known to affect the rnt gene, resulting in a decreased level of RNase T (4). Despite this problem, these data show that the addition of PAP$^-$ and/or PNP$^-$ mutations to a CCA$^-$ strain results in even slower growth, indicating that the functions of tRNA nucleotidyltransferase, poly(A) polymerase I, and PNPase must overlap in some common process in vivo. Inasmuch as the only known function of tRNA nucleotidyltransferase in E. coli is the repair of the -CCA end of tRNA, these findings suggest that the other two enzymes participate in this process as well.

In support of this conclusion, we found that overexpression of poly(A) polymerase I in the CCA$^-$ background (strain CCA$^-$/pJL89) led to faster growth (Table I), indicating that overexpression of poly(A) polymerase can partially compensate for the absence of tRNA nucleotidyltransferase, as would be expected if their in vivo functions overlapped. Overexpression of PNPase (strain CCA$^-$/pKAK7), in contrast, did not have such an effect, suggesting that it may already be present at sufficient levels; in fact, too much PNPase may even be inhibitory to growth of some strains.$^3$

| Strain relevant phenotype | Doubling time$^*$ |
|---------------------------|------------------|
| Wild-type                 | 27               |
| CCA$^-$                   | 42               |
| PAP$^-$                   | 34               |
| PNP$^-$                   | 32               |
| CCA$^-$·PAP$^-$           | 70               |
| CCA$^-$·PNP$^-$           | 56               |
| PAP$^-$·PNP$^-$           | 55               |
| CCA$^-$·PAP$^-$·PNP$^-$   | 90               |
| CCA$^-$·pBR322            | 44               |
| CCA$^-$·pJL89             | 35               |
| CCA$^-$·pKAK7             | 45               |

$^*$ Many of these strains tend to revert to faster growing forms. The doubling times shown are the maximum values observed reproducibly.

$^2$ N. B. Reuven, Z. Zhou, and M. P. Deutscher, unpublished observations.

$^3$ S. Pandit and M. P. Deutscher, unpublished observation.
Cells were grown at 37 °C to an optical density at 600 nm of 1, and sonicated extracts from the indicated strains were prepared and assayed for AMP incorporation into tRNA-C-C as described under "Experimental Procedures." One unit of activity is defined as that which incorporates 1 nmol of AMP in 1 h at 37 °C. For relative specific activity, the wild type was set at 100.

| Strain relevant phenotype | Specific activity | Relative specific activity |
|--------------------------|------------------|--------------------------|
| Wild type                | 114              | 100                      |
| CCA                      | 20               | 17                       |
| PAP                      | 85               | 75                       |
| PNP                      | 128              | 112                      |
| CCA ,PAP                 | <0.1             | <0.1                     |
| CCA ,PNP                 | 35               | 31                       |
| PAP ,PNP                 | 114              | 100                      |
| CCA ,PAP ,PNP            | <0.1             | <0.1                     |

**Effect of tRNA Nucleotidyltransferase, Poly(A) Polymerase I, and PNPase on AMP Incorporation into tRNA-C-C in Vivo**—To assess whether poly(A) polymerase I and PNPase could be involved in the repair of tRNA, extracts were prepared from strains lacking these enzymes, alone or in combination, and assayed for their ability to incorporate AMP into tRNA-C-C (Table II). Assays were carried out under conditions optimal for tRNA nucleotidyltransferase. Using these conditions, >80% of the total AMP-incorporating activity present in a wild-type extract was lost when this enzyme was absent. However, a significant level of residual AMP incorporation remained, and this was due entirely to poly(A) polymerase I, as an extract from a CCA ,PAP double mutant was devoid of activity.

Interestingly, removal of PNPase activity from the wild-type, CCA , or PAP background uniformly led to an elevation of AMP-incorporating activity, indicating that under these in vitro assay conditions, PNPase was probably functioning as a degradative enzyme and destroying the product. As is well known, PNPase can act as either a synthetic or a degradative enzyme in vitro depending on the relative concentrations of nucleoside diphosphates and P, and the specific assay conditions (19). In fact, under conditions favoring the synthetic reaction, PNPase adds stretches of A residues to tRNA-C-C (data not shown).

Taken together, these data show that tRNA nucleotidyltransferase and poly(A) polymerase I are the major enzymes responsible for AMP incorporation into tRNA-C-C in vitro. PNPase, on the other hand, appears to remove the AMP residues incorporated by the former two enzymes. Based on their in vitro activities, it is clear that poly(A) polymerase and PNPase could influence the process of tRNA end repair especially in cells lacking tRNA nucleotidyltransferase, supporting the conclusions drawn from the growth experiments presented above that these enzymes overlap functionally in vivo.

**Effect of tRNA Nucleotidyltransferase, Poly(A) Polymerase I, and PNPase on the 3′ Termini of tRNA**—To directly determine the importance of each of the enzymes for tRNA end repair, tRNA was isolated from each of the mutant strains, and the status of their 3′ termini was determined based on the levels of AMP incorporation and aminoaclylation (Table III). AMP incorporation is a reliable indicator of defective tRNA when 1 or 2 residues are missing from the -CCA sequence, but cannot be used when 3 or more residues are absent (4, 16). For the more defective tRNAs, aminoaclylation provides a more accurate picture of the state of the 3′ termini. As is already known, cells lacking tRNA nucleotidyltransferase accumulate defective tRNA amounting to 10–15% of the tRNA population based on AMP incorporation (4, 5, 7), and the data presented here confirm this information. The amount of defective tRNA in a CCA strain based on aminoaclylation levels was consistent with this value (Table III). The absence of either poly(A) polymerase I or PNPase by itself did not increase the amount of defective tRNA compared with the wild type. The combined absence of both enzymes also had relatively little effect on the level of defective tRNA based on AMP incorporation. These data indicate that poly(A) polymerase I and PNPase are relatively unimportant for tRNA repair when tRNA nucleotidyltransferase is present. On the other hand, these enzymes become quite important when tRNA nucleotidyltransferase is absent. Thus, the amount of defective tRNA in the CCA ,PAP and CCA ,PNP double mutant strains was increased compared with the CCA strain based on both AMP incorporation and aminoaclylation (Table III). The effect on the triple mutant was even more pronounced. In the latter case, the amount of defective tRNA based on aminoaclylation levels increased to >50%. Paradoxically, AMP incorporation actually appears to decrease; however, this is due to the fact that a portion of the tRNA population has been shortened past the point at which tRNA nucleotidyltransferase can add back an AMP residue and therefore does not show up as defective tRNA in this assay (see the Northern analysis presented below). Control experiments showed that the elevated amount of defective tRNA in the triple mutant was not due to increased RNase T activity in this strain, to contamination of the tRNA preparation with other nucleic acids that could not accept amino acids, or to the presence of an aminoaclylation inhibitor in the tRNA preparation. These data demonstrate directly that repair of the 3′-end of tRNA in the absence of tRNA nucleotidyltransferase is dependent on both poly(A) polymerase I and PNPase.

Moreover, overexpression of poly(A) polymerase (strain CCA ,pBR222) decreases the amount of defective tRNA based on AMP incorporation (Table III), suggesting a direct role for this enzyme in the repair process. Overexpression of PNPase (strain CCA ,pKAK7), on the other hand, has no effect on the amount of defective tRNA in a CCA strain (Table III), supporting the conclusion that it may already be present at sufficient levels for its role in tRNA repair.

**Northern Analysis of tRNAs from Strains Lacking tRNA Nucleotidyltransferase, Poly(A) Polymerase I, and/or PNPase**—

### Table II

| Source of tRNA | AMP incorporation[^a] | Incomplete termini | Relative aminoaclylation | Incomplete aminoaclylation |
|---------------|------------------------|--------------------|--------------------------|---------------------------|
| Wild type     | <0.2 mg/ml             | <1                 | 100                      | 0                         |
| CCA ,PAP      | 3.9 mg/ml              | 13                 | 82                       | 18                        |
| PAP           | <0.2 mg/ml             | <1                 | 94                       | 6                         |
| PNP           | 0.3 mg/ml              | 1                  | 18                       | 16                        |
| CCA ,PAP ,PNP | 4.2 mg/ml             | 14                 | 45                       | 55                        |

[^a]: Many of these strains tend to revert to faster growing forms. The incorporation values presented are for the most defective tRNA prepa- rations. For all strains, tRNA was prepared only if a strain grew with its typical doubling time (see Table I).
residual repair of 3′ end of tRNA

The Northern analyses provide further evidence that the absence of poly(A) polymerase and PNPase in CCA− strains increases the amount of defective tRNA present. Moreover, these data confirm what was previously shown for CCA− strains (7), that not all tRNA species are affected in an identical manner by the absence of repair enzymes.

DISCUSSION

The information presented here, based on effects on growth, enzyme activity, and the structure of tRNA, supports the conclusion that the residual tRNA end repair observed in cells lacking tRNA nucleotidyltransferase (7, 8) depends on both poly(A) polymerase I and PNPase, two other template-independent enzymes. In wild-type cells, the level of tRNA molecules with defective 3′ termini is extremely low, amounting to <1% (Ref. 7 and this study), implying that tRNA nucleotidyltransferase is very efficient in repairing defective tRNA molecules. In fact, mutant cells with only ~20% of the normal amount of this enzyme do not accumulate defective tRNA (10).

Thus, it is not surprising that removal of poly(A) polymerase I and PNPase does not affect tRNA 3′-ends when tRNA nucleotidyltransferase is present. The slowed growth of the PAP−,PNP− double mutant strain is probably due to the role of these enzymes in mRNA decay (21). On the other hand, these enzymes make a substantial contribution to maintaining the function of the tRNA population when tRNA nucleotidyltransferase is absent. The additional removal of poly(A) polymerase and PNPase leads to a major increase in the amount of defective tRNA and consequently to extremely slow growth. Nevertheless, it should be emphasized that compared with tRNA nucleotidyltransferase, the residual repair in its absence is quite slow.

Based on its in vitro activity, the function of poly(A) polymerase I in tRNA end repair is most likely the re-addition of the 3′-terminal AMP residue. Interestingly, poly(A) polymerase also shows significant sequence homology to tRNA nucleotidyltransferase (20). However, unlike tRNA nucleotidyltransferase, which adds only a single AMP residue to tRNA under normal conditions, poly(A) polymerase would be expected to incorporate runs of AMP residues into the defective tRNA termini. These multiple AMP residues would need to be removed to generate mature functional tRNAs with terminal -CCA sequences. Such a processing event may be no different from that in which normal tRNA precursors with extended 3′ termini are subjected to the actions of multiple exoribonucleases (18); however, recent work with RNA I and several mRNAs (21–24) suggests that poly(A) tail shortening, in contrast, is mediated primarily by only two enzymes, PNPase and RNase II. Our in vitro measurements of AMP-incorporating activity are consistent with the notion that PNPase participates in removal of the multiple AMP residues generated by poly(A) polymerase. Such a role would be one explanation of the need for PNPase in the tRNA end repair process.

Thus, our data are most consistent with a model in which a second, slower pathway for tRNA end repair exists that becomes important when the pathway dependent on tRNA nucleotidyltransferase is eliminated. This second pathway utilizes poly(A) polymerase I to incorporate multiple AMP residues into tRNA-C-C and uses PNPase to remove most of the extra AMP moieties. Other exoribonucleases may be needed to remove the last few AMP residues after normal tRNA maturation (18). In this model, both poly(A) polymerase I and PNPase are essential, explaining why removal of either enzyme leads to slower growth. Whether poly(A) polymerase II, a recently discovered enzyme (25), has any role in this process is not known.

However, the role of PNPase may not be completely explained by this straightforward model. For example, it is not clear why removal of PNPase from the CCA−,PAP− double...
mutant to generate the triple mutant leads to slower growth and to a substantial increase in shortened tRNA. A similar question can be raised regarding the increased AMP incorporation into tRNA from the CCA\(^-\)\(\cdot\)PNP\(^-\) double mutant compared with that from the CCA\(^-\) strain. In both of these situations, the removal of PNP\(\)ase results in more defective shortened tRNA, an unexpected effect if the only role of the enzyme is to degrade the poly(A) tail. One possibility explaining these observations is that PNP\(\)ase can also participate in the synthetic phase of the repair process, especially when both tRNA nucleotidyltransferase and poly(A) polymerase are absent. PNP\(\)ase does have synthetic capabilities (19), but to our knowledge, these have not yet been observed in vitro. Further work will be necessary to explore this interesting possibility.

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