The EMBO Journal vol.6 no.8 pp.2499–2503, 1987

Bacteriophage T4 anticodon nuclease, polynucleotide kinase and RNA ligase reprocess the host lysine tRNA

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Communicated by Y.Groner

Host tRNAs cleaved near the anticodon occur specifically in T4-infected *Escherichia coli prr* strains which restrict polynucleotide kinase (*pnk*) or RNA ligase (*rli*) phage mutants. The cleavage products are transient with *wt* but accumulate in *pnk*$^-$ or *rli*$^-$ infections, implicating the affected enzymes in repair of the damaged tRNAs. Their roles in the pathway were elucidated by comparing the mutant infection intermediates with intact tRNA counterparts before or late in *wt* infection. Thus, the T4-induced anticodon nuclease cleaves lysine tRNA 5′ to the wobble position, yielding 2′:3′-P> and 5′-OH termini. Polynucleotide kinase converts them into a 3′-OH and 5′ P pair joined in turn by RNA ligase. Presumably, lysine tRNA depletion, in the absence of polynucleotide kinase and RNA ligase mediated repair, underlies *prr* restriction. However, the nuclease, kinase and ligase may benefit T4 directly, by adapting levels or decoding specificities of host tRNAs to T4 codon usage.

Key words: E. coli prr locus/M13-lys-tDNA recombinant/RNA processing/RNA sequence/T4 *stp* gene

Introduction

Polynucleotide kinase (Richardson, 1965), the product of bacteriophage T4 *pseT* (or *pnk*) gene (Depew and Cozzarelli, 1974; Sirotkin *et al.*, 1978), catalyses *in vitro* three different enzymatic reactions: (i) phosphorylation of 5′-OH polynucleotide termini (Richardson, 1965), (ii) hydrolysis of 3′-terminal phosphomonoesters (Becker and Hurwitz, 1967; Cameron and Uhlenbeck, 1977) and (iii) hydrolysis of 2′:3′-cyclic phosphodiesters (O.C. Uhlenbeck, personal communication). Combined, these reactions convert a pair of 2′:3′-P> and 5′-OH cleavage termini, which can be generated by a ribonuclease, into a 3′-OH and 5′-P pair. The latter pair constitute a substrate of RNA ligase (Silber *et al.*, 1972), the product of gene 63 or *rli* (Snopek *et al.*, 1977; Runnels *et al.*, 1982).

Polynucleotide kinase, RNA ligase and an anticodon-specific nuclease were implicated in cleavage and reunion of host tRNA. This model is supported by the specific occurrence of two tRNAs severed in their anticodon loops (vulnerable tRNAs A and B) during T4 infection of *Escherichia coli* CTr5X (David *et al.*, 1982a), a host strain restrictive to *pseT*–*pnk*$^-$ and *rli*$^-$ phage (Depew and Cozzarelli, 1974; Depew *et al.*, 1975; Sirotkin *et al.*, 1978; Runnels *et al.*, 1982). The CTr5X-specific tRNA fragments appear transiently in T4 *wt* infection but accumulate with *pseT*$^-$ or *rli*$^-$ mutants (David *et al.*, 1982b). Formation of these fragments is abolished by *stp* mutations in the phage (Kaufmann *et al.*, 1986) which alleviate *pseT*$^-$ and *rli*$^-$ restriction on *E. coli* CTr5X as well (Depew and Cozzarelli, 1974; Depew *et al.*, 1975; Sirotkin *et al.*, 1978; Runnels *et al.*, 1982). These facts suggest that the failure to repair the anticodon cleavages underlies the restriction of *pseT* and *rli* mutants on *E. coli* CTr5X and associate *stp* with the anticodon nuclease function. However, the manifestation of the anticodon nuclease activity depends, in addition to *stp*, on the host locus responsible for *pnk*$^-$ and *rli*$^-$ restriction which is termed *prr* (Abdul Jabbar and Snyder, 1984; Kaufmann *et al.*, 1986). Finally, a host tRNA species that corresponds in its T1-oligonucleotides fingerprint to vulnerable $tRNA_A$ fragments, sustains *wt* but not *rli*$^-$ infection. This result is consistent with the actual repair of the anticodon nuclease cleavage product by RNA ligase, to restore the original or a slightly altered tRNA (Kaufmann and Amitsur, 1985).

Depletion of one or more needed host tRNAs in T4-infected *prr* strains can be envisaged as a potential host restriction mechanism and the polynucleotide kinase–RNA ligase mediated repair of the damaged tRNAs as a phage rebuttal. However, *stp*, *pnk* and *rli* may have evolved primarily to benefit the phage. Further knowledge about the nature of the vulnerable tRNA species and the reprocessing reactions they undergo could serve as a clue in the quest for such T4-beneficial roles.

Data shown here indicate that anticodon nuclease cleaves lysine tRNA 5′ to the wobble position, producing 2′:3′-cyclic P and 5′-OH cleavage termini. These termini are rearranged by polynucleotide kinase to provide a 3′-OH and 5′-P donor–acceptor substrate that is joined by RNA ligase.

Results

Nucleotide sequence of the major vulnerable tRNA

The abundant cleavage product of the anticodon nuclease is referred to as vulnerable $tRNA_A$. The forms accumulating in the *pseT*$^-$ or *rli*$^-$ infections are referred to as prekinase or preligase intermediates, respectively. The preligase vulnerable $tRNA_A$ fragments were sequenced as such or in their *in vitro* ligated form, as described in Materials and methods. In the sequence, shown as a clover-leaf, the cleavage junction is 5′ to the wobble position (Figure 1). The sequence resembles that described for *E. coli* B lysine tRNA (Chakraburtty *et al.*, 1975), although the identity of modified residues 34, 37 and 47 of the CTr5X species remains to be established. The sequence reported by us before for vulnerable $tRNA_A$ (David *et al.*, 1982) was mistaken.

Lysine tRNA metabolism during T4 infection of E. coli CTr5X

That vulnerable $tRNA_A$ fragments were derived from lysine tRNA or a related species was confirmed by monitoring intact tRNAs and tRNA fragments hybridizing with the M13-lysine to tDNA clone mRL8601 or the Lys–Val_1 clone mRL8618 (Materials and methods) during infection of *E. coli* CTr5X with *wt*, *pnk*$^-$ or *rli*$^-$ phage. Intact tRNAs hybridizing to mRL8601 were resolved by gel electrophoresis into two bands of comparable intensity termed L1 and L2 (Figure 2A), presumably two related lysine tRNA variants. During *wt* infection, band L1 subsided to a minimum of ~50% by 8–12 min and increased to near original levels by 24 min (Figure 2A). This pattern reciprocates that of the vulnerable $tRNA_A$ fragments (5′-A and 3′-A) which

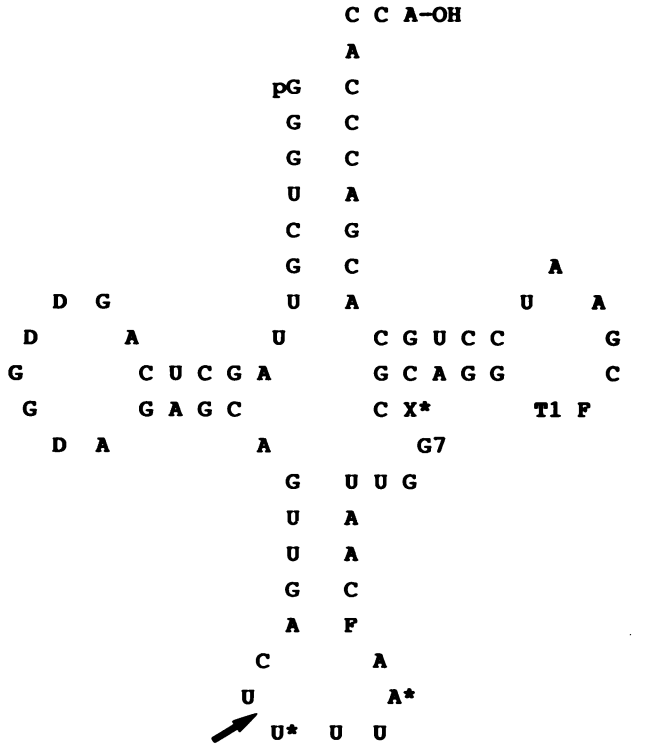

Fig. 1. Nucleotide sequence of vulnerable $tRNA_A$. The arrow indicates the junction between the 5′ and 3′ fragments. Modified nucleoside nomenclature (Sprinzl *et al.*, 1985): A* is probably A7, *N*[(9-D-ribofuranosylpurin-6-yl)carbamoyl]threonine; D, dihydrouridine; F, pseudouridine; G7, 7-methylguanosine; T1, 5-methyluridine, U* is probably U8; 5-methylaminomethyl-2-thiouridine, X* is probably X, 3-(3-amino-3-carboxypropyl)uridine.

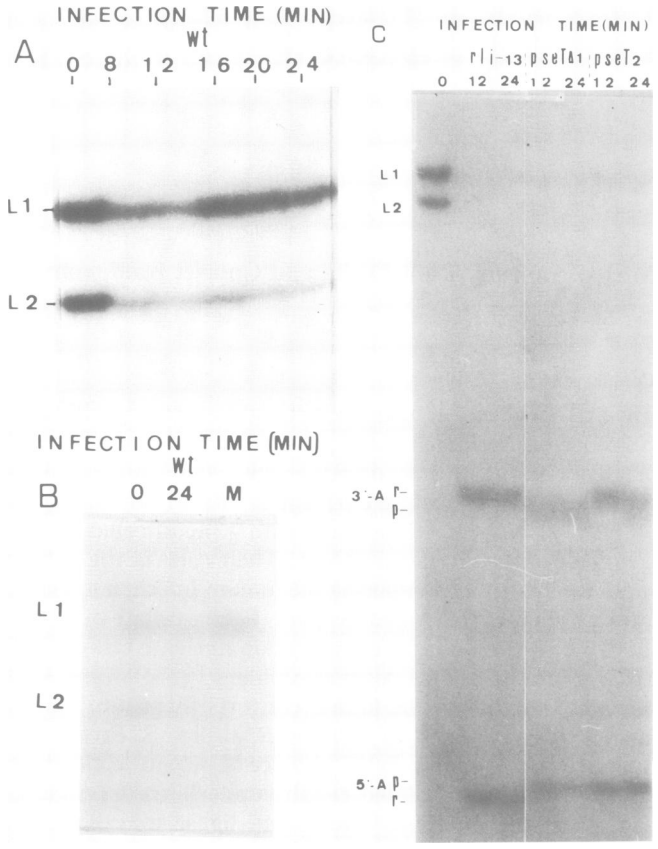

Fig. 2. Host lysine tRNA metabolism during T4 infection of *E. coli* CTr5X. Host tRNA was uniformly labelled with [^{32}P]Pi. Aliquots were isolated from uninfected cells or at the indicated time points of infection with T4D (*wt*) or strains *pseTΔ1*, *pseT2* or *rli-13* and hybridized with ssDNA of an M13 lysine−tDNA clone mRL8601. The hybrid was separated from bulk tRNA by agarose gel electrophoresis, dissociated in formamide and fractionated by polyacrylamide gel electrophoresis as detailed in Materials and methods. **(A)** Intact tRNA from *wt*-infected cells, tRNA region shown. **(B)** Similar to (A) with *in vitro* ligated vulnerable $tRNA_A$, designated M. **(C)** Intact lysine tRNA molecules and lysine tRNA fragments from uninfected cells (○) or from the indicated mutant infections. V, valine $tRNA_1$; L1 and L2, two lysine tRNA variants; 5′-A and 3′-A, vulnerable $tRNA_A$ fragments; p and r, prekinase and preligase fragment forms.

reach a maximum between 8−12 min and then begin to decline (David *et al.*, 1982a,b). Furthermore, the *in vitro* ligated vulnerable $tRNA_A$ fragments migrated with L1 (Figure 2B). In contrast, band L2 was severely reduced after 8 min and was not restored later (Figure 2A). These changes in bands L1 and L2 were underscored by the constancy of the valine $tRNA_1$ bands when the Lys−Val_1 tDNA probe mRL8618 was used (not shown).

During infections with strains *rli-13*, *pseTΔ1* and *pseT2*, tRNAs L1 and L2 disappeared while the vulnerable tRNA fragments 3′-A and 5′-A accumulated (Figure 2C). The reasons for the different mobilities of prekinase (*pseTΔ1*), preligase (*rli-13*) or mixed (*pseT2*) fragment pairs are dicussed more fully below.

In conclusion, vulnerable $tRNA_A$ fragments emanated from lysine tRNA of band L1. They were subsequently repaired in reactions catalysed by polynucleotide kinase and RNA ligase, to restore L1. The nature and metabolic fate of species L2 were not further investigated.

Cleavage termini configurations of prekinase and preligase forms

Vulnerable $tRNA_A$ prekinase 5′-fragment (5′-A) moved on denaturing gels faster than the preligase counterpart (Figure 3). This difference was abolished by treating the prekinase form with purified polynucleotide kinase at pH 6.0, conditions optimal for the intrinsic 3′-phosphatase (Cameron and Uhlenbeck, 1977). The preligase species was not affected by this treatment (Figure 3).

The 3′-phosphatase treatment released one Pi equivalent from prekinase but not preligase 5′-A chain (Table I), suggesting the existence of 3′-P and 3′-OH ends, respectively. Nonetheless, exhaustive alkaline phosphatase digestion released from each form only one Pi equivalent (Table I), probably from the 5′ end. Hence the 3′-P released by the *pseT* protein was inaccessible to alkaline phosphatase.

This conclusion was supported by the failure to release Pi from the prekinase 5′-A chains by nuclease P1, an enzyme that hydrolyses 3′-phosphomonoesters. However, the prekinase but not preligase digest contained a product migrating with R_{pG} of 0.75 (Figure 4). This compound, which must have emanated from the 3′ end, was identified as uridine-5′-phosphate, 2′:3′-cyclic phosphate (pUp>). This, by (i) RNase A hydrolysis which converted it into a compound migrating with 3′-5′-uridine diphosphate (pUp) and (ii) subsequent digestion with nuclease P1 which yielded a mixture of pU and Pi (not shown). These data demonstrated the presence of a terminal 2′:3′-P> in the prekinase species and its *in vivo* removal by the phosphodiesterase (O.C.Uhlenbeck, personal communication) and 3′-phosphatase (Cameron and Uhlenbeck, 1977) resident in the *pseT* protein.

The prekinase and preligase forms of fragment 3′-A were compared at the level of their respective 5′-terminal dodecanucleotides, generated by RNase T1. The prekinase T1-dodecanucleotide migrated slower than the preligase species (Figure 5A),

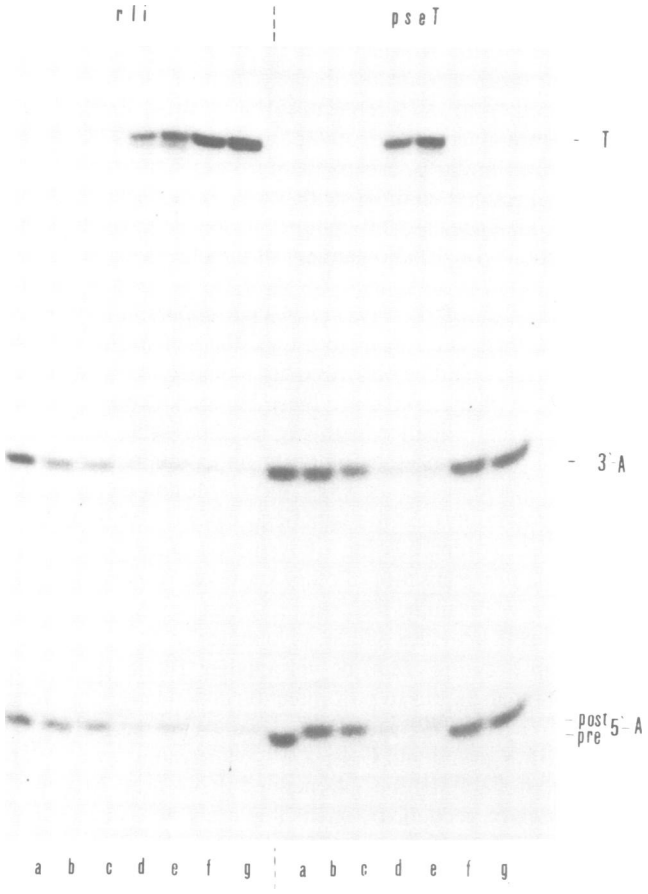

Fig. 3. *In vitro* ligation of vulnerable $tRNA_A$ fragments. Uniformly labelled vulnerable $tRNA_A$ fragments of the indicated infections (rli, *rli-13*; pseT, *pseTΔ1*) in equimolar proportions were subjected to the following enzymatic treatments and separated by denaturing gel electrophoresis as in Materials and methods. **(lane a)** Untreated; **(lane b)** after 1 h incubation with polynucleotide kinase at pH 6.0; **(lane c)** additional 1 h incubation at pH 7.5; **(lane d)** further incubation with RNA ligase 1 h or **(lane e)** 2 h. Lanes **(f)** and **(g)** correspond to **(d)** and **(e)** but polynucleotide kinase was omitted. T, ligated fragments; 3′-A and 5′-A, 3′- and 5′-fragments of vulnerable $tRNA_A$; pre and post: prekinase and postkinase forms of 5′-A.

Table I. End groups of prekinase and postkinase forms of vulnerable tRNA fragment 5′-A

T4 strain	Molar equivalents of released end group		
	3′-phosphatase (*pnk* protein) (Pi)	Alkaline phosphatase (Pi)	Nuclease P1 (pUp>)
pseT2	0.8	n.d.	n.d.
pseTΔ1	1.0	1.0	0.7
rli-13	>0.1	0.9	>0.1

The various forms of fragment 5′-A, uniformly labelled with [^{32}P]Pi, were exhaustively digested with alkaline phosphatase, 3′-phosphatase (polynucleotide kinase) or nuclease P1, as in Materials and methods. Inorganic phosphate released by the phosphatases was separated from the polynucleotide by chromatography on PEI−cellulose and the spots excised and counted. The amount of pUp> released was determined from the ratio of radioactivity in the spot containing it (Figure 4B) to other mononucleotides of the digest.

commensurate with the presence of 5′-phosphoryl only in the latter. Furthermore, incubation of the prekinase species with polynucleotide kinase and ATP prior to the RNase T1 digestion

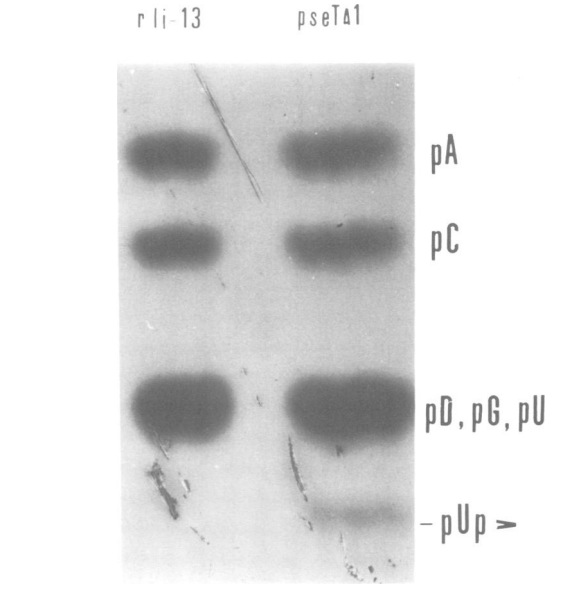

Fig. 4. Release of pUp> end group from prekinase 5′-A chains. Fragment 5′-A was isolated from the indicated mutant infection, digested with nuclease P1 and the digest separated by cellulose thin layer developed with isobutyric acid:0.5 M ammonia (66:34).

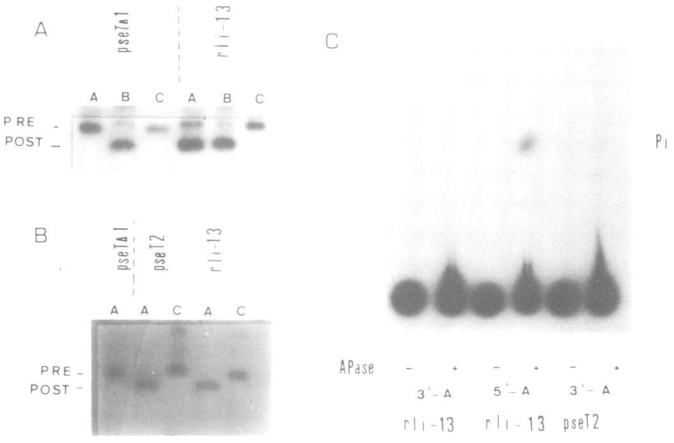

Fig. 5. (A) Analysis of 5′-end group in 3′-A prekinase and postkinase fragments. The 3′-A fragment was isolated from cells infected with the indicated T4 strains and subjected to **(A)** no treatment; **(B)** incubation with polynucleotide kinase and ATP; **(C)** incubation with alkaline phosphatase. The RNA was extracted with phenol, digested with RNase T1 and separated by electrophoresis on 20% polyacrylamide−7 M urea gel. Prekinase and preligase forms of the 5′-terminal T1-dodecanucleotide are indicated by (pre) and (post), respectively, **(B)** Comparison of 5′ end groups of 3′-A from *pseTΔ1*, *pseT2* and *rli-13* infections. **(C)** Pi release from vulnerable tRNA fragment ends. About 1500 c.p.m. of the indicated fragment were chromatographed on PEI−cellulose thin layer before or after incubation with alkaline phosphatase (mixtures A and C of **A**). Fragment 5′-A was included as a control releasing one chain equivalent of Pi.

abolished this difference while prior treatment with alkaline phosphatase had no effect. Conversely, the preligase dodecanucleotide was not affected by prior incubation of original 3′-A with polynucleotide kinase but prior alkaline phosphatase treatment shifted it to the postion of the prekinase band. Therefore, the prekinase 3′-A chain contained a 5′-OH, the preligase a 5′-P end. Nevertheless, the phosphatase did not release labelled Pi from the preligase species (Figure 5C), in agreement with the fact that kination of the preligase species occurred during infection in nonlabelled

medium. This result ruled out direct transfer of the 3′-phosphoryl to the 5′ end or a different mode of nuclease cleavage in the *rli-13* infection.

Surprisingly, fragment 3′-A of mutant *pseT2* also contained a 5′-phosphoryl group, like the preligase *rli-13* species, as judged from the behaviour of the *pseT2* dodecanucleotides (Figure 5B). Thus, although both the *in vitro* activities of polynucleotide kinase and 3′-phosphatase are inactivated by the *pseT2* mutation (Sirotkin *et al.*, 1978), *pseT2* is leaky *in vivo* with regard to polynucleotide kinase. In addition, 5′-kination of the one cleavage terminus can occur independent of the 3′-dephosphorylation of the other. The mixed 5′-prekinase and 3′-preligase pair of *pseT2* is shown in Figure 2C.

Conversion of the prekinase form into an RNA ligase substrate

The cleavage termini configurations of vulnerable $tRNA_A$ intermediates were 2′:3′-P> and 5′-OH in the *bona fide* prekinase species (*pseTΔ1*) and 3′-OH and 5′-P in the preligase counterpart (*rli-13*). This suggested that the *pseT* gene product converted *in vivo* the prekinase form into an RNA ligase substrate. These reactions could in fact be reproduced *in vitro*. Thus, the preligase pair could be ligated in a reaction catalysed by RNA ligase while the prekinase pair became an RNA ligase substrate only after prior incubation with polynucleotide kinase (Figure 3).

Discussion

Anticodon nuclease catalyses a step in an RNase A-type reaction

The anticodon nuclease cleavage that generates the prekinase intermediate constitutes a first step in an RNase A reaction type (cf. Fersht, 1984). The second step, hydrolysis of the 2′:3′-cyclic phosphodiester, is mediated by the *pseT–pnk* protein *in vivo* and *in vitro*. The failure to hydrolyse the cyclic phosphodiester could be an inherent property of the anticodon nuclease. Alternatively, a conformational change in the tRNA, elicited by the cleavage event, could remove the 2′:3′-P> from the fixed active site of a particulate anticodon nuclease. Interestingly, *stp* mutations, which inactivate the anticodon nuclease (Kaufmann *et al.*, 1986), affect a small reading frame of 29 amino acids containing all the functional residues that are implicated with RNase A catalysis (Chapman, Morad, Kaufmann, Gait, Jorrissen and Snyder, submitted).

In vivo reactions of T4 polynucleotide kinase and RNA ligase

The 2′:3′-P> and 5′-OH termini pair generated by the anticodon nuclease is rearranged *in vivo* by the triple activities of polynucleotide kinase to furnish a 3′-OH and 5′-P RNA ligase acceptor–donor substrate pair, as judged from comparing the structures and substrate properties of the intermediates of pnk^- and rli^- infections. That the latter intermediate is actually ligated *in vivo* is suggested by the restoration of lysine tRNA and disappearance of its fragments late in *wt* infection, contrary to the depletion of lysine tRNA and accumulation of its fragments in the rli^- infection (Figure 2). These results confirm the roles predicted for polynucleotide kinase and RNA ligase in host tRNA cleavage and reunion (Kaufmann and Kallenbach, 1975; David *et al.*, 1979, 1982a,b; Kaufmann and Amitsur, 1985; Kaufmann *et al.*, 1986). We term these cleavage–ligation reactions reprocessing, to indicate that they occur with mature and functional RNA in response to changing cellular circumstances. Whether these reprocessing reactions also entail a slight structural change somewhere in the lysine tRNA molecule remains an open question.

How does host tRNA reprocessing benefit T4?

Depletion of lysine tRNA in $pseT^-$ or rli^- infected *E. coli* CTr5X can itself account for the cessation of late protein synthesis and defects in DNA replication during these abortive infections (Depew and Cozzarelli, 1974; Sirotkin *et al.*, 1978; Runnels *et al.*, 1982). Similarly, escape from *prr* restriction is probably caused by (i) restoration of lysine tRNA and other species affected by the anticodon nuclease through polynucleotide kinase and RNA ligase reactions, or (ii) abolishment of the cleavages by *stp* mutations.

This interpretation portrays *stp* as a participant in host restriction and *pnk–rli* as a means to escape the self-inflicted lesion. However, these could be borrowed tasks of enzymes that primarily benefit the phage. What are these beneficial roles? The fact that the cleavage–ligation pathway is dispensable even on *E. coli prr* strains (i.e. inactivation of the anticodon nuclease by an stp^- mutation suppresses the pnk^- and rli^- lesion on *prr* strains) raises the possibility that the beneficial roles of *stp*, *pnk* and *rli* are unrelated to reprocessing of lysine or other host tRNAs. Nonetheless, in the absence of other known *in vivo* roles of these T4 products, we examine the possibility that reprocessing of host tRNA by itself provides subtle advantages to the phage.

Thus, reprocessing could be yet another T4 device to adapt the translation apparatus to post-infection codon usage (cf. Scherberg and Weiss, 1972; Sueoka and Kano-Sueoka, 1970; Ikemura, 1981a,b; Rand and Gait, 1984; Maruyama *et al.*, 1986). Others are probably (i) the T4-encoded tRNAs (cf. McClain *et al.*, 1972) which recognize codons that are scarce in *E. coli* but abundant in T4, and (ii) the T4-induced removal of the host leucine $tRNA_1$ (Kano-Sueoka and Sueoka, 1968; Yudelevich, 1971), a species that decodes a predominant *E. coli*, but not T4, leucine codon. Exceptional to this pattern is the AAU over AAC (Asn) preference of T4 which is reversed to that of *E. coli* but is not known to be compensated by a phage-encoded cognate tRNA or by removal of an excessive host tRNA. This and perhaps more subtle codon usage differences, such as lesser preference of AAG over AAA (Lys) by T4, could be remedied through modulation of levels or decoding specificities of reprocessed host tRNAs.

Materials and methods

Bacterial and phage strains

The sources of *E. coli* and T4 strains used in this study were described by Kaufmann *et al.* (1986).

Isolation of vulnerable tRNA fragments

Vulnerable tRNA fragments were prepared essentially as before (David *et al.*, 1982a). However, in scaled-up preparations, the growth medium contained 1–2 μCi/ml of [^{32}P]Pi, sufficient to monitor the fragments but negligible in *in vitro* end-labelling. Typically, 800 ml of *E. coli* CTr5X cells were infected with T4 *rli-13* for 20 min at 30°C. The tRNA was extracted with phenol and further purified on a small DEAE–cellulose column and fractionated by 15% polyacrylamide–7M urea gel electrophoresis. The tRNA fragments were traced by autoradiography and extracted from the gel. About 10–20 μg each of the vulnerable $tRNA_A$ fragments were obtained.

RNA sequencing

The RNA sequencing procedures employed are described by Silberklang *et al.* (1979) and Diamond and Dudock (1983). To determine 5′-end groups, uniformly labelled fragments were digested with RNase T2 or 5′-^{32}P-labelled fragments with nuclease P1 and the relevant pNps or pNs of the respective digests identified chromatographically. Determination of 3′-end groups were performed by labelling with [5′-^{32}P]pCp in a reaction catalysed by RNA ligase (England and Uhlenback, 1978), followed by RNase T2 hydrolysis and chromatographic identification of the labelled Nps. Base composition was determined on nuclease P1 digests of uniformly labelled fragments separated by two-dimensional cellulose t.l.c.

(Nishimura, 1972). Direct sequencing of end-labelled fragments or the *in vitro* ligated form was carried out by partial cleavage with base-specific RNases followed by gel electrophoresis (Donis-Keller *et al.*, 1977) essentially as in Silberklang *et al.* (1979), with addition of the C-preferring ribonuclease CL3 (Boguski *et al.*, 1980). Positions of nucleotides not distinguished by base-specific RNases were determined according to Stanley and Vassilenko (1978). Nucleoside diphosphate derivatives of some modified bases were converted with nuclease P1 into pNs and further analysed by two-dimensional t.l.c.

Enzymatic treatments of tRNA fragments

Complete digestions with nuclease P1, RNase T2 and alkaline phosphatase were adapted from the RNA sequencing protocols. Digestion with 3′-phosphatase was carried out in 10 μl of 50 mM ammonium acetate buffer, pH 6.0; 10 mM $MgCl_2$, 1 mM dithiothreitol containing 3000–5000 c.p.m. of uniformly labelled tRNA fragment. 1 μg carrier tRNA and 2 units of T4 polynucleotide kinase. *In vitro* ligation of fragments was carried out in 50 μl of 50 mM Tris–HCl buffer, pH 7.5; 10 mM $MgCl_2$, 1 mM dithiothreitol containing 3400 c.p.m. of uniformly labelled 5′-A and 4200 c.p.m. of 3′-A and 1 unit of purified T4 RNA ligase. Incubation was at 30°C. In scaled-up ligations the mixture contained 680 and 840 ng of these fragments, respectively. The complete *in vitro* ligation mixtures of the prekinase intermediate and of the appropriate controls were started in 20 mM ammonium acetate buffer, pH 6.0; 10 mM $MgCl_2$ and 1 mM dithiothreitol containing 1 unit of polynucleotide kinase and uniformly labelled fragments as above. Following 1 h incubation at 37°C the mixture was made up to 1 mM ATP and 50 mM Tris–HCl buffer, pH 8.0; and incubation continued for another hour. Then, 1 unit RNA ligase was added and incubation continued for up to 2 h.

Monitoring lysine tRNA with M13 clones

The vulnerable tRNAs hybridize with two sets of *E. coli* K-12 DNA restriction fragments corresponding to the lysine tRNA–valine $tRNA_1$ loci (Levitz, Paz and Kaufmann, unpublished results), *lysT* (Yoshimura *et al.*, 1984) and *lysN* loci (Uemura *et al.*, 1985). A 1.4-kb *Pst*I fragment containing *lysT* was cloned into phage M13mp11 (Messing, 1983). Recombinants were screened with 5′-^{32}P-labelled vulnerable tRNA fragments and a positive clone, mRL86, further characterized by dideoxy chain termination DNA sequencing (Sanger *et al.*, 1977). Among subclones arising by spontaneous deletions, mRL8601 retained the promoter and a proximal lysine tRNA gene while mRL8618 contained in addition a portion of valine $tRNA_1$ (the latter served to normalize the fluctuating lysine tRNA level against the constant valine $tRNA_1$). To isolate intact vulnerable tRNA and derived cleavage products, 10 μg ssDNA of the M13 clone were incubated with total tRNA of 2×10^8 cells in 100 μl of 0.75 M NaCl, 0.1 M Tris–HCl buffer, pH 7.8; 5 mM sodium EDTA in 50% formamide containing 0.1% sodium dodecyl sulfate, for 15 min at 25°C. Following ethanol precipitation and separation from bulk tRNA by electrophoresis of 0.7% low melting agarose in 0.1 M Tris-borate buffer, pH 8.3, 2.5 mM sodium EDTA. The hybrid was extracted from the melted gel with phenol and precipitated. The RNA was released by dissociating the hybrid in formamide and fractionated by electrophoresis on 15% nondenaturing polyacrylamide gel.

Acknowledgements

This work was supported by US National Institutes of Health grant GM34124.

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Received on March 31, 1987; revised on May 22, 1987