Host transfer RNA cleavage and reunion in T4-infected Escherichia coli CT5x

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Received 1 April 1985; Revised and Accepted 30 May 1985

ABSTRACT
T4 mutants lacking polynucleotide kinase (pnn-) or RNA ligase (rll-) do not grow on E. coli CT5x. During the abortive infections there accumulate host tRNA fragments that match into two species severed 3' to the anticodon. The CT5x-specific fragments appear only transiently with wt phage, implicating the affected enzymes in phosphoryl group rearrangement and religation [David et al. (1982) Virol. 123, 480]. In a search for the vulnerable host tRNAs and putative religation products, tRNA ensembles from uninfected E. coli CT5x or cells infected with various phage strains were fractionated and compared. A tRNA species absent from rll- infected cells but present in uninfected cells or late in wt infection was thus detected. RNase T1 finger prints of this species, isolated before or after wt infection, were compared with that of an in vitro ligated pair of CT5x-specific fragments. The results indicated that this tRNA is cleaved upon infection and later on restored to its original or to a very similar form, by polynucleotide kinase and RNA ligase reactions. It is suggested that depletion of such vulnerable host tRNA species underlies the restriction of pnn- or rll- phage on E. coli CT5x.

INTRODUCTION
Although T4 polynucleotide kinase (1) and RNA ligase (2) were characterized biochemically (3, 4) and genetically (5), their physiological roles are not well understood. Using E. coli CT5x, a host strain restricting specifically pnn- and rll- phage (6-8), the affected enzymes were shown to be involved in host tRNA metabolism (9-11). The following observations underscore this conclusion. First, fragments that match into two tRNA structures severed 3' to the anticodon appear transiently during wt infection of E. coli CT5x but not in permissive host strains (9). Second, the CT5x specific tRNA fragments accumulate with pnn- and rll- infections, implicating polynucleotide kinase and RNA ligase in further processing, presumably through phosphoryl group rearrangement of the cleavage termini followed by their religation (10). Thirdly, suppressor mutations in stp that allow growth of pnn- or rll- phage on E. coli CT5x (6-8), inhibit
the anticodon loop cleavages (11). Finally, the E. coli CTr5x locus restricting pkn- and ril- mutants (prr, ref. 12) cotransduces with the ability to generate host tRNA cleavage patterns characteristic of E. coli CTr5x (11), indicating that prr determines the vulnerability of certain host tRNAs to the phage anticodon nuclease.

In studying the relation of the CTr5x-specific tRNA cleavages and further processing reactions to the host restriction mechanism and the phage ability to escape it, we looked for host tRNA species from which the anticodon nuclease cleavage products originate as well as for the putative repair products. Here we report the detection of such compounds.

MATERIALS AND METHODS

Materials - Benzoylated DEAE-cellulose (BDC) was purchased from Boehringer GmbH, Mannheim; RNase T1 and T4 RNA ligase from P-L Biochemicals Inc. Milwaukee; DEAE-cellulose (DE-52) from Whatman LTD England, cellulose acetate strips from Schleicher and Schuell Inc., Keene NH; and polyethyleneimine thin layer plates (CEL 300 PEI) from Macherey and Nagel GmbH, Dueren. Phage and bacterial strains - E. coli CTr5x (6), T4 ril-13 (8) were obtained from Larry Snyder, Michigan State University-East Lansing. The pseT2 (3'-phosphatase-polynucleotide kinase deficient, refs. 6&7) revertant lacking the anticodon nuclease (11) was identified as an stp-, pseT2 double mutant (Snyder, pers. comm.). Isolation of host tRNA vulnerable to T4 anticodon nuclease - E. coli CTr5x RNA was pulse labeled with 32P-Pi before infection and was extracted from uninfected cells or from cells infected for 20 min at 30° with the indicated phage strain, as previously described (9). The production of host tRNA fragments was monitored by separating aliquots by polyacrylamide gel electrophoresis (9), to ascertain the effectiveness of infection. In the case of the anticodon nuclease mutant that does not produce the CTr5x specific tRNA fragments, infection could be monitored by the appearance of the host leucine tRNA1 fragments that are generated in many E. coli strains by a different T4 activity (9,11,13,14). Each of the RNA preparations was applied to a DEAE-cellulose column (0.3 ml) equilibrated with 0.01 M Tris-HCl buffer, pH 7.5. The column was washed with 5 ml of 0.25 M NaCl in pH 7.5 buffer and the crude tRNA eluted with 1 ml of 1 M NaCl in this buffer. The tRNA was precipitated with ethanol, dissolved in 0.1 ml of 0.01 M Tris-HCl buffer, pH 8.8; and incubated for 10 min at 37°. The deacylated tRNA was diluted in 5 ml of 20 mM sodium acetate buffer, pH 5.5; and applied to a
BDC column (1.0 ml) equilibrated with the pH 5.5 buffer. The column was washed successively with 6 ml ea. of 0.6 M NaCl, 0.8 M NaCl and 1.5 M NaCl+10% ethanol, in the pH 5.5 buffer. The tRNA of the various fractions was concentrated on DEAE-cellulose as above and applied to a 60 cm long and 0.75 mm thick slab of 15% polyacrylamide-7M urea gel in 25 mM Tris borate buffer, pH 8.3; 0.7mM sodium EDTA. Electrophoresis was for 24 hours at 2000 V at 25° ambient temperature. The gel was autoradiographed and the region of the shorter tRNA molecules of ca. 75-80 nucleotides was excised from it. The strip was equilibrated with 0.25 mM Tris borate buffer, pH 8.3 containing 0.001% of xylene cyanol, placed between 40 cm long glass plates and a 22% gel was cast underneath. Electrophoresis in the second dimension was performed within 30 min of gel polymerization and lasted 36 hours at 25° ambient temperature. The voltage was gradually increased from 800 to 1500V during the first 3 hours. The gel was autoradiographed and tRNA spots found in fractions from uninfected cells, wt or stp- infected cells but absent with rII-13 were excised, crushed and suspended in 0.2 ml of 0.3M sodium acetate buffer pH 5.5, containing 0.2% sodium lauryl sulfate and 0.02 mg/ml carrier tRNA. The suspension was shaken 10 min and extracted with an equal volume of aqueous phenol. Under these conditions, greater than 90% of the radioactive tRNA was found in the aqueous phase, free of gel particles. RNA finger print analysis - Digestion of the tRNA or tRNA fragments with ribonuclease T1, electrophoresis of oligonucleotides on cellulose acetate strips in 5% pyridine acetate buffer, pH 3.5 in 7M urea, and subsequent chromatography on PEI-cellulose thin layers using pyridine:formic acid:water 7.5:7.5:85 was essentially as in ref. 15 and 16. In vitro ligation of vulnerable tRNA fragments - E. coli CTr5x-specific tRNA fragments II and VI, isolated from wt infected cells 6 min post-infection (9), were mixed and incubated in vitro at 25° with purified T4 RNA ligase in a reaction mixture (0.01 ml) containing: 0.2-0.5 pmol of each fragment, 1 mM ATP, 5 mM MgCl₂, 1 mM dithiothreitol, 50 mM Tris-HCl buffer, pH 7.5; and 0.5 units of enzyme. The reaction course was monitored by electrophoresis on 15% polyacrylamide-7M urea gel, E. coli CTr5x- vulnerable tRNA (vulA, see below) serving as a marker. Under these conditions, the fragments were ligated into a form migrating with the vulnerable tRNA marker. Ligation was ca. 80% complete within 5 min (Fig. 1). The residual fragments constitute the pre-kinase intermediate that can not be ligated. In contrast, the fragment pair from late in rII- infection is quantitatively ligated (unpublished results). The in vitro ligated tRNA was extracted from the gel and finger printed.
RESULTS
Total host tRNA preparations from uninfected E. coli CTr5x, or from cells infected with various phage strains were compared, in search for the precursors of the CTr5x-specific RNA fragments and the putative reigation products. It was expected that uninfected cells and cells infected with stp phage (lacking the anticodon nuclease, ref. 11) would contain the vulnerable tRNA species whereas late wt infection would yield the reigated form. In contrast, rII infected cells, in which the fragments accumulate, were expected to contain neither the vulnerable tRNA substrates nor the putative reigated products.

To label the host tRNA selectively, E. coli CTr5x cells were pulse-labeled with $^{32}$P-PI prior to infection. The tRNA was extracted from uninfected cells or from cells infected for 20 min at 30° with the appropriate phage strain and fractionated as described in METHODS. Comparison of the 0.8M NaCl BDC fractions from the various tRNA preparations by two dimensional polyacrylamide gel electrophoresis revealed a tRNA spot, designated vulA,
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Incubation Time (min) 0 1 5 20

Figure 2 - *In vitro* ligation of the CTr5x specific fragments II and VI. E. coli CTr5x-specific tRNA fragments II & VI (9) were ligated with T4 RNA ligase as described in METHODS. Aliquots were analyzed at the indicated time points by polyacrylamide gel electrophoresis. M - vulnerable tRNA marker, L - ligated form.

that was found at identical positions in the electrophograms of uninfected cells or cells infected with the pnk~ , stp~ double mutant or the wt phage; but was missing from the rII~ electrogram (shown in Fig. 1 for wt & rII~). No differences were detected between other BOC fractions. Based on the results, it was assumed that the spot missing from the 0.8M BOC electrogram of the rII-13 preparation corresponds to a pair of the CTr5x-specific tRNA fragments (probably the abundant pair - II&VI), which appear transiently in wt infection and accumulate with pnk~ and rII~ mutants (9,10). In fact, the *in vitro* ligated form of fragments II&VI and Vul A tRNA from uninfected cells featured identical electrophoretic mobilities (Fig. 2). The assumption that Vul A and fragments II&VI are closely related was confirmed by comparing RNase T1 finger prints of Vul A from both the uninfected and the wt preparations with the finger prints of the CTr5x-specific tRNA fragments II and VI; as well as with the finger prints of the *in vitro* ligated form of fragments II&VI. Thus, the upper portions of the finger prints of the two Vul A preparations, containing the major oligonucleotides, were identical and contained the spots seen in the separate finger prints of fragment II and VI. This oligonucleotide set was also seen with the finger print of the *in vitro* ligated form of fragments II&VI (Fig. 3). Preliminary results point to the existence of a minor
E. coli CTr5x species, designated vulB, that is related to the minor pair of the CTr5x specific fragments (III&V) in a similar manner.

**DISCUSSION**
A simple interpretation of the data is that at least one E. coli CTr5x tRNA species is cleaved by the T4 induced anticodon nuclease and further processed by polynucleotide kinase and RNA ligase to restore the original or a very similar species. We base this conclusion on the great similarity between the vul A tRNA species from uninfected cells, from late in wt infection as well as the in vitro ligated fragments II&VI; in their chromatographic and electrophoretic behavior and RNase T1 finger print patterns.

How are these tRNA cleavage and reunion reactions related to the host restriction mechanism and to the phage ability to escape it? Since stp mutations that suppress mutations in pnk or rll (6-8) also inhibit the cleavages of the vulnerable tRNAs (11), it is clear that T4 development on E. coli CTr5x does not depend on these cleavages. Hence, the phage does not require the destruction of the vulnerable host tRNA, its alteration or replacement by a phage coded species. It seems, rather, that the restriction of pnk- or rll- phage is due to the loss of one or more of the vulnerable host tRNA species. Alternatively, the restriction could result from an inhibitory effect exerted by the severed tRNA molecules, acting perhaps as tRNA analogs. However, we favor the model of restriction through tRNA depletion, for the following reasons. First, vul A tRNA is virtually depleted during rll- infection. In contrast, the maximal level of anticodon nuclease reaction products seen during wild type infection never reaches that accumulating with pnk or rll mutants (10,11), indicating that a certain level of the vulnerable tRNAs is maintained throughout the wt infection. Second, the anticodon nuclease reaction products persist during a considerable portion of the wt infection period. It follows that the damaged tRNA could exert an inhibitory effect only above a threshold concentration and/or at a critical time point. This problem is accentuated by the existence of pnk- false revertants with only a partial, sometimes hardly detectable deficiency in anticodon nuclease (11). Thus, suppression of the pnk- phenotype can be explained in that a weaker anticodon nuclease activity is perhaps insufficient to deplete the vulnerable tRNAs before mature phage has been produced.

Although, for reasons discussed above, anticodon nuclease, polynucleotide
Figure 3. Upper portions of RNase T1 finger prints of the anticodon nuclease products fragments II and VI; vul A tRNA from uninfected cells, vul A from late in wt infection and of the in vitro ligated (11g) form of fragments II&VI.
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kinase and RNA ligase are not needed by the phage for the alteration, destruction or replacement of E. coli CTr5x tRNAs, these enzyme may figure in such roles in other, yet unexplored T4 host strains.

Facts suggesting the existence of additional RNA cleavage and ligation processes in prokaryotes were recently reported. Thus, an RNA ligase activity, joining yeast tRNA splicing intermediates by a 2'-5' phosphodiester bond was detected in extracts from various bacteria (17) and some archaeabacterial tRNA genes were found to contain introns (18). Another striking discovery was of a 1017 bp intron within the T4 thymidylate synthase gene. It was suggested that splicing of a primary thymidylate synthase transcript, mediated perhaps by polynucleotide kinase and RNA ligase, generates a functional mRNA (19).

Yet, while the T4 induced host tRNA cleavage and reunion pathway (termed by us reprocessing pathway) may be related to RNA splicing, it also differs in two important respects. First, RNA reprocessing does not entail the removal of an intron. Second, the reprocessing substrate is a stable, presumably functional tRNA molecule.

ACKNOWLEDGEMENTS
We thank Larry Snyder for strains and for communicating results prior to publication. This work was supported by U.S. National Institutes of Health Grant 1 R01 GM34124-01.

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