Bacteriophage T4-induced anticodon-loop nuclease detected in a host strain restrictive to RNA ligase mutants

(polynucleotide kinase/leucine $tRNA_1$ nuclease/bacteriophage T4 tRNAs/*Escherichia coli* CTr5x isoleucine tRNA)

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ABSTRACT **The fate of host tRNAs during T4 bacteriophage infection was investigated with *Escherichia coli* CTr5x, the only known host strain that is restrictive to RNA ligase and polynucleotide kinase mutants. Three CTr5x tRNA species were cleaved during infection. One was leucine $tRNA_1$, which was cleaved in the extra arm, as reported elsewhere for *E. coli* B infected with bacteriophage T2 or T4. The other two were specific to *E. coli* CTr5x and were not cleaved in various other hosts. One of the cleaved CTr5x-specific tRNAs had an anticodon sequence of the *E. coli B* "major" isoleucine tRNA but otherwise little sequence homology. Both CTr5x-specific tRNAs were cleaved by a distinct T4-induced endonuclease, other than that of leucine $tRNA_1$, because the CTr5x-specific cleavages (*i*) were induced later in infection, (*ii*) persisted with a T4 mutant deficient in leucine $tRNA_1$ endonuclease, and (*iii*) occurred in the anticodon loop. The specific manifestation of the anticodon-directed endonuclease activity in T4-infected *E. coli* CTr5x suggests roles for RNA ligase and polynucleotide kinase in processing of host tRNA species.**

RNA ligase from T4 bacteriophage [polyribonucleotide synthetase (ATP); poly(ribonucleotide):poly(ribonucleotide) ligase (AMP-forming), EC 6.5.1.3; ref. 1] has been well characterized *in vitro* (2, 3), but its role in phage infection still needs to be determined (4–6). We have previously suggested that RNA ligase and additional T4 enzymes, such as polynucleotide kinase (EC 2.7.1.78; ref. 7) and a tRNA-specific endonuclease (8, 9), may constitute a pathway of host tRNA breakage and reunion (refs. 5 and 10 and Fig. 7). To examine this hypothesis we followed the fate of host tRNAs during T4 infection of *Escherichia coli* CTr5x (11). This host strain, and CT196, from which it was derived (11), are to date the only strains known to restrict polynucleotide kinase–3′-phosphatase (11–13) and RNA ligase (ref. 6 and unpublished results) mutants. It was expected, therefore, that *E. coli* CTr5x might feature a specific pattern of tRNA processing during T4 infection.

We describe here a T4-induced endonuclease activity directed towards the anticodon loops of two *E. coli* CTr5x tRNAs. Its specific manifestation in *E. coli* CTr5x reinforces the idea about roles of RNA ligase and polynucleotide kinase in host tRNA metabolism.

METHODS

Bacterial and Viral Strains. *E. coli* CTr5x, a cross of *E. coli* K-12 and CT196 (11), was obtained from L. Snyder; strains CT196, CT397, CT439, CT447, CT460, and CT511 were from W. B. Wood and W. H. McClain. T4 strain L8-7, a mutant deficient in leucine $tRNA_1$ endonuclease (W. H. McClain, personal communication) was obtained from W. H. McClain.

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Analysis of Host-tRNA Cleavage During Infection. Bacteria were grown at 30°C in LP medium (14) supplemented by L-tryptophan at 20 μg/ml, 0.1 mM $CaCl_2$, and 0.4% glucose. When at 2–3 × 10^7 cells per ml, the culture was made up to 0.1 mCi/ml (1 Ci = 3.7 × 10^{10} becquerels) in [^{32}P]P_i and growth continued to 2–4 × 10^8 cells per ml. The cells were centrifuged, resuspended in an equal volume of nonlabeled phosphate-rich SM9 medium (15), further grown for 15 min, and then infected with the T4 strain of choice at a multiplicity of infection of 6–12. The degree of infection was determined by plating aliquots before and during infection and counting bacterial colonies; it was found to be greater than 90% under these conditions. Culture aliquots were withdrawn either before or at progressive infection times, chilled by mixing with 0.3 vol of frozen LP medium, and centrifuged. Low molecular weight RNA was extracted with phenol at 25°C in the presence of carrier rRNA at 100 μg/ml and precipitated with ethanol as described by Ikemura (16). The extracted RNA was separated on a 15% polyacrylamide/7 M urea gel slab in 25 mM Tris borate buffer, pH 8.3 (16). The gels were autoradiographed with preflashed film and intensifier screens.

RNA Sequence Analysis. RNase T1 two-dimensional analysis was carried out according to Brownlee (17) on ^{32}P-labeled fragments extracted from the gels described above. Readout RNA sequence determination procedures were performed on nonlabeled RNA fragments from scaled-up preparations. These fragments were end-labeled with RNA ligase or polynucleotide kinase and their sequences were determined essentially according to the procedures of Donnis-Keller *et al.* (18) and Lockard *et al.* (19). To identify modified nucleotides, the procedure of Gupta and Randerath was employed (20). The detailed sequence derivation of CTr5x tRNAs whose cleavage was specifically induced by T4 phage will be published elsewhere.

RESULTS

Unique Host tRNA Cleavage Pattern in T4-Infected *E. coli* CTr5x. Infection of *E. coli* B by T2 or T4 phages results in cleavage of the host's leucine $tRNA_1$ (8, 9). In contradistinction, infection of *E. coli* CTr5x resulted in the cleavage of at least *three* host tRNAs. Thus, when ^{32}P-labeled CTr5x tRNA was examined by denaturing gel electrophoresis, six RNA bands of ca. 35–50 nucleotides were found to appear during infection (Fig. 1). Two of these bands, I and IV, were generated earlier than the others and were identified by RNase T1 analysis (Fig. 2) as leucine $tRNA_1$ fragments 1–48 and 49–87 (9). The CTr5x-typical fragments appeared later in infection and also persisted into later infection times. They seemed to have been produced from tRNA molecules, because they could be matched by their lengths and relative intensities into pairs of "tRNA halves" (bands II + VI and III + V). Bands II and VI reached intensities comparable to those of fragments I and IV and probably stemmed from an abundant tRNA species, whereas bands III

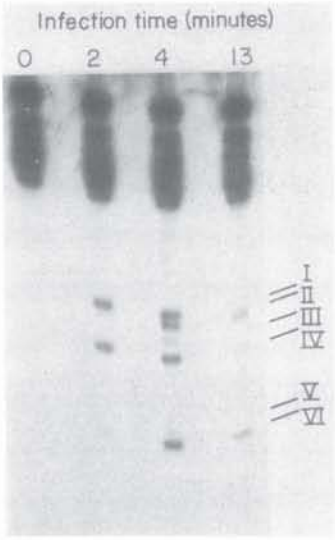

FIG. 1. Cleavage pattern of *E. coli* CTr5x tRNAs during T4 infection. *E. coli* CTr5x cells were pulse-labeled with [^{32}P]P_i, chased with nonlabeled P_i, and infected with T4 phage. Low molecular weight RNA was extracted and separated by denaturing gel electrophoresis at the indicated infection times.

and V were produced in minor quantities (16). These bands were not derived from leucine $tRNA_1$, as indicated by further analyses (see below).

The particular cleavage pattern of host tRNAs observed with T4-infected *E. coli* CTr5x (Fig. 1) was also seen with its parent strain CT196 but not with a number of other *E. coli* hosts. Among the strains tested were *E. coli* C600, MRE600, CR63, and strains CT397, CT439, CT447, CT460, and CT511. The latter hosts (CT397 through CT511) as well as CT196 and CTr5x restrict T4 mutants lacking tRNA genes (psu_b^-), (refs. 21–23; W. H. McClain, personal communication; and unpublished results). It should be emphasized that, while CT196 and CTr5x belong to the group of psu_b^- restricting hosts, they are unique in restricting rli^- and pnk^- mutants (refs. 6 and 11 and unpublished results). The cleavage patterns of host tRNAs are shown for some of these strains in Fig. 3. Most of these strains exhibited a pattern similar to that of *E. coli* B, except for *E. coli* CT447, which yielded fragment bands additional to those of leucine $tRNA_1$ but differing from the CTr5x-specific products. The identity of these bands has not been established yet.

The CTr5x-Specific tRNAs Are Cleaved in the Anticodon Loop. The CTr5x-specific cleavage products (bands II, III, V, and VI, Fig. 1) were further characterized by RNA sequence analyses (17–20). The sequences of fragments II and VI shown in Fig. 4 are compatible with their matching into a pair of tRNA halves. Thus, fragment VI corresponds to the 5′ segment, beginning with pG . . . and ending with the anticodon sequence . . . G-A-U-OH. Fragment II corresponds to the 3′ segment, beginning with the next-to-anticodon pA . . . and ending with . . . C-C-A-OH. From the anticodon sequence it may be inferred that fragments II and VI were derived from an AUU/C decoding isoleucine tRNA species (24) of *E. coli* CTr5x through cleavage of the anticodon loop. There exist further sequence homologies between II and VI and both "major" (AUU/C-decoding) (24) and "minor" (AUA-decoding) (25) isoleucine tRNAs of *E. coli* B, such as in the D stem [which is shared, however, with a number of *E. coli* tRNA species (26)] and a few other noncanonical residues. However, notable differences are found in the anticodon and aminoacyl stems and in the absence of the carbamoylthreonine (25) substitution in A-37, the first residue of fragment II. Preliminary sequence data indicated that fragments III and V were derived from another tRNA species that was also cleaved 3′ to the anticodon. The complete sequences of the CTr5x-specific cleavage products and their derivation will be published elsewhere.

A T4 Mutant Deficient in Leucine $tRNA_1$ Endonuclease Still Generates the CTr5x Typical tRNA Halves. The CTr5x typical tRNA halves differed from those of leucine $tRNA_1$ in their time of appearance as well as in their cleavage site, anticodon loop versus variable arm. These differences suggested the existence of a second T4-induced endonuclease, additional to that cleav-

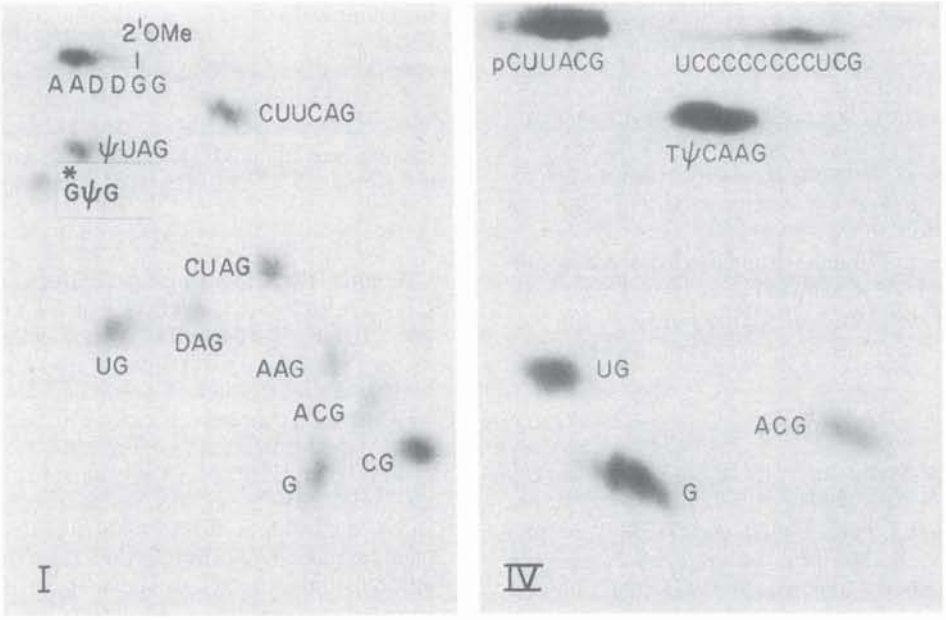

FIG. 2. RNase T1 fragment analysis. *E. coli* CTr5x leucine $tRNA_1$ fragments I and IV (Fig. 1) were extracted from the gel and analyzed by two-dimensional electrophoresis and chromatography. The oligonucleotides were identified by comparison with the RNase T1 pattern of *E. coli* B leucine $tRNA_1$ (9) and by determination of the composition of individual oligonucleotides. The 5′ pGp and 3′ UpC-OH termini were probably to the left and right of the range shown, respectively.

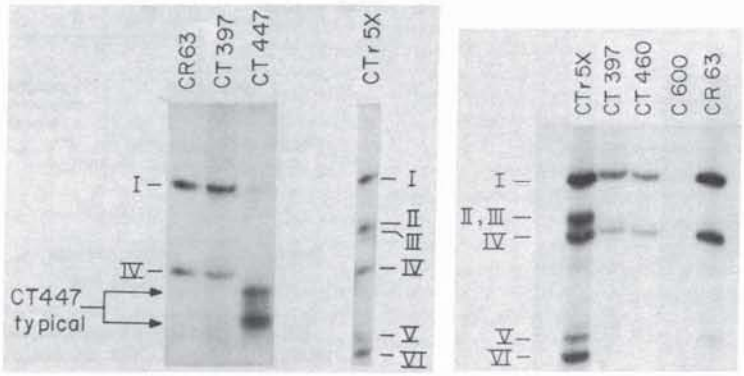

FIG. 3. Comparison of low molecular weight RNA patterns from various *E. coli* strains infected with T4 bacteriophage.

ing leucine $tRNA_1$ (9). To examine this possibility we employed a mutant of T4 phage, strain L8-7, which is deficient in the leucine $tRNA_1$ endonuclease (W. H. McClain, personal communication). As shown, this mutant still generated in *E. coli* CTr5x the later appearing bands, II, III, V, and VI in normal quantities, whereas the amounts of fragments I and IV of leucine $tRNA_1$ were much reduced (Fig. 5). Thus, the L8-7 mutation selectively impaired the leucine $tRNA_1$ endonuclease function, leaving the anticodon-directed activity unaffected.

Anticodon-Directed Endonuclease Is Translated Later Than That of Leucine $tRNA_1$. Further evidence for the existence of a separate T4-induced endonuclease, directed against the anticodon loop, was obtained through chloramphenicol inhibition studies. In this experiment, the drug was added at progressive infection times and its effect on the production of fragments was monitored at a fixed later time point. As shown in Fig. 6, addition of the drug before infection inhibited the cleavage of leucine $tRNA_1$, as reported by Yudelevich (9), and of the other two tRNA species. Addition after 1 or 2 min selectively inhibited the appearance of the CTr5x-specific bands II, III, V, and VI, whereas addition at later time points did not affect the appearance of any of the tRNA fragments. In conclusion, the anticodon-directed nuclease activity depends on a translation event that is induced after infection and later than that of leucine $tRNA_1$ endonuclease.

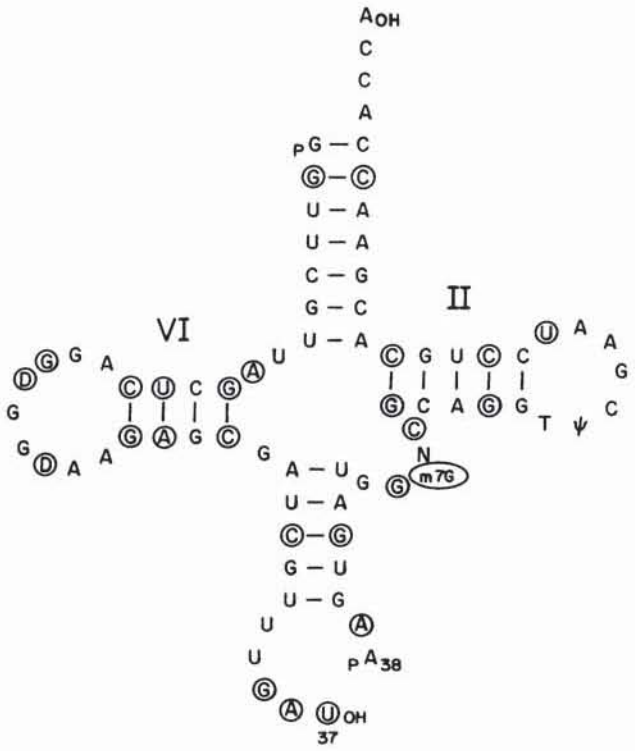

FIG. 4. Tentative nucleotide sequence of *E. coli* CTr5x tRNA fragments II and VI. Noncanonical residues homologous with *E. coli* B "major" isoleucine tRNA (24) are encircled.

DISCUSSION

The anticodon-directed endonuclease activity manifested in *E. coli* CTr5x after bacteriophage T4 infection is most likely coded for by the phage genome, because its induction depends upon a translational event that begins after 2 min of infection (Fig. 6). This activity seems to reside in a distinct enzyme other than leucine $tRNA_1$ endonuclease (9). Our conclusion rests on (*i*) the different time schedules in which these two activities are induced (Figs. 1 and 6), (*ii*) the selective impairment of one activity by mutation (Fig. 5), and (*iii*) the difference between the tRNA cleavage sites, extra arm (9) versus anticodon loop (Fig. 4). Nonetheless, the two activities may still be considered molecularly related. For example, the later-appearing enzyme could be derived from the former through a phage-induced protein modification. Eventual isolation and mapping of mutations in both functions as well as enzymological studies are needed to clarify this point. Further studies are also required

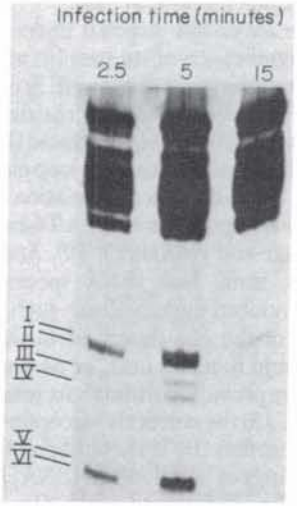

FIG. 5. Cleavage pattern of tRNA from *E. coli* CTr5x infected with a T4 mutant deficient in leucine $tRNA_1$ endonuclease. The cleavage of *E. coli* CTr5x tRNAs after infection with T4 L8-7 was monitored as described in the legend to Fig. 1.

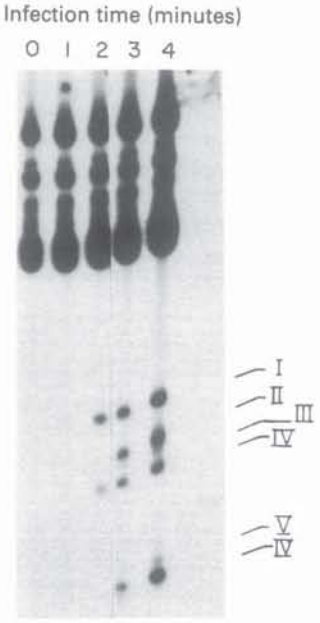

FIG. 6. Effect of chloramphenicol addition at various infection times on the induction of *E. coli* CTr5x tRNA cleavage. The cleavage of host tRNA in T4-infected *E. coli* CTr5x was monitored 4.5 min after infection following the addition of chloramphenicol (9) at the indicated infection times.

to reveal what aspect of the *E. coli* CTr5x environment allows the specific expression of the anticodon-directed nuclease activity that remained silent or less active in the other T4 host strains tested (Fig. 3). One possible explanation is that specific structural traits of the cleaved CTr5x tRNAs make them a substrate for the enzyme. The differences in the nucleotide sequences between the major isoleucine tRNA of *E. coli* B (25, 27) and the CTr5x fragments II + VI (Fig. 3), in particular the lack of modification at A-38, are consistent with the latter idea (if these fragments are legitimate cleavage products of an isoleucine tRNA species rather than derivatives of another species). Alternatively, the anticodon nuclease could originate from a CTr5x-specific protein through a T4-induced modification, analogous to the *mod* and *alt* functions of T4 phage, which modify the host's RNA polymerase (27, 28).

The role of the anticodon-directed endonuclease in T4 infection and the significance of its specific manifestation in *E. coli* CTr5x are not understood at present. A correlation may be drawn, however, between the unique restrictive phenotype of *E. coli* CTr5x towards polynucleotide kinase (11) and RNA ligase (6) mutants, and the specific anticodon loop cleavages that occur in this host (Figs. 1 and 3). Such a correlation lends further support to our previous hypothesis about a T4-induced pathway of host tRNA breakage and reunion (5, 10). According to this hypothesis (Fig. 7), some host tRNA species are specifically cleaved by a T4-induced endonuclease such as leucine $tRNA_1$ endonuclease (9) or the one described in this paper. Polynucleotide kinase could function next, or perhaps after additional unknown steps, by providing through its joint phosphatase–kinase activities (12, 13) the correct 3′-acceptor–5′-donor termini of a RNA ligase reaction (1). It should be noted, however, that the cleavage products of both leucine $tRNA_1$ (9) and the CTr5x-specific tRNAs (Fig. 3) featured a 3′-OH and 5′-PO_4 termini pair. It is not clear yet whether this outcome was directly due to a primary endonuclease cut or reflected also subsequent reactions catalyzed by polynucleotide kinase. Supporting these ideas are our recent findings that the CTr5x-specific tRNA fragments disappear during wild-type T4 infections, but not in *rli*$^-$ and *pnk*$^-$ T4 infections. Furthermore, among *pnk*$^-$ pseudorevertants able to form plaques on *E. coli* CTr5x, we found one strain that lacks the anticodon nuclease activity (unpublished results).

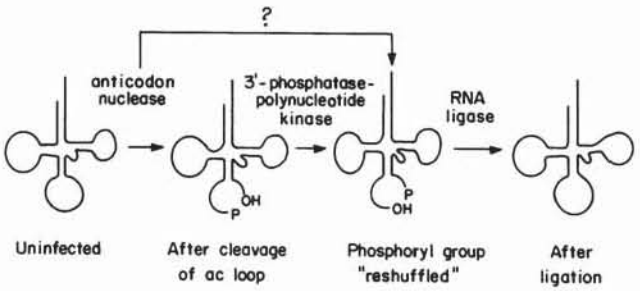

FIG. 7. Putative T4-induced pathway of host tRNA breakage and reunion in *E. coli* CTr5x. ac, Anticodon.

Other T4 genes that could be related to host strain-specific tRNA cleavages are those coding for tRNAs (29, 30). Thus, for example, the T4 tRNAs could replace the cleaved host species. These genes (psu_b^- locus, ref. 21) are dispensable in many T4 host strains but there exist several strains in which one or more of the T4-coded tRNAs are needed (22, 23). *E. coli* CTr5x and CT196 belong to this group (W. H. McClain, personal communication; and unpublished results), and their specific tRNA cleavages could also account for psu_b^- restriction. However, because the CTr5x-specific cleavages are related to *rli*$^-$ and *pnk*$^-$ restrictions, it would be easier to explore the possible relationship between psu_b^- restriction and the specific host tRNA cleavages with a simpler system, such as that of *E. coli* CT447. The additional fragments found in *E. coli* CT447 (Fig. 3) were not derived from leucine $tRNA_1$ or from the CTr5x-specific cleavage products, because they occurred after infection with T4 mutants lacking either the extra arm or anticodon nuclease (our unpublished results). It seems, thus, that the CT447-specific cleavages should be considered as a clue for the functions of T4-tRNA genes.

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