

Phage T4-coded Stp: Double-Edged Effector of Coupled DNA and tRNA-Restriction Systems

Michal Penner, Ilan Morad, Larry Snyder and Gabriel Kaufmann*

Department of Biochemistry
Tel Aviv University, Israel
69978

The optional *Escherichia coli* *pr*r locus encodes two physically associated restriction systems: the type IC DNA restriction-modification enzyme *Eco*pr*r*I and the tRNA^{Lys}-specific anticodon nuclease, specified by the PrrC polypeptide. Anticodon nuclease is kept latent as a result of this interaction. The activation of anticodon nuclease, upon infection by phage T4, may cause depletion of tRNA^{Lys} and, consequently, abolition of T4 protein synthesis. However, this effect is counteracted by the repair of tRNA^{Lys} in consecutive reactions catalysed by the phage enzymes polynucleotide kinase and RNA ligase. Stp, a short polypeptide encoded by phage T4, has been implicated with activation of the anticodon nuclease. Here we confirm this notion and also demonstrate a second function of Stp: inhibition of *Eco*pr*r*I restriction. Both effects depend, in general, on the same residues within the N-proximal 18 residue region of Stp. We propose that Stp alters the conformation of *Eco*pr*r*I and, consequently, of PrrC, allowing activation of the latent anticodon nuclease. Presumably, Stp evolved to offset a DNA restriction system of the host cell but was turned, eventually, against the phage as an activator of the appended tRNA restriction enzyme.

Keywords: anticodon nuclease; tRNA^{Lys}; RNA ligase; polynucleotide kinase; *hsd*

*Corresponding author

Introduction

Several phage T4-exclusion systems have been described that are encoded by DNA elements parasitising the *Escherichia coli* chromosome. In each of these systems a latent host function, encoded by the optional DNA element, is activated by phage infection to eliminate a vital cellular activity. Consequently, the viral infection is contained (Snyder & Kaufmann, 1994). The *pr*r exclusion system (Abdul-Jabbar & Snyder, 1984), which belongs to this category, was discovered in the search for physiological roles of two T4-encoded enzymes: 3'-phosphatase-polynucleotide kinase (encoded by the *pseT/pnk* gene: Depew & Cozzarelli, 1974; Cameron & Uhlenbeck, 1977; Sirotkin *et al.*, 1978) and RNA ligase (encoded by *g63/rli*: Snopek *et al.*, 1977; Runnels *et al.*, 1982). These enzymes are dispensable for T4 growth on common *E. coli* laboratory strains but indispensable on the clinical isolate of *E. coli*

strain CT196 and derivatives (Depew & Cozzarelli, 1974; Sirotkin *et al.*, 1978; Runnels *et al.*, 1982). The latter strains carry a cryptic DNA element integrated at 29 minutes on the *E. coli* map encoding a unique restriction system named *pr*r (*pnk*, *rli* restriction: Abdul-Jabbar & Snyder, 1984). When phage T4 infects *pr*r-containing (*pr*r⁺) strains it activates a pre-existing, latent ribonuclease directed against the anticodon loop of tRNA^{Lys}, hence the name anticodon nuclease (ACNase). The resultant 2',3'-cyclic P and 5'-OH cleavage termini are subsequently repaired in consecutive reactions catalysed by polynucleotide kinase and RNA ligase. Therefore, in the absence of either enzyme, tRNA^{Lys} is depleted (Amitsur *et al.*, 1987), late T4 protein synthesis is inhibited and the infection is aborted (Sirotkin *et al.*, 1978).

Manifestation of ACNase activity in T4-infected *E. coli* cells depends on the products of both the host *pr*r locus and the phage *stp* gene (Kaufmann *et al.*, 1986). The DNA region responsible for *pr*r restriction has been cloned and partially sequenced. It comprises four open reading frames (orfs) encoding positive (*pr*rC) and negative (*pr*rABD) ACNase functions (Levitz *et al.*, 1990). Transcriptional induction of *pr*rC from a multicopy plasmid in uninfected *E. coli* elicits translation of a 45 kDa PrrC polypeptide and expression of ACNase activity (Morad *et al.*, 1993). However, this effect is not lethal,

Permanent address: L. Snyder, Department of Microbiology, Michigan State University, East Lansing, MI 48824-1101, USA.

Abbreviations used: ACNase, anticodon nuclease; orf, open reading frame; R-M, restriction modification; PCR, polymerase chain reaction; IPTG, isopropyl- β -D-thiogalactoside; NPR, CPR, N and C-proximal.

probably due to continued synthesis of tRNA^{Lys} in the uninfected host cell. The nature of the *prxABD* genes which mask the activity of PrrC was suggested by a sequence homology with the *hsdMSR* genes encoding the plasmid-borne, type IC restriction-modification (R-M) enzyme *EcoR124I* (Linder *et al.*, 1990). Subsequent *in vivo* and *in vitro* studies have shown that the *prxABD* genes encode a type IC R-M enzyme of novel specificity, termed *EcoprrI* (Tyndall *et al.*, 1994). A physical interaction between *EcoprrI* and PrrC proteins has been demonstrated by co-immunoprecipitation (Amitsur *et al.*, 1992; Morad *et al.*, 1993). Earlier genetic experiments had indicated that an R-M system is linked to *prr* (Abdul-Jabbar & Snyder, 1984).

The phage determinant of ACNase, *stp*, was originally defined as the suppressor locus of *prr*-restriction (Depew & Cozzarelli, 1974; Sirotkin *et al.*, 1978; Runnels *et al.*, 1982). Mutations in *stp* abolish the activation of ACNase and thus render *prnK* and *rli* non-essential for T4 growth on *prr*⁺ hosts (Kaufmann *et al.*, 1986). Complementation experiments indicated that *stp* encodes a diffusible product. The gene has been mapped to the non-essential D-region, between *ndd* and *ac* (Depew *et al.*, 1975). Molecular cloning and DNA sequencing have revealed there a minuscule orf coincident with *stp* mutations (Chapman *et al.*, 1988; Bouet *et al.*, 1994; L. Ripley, personal communication). Synthetic polypeptides fashioned according to the *stp* orf activate latent ACNase in extracts of *E. coli prr*⁺ (Amitsur *et al.*, 1989, 1992) but have no effect on the

prrC-encoded core ACNase activity (Morad *et al.*, 1993). This suggested that *Stp* alters the conformation of *EcoprrI* and consequently of PrrC, allowing the activation of ACNase. However, because this reaction is potentially damaging to the phage, the primary role of *Stp* could be different. We show here that *Stp* also inhibits the restriction activity of *EcoprrI*. Moreover, the two functions of *Stp* depend in general on the same residues within the conserved N-proximal portion of the polypeptide, suggesting a common underlying mechanism.

Results

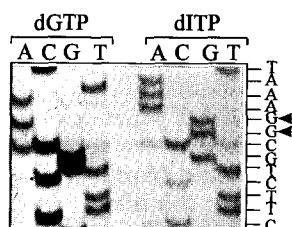
Corrected sequence of *stp*

Nucleotide sequencing of *stp* was performed on T4 DNA fragments cloned in a plasmid or amplified directly by the polymerase chain reaction (PCR). This revealed, following codon K20, two adjacent G bases (Figure 1A) that had escaped detection previously (Chapman *et al.*, 1988). These missing G bases were visualised in dideoxy-sequencing reactions containing dITP instead of dGTP (Figure 1B). Their concealment is attributed to a stable CUUCGG hairpin (Turek *et al.*, 1988) in which they reside (Figure 1C). However, this structure seems unrelated to *Stp*'s function because it is missing from an ACNase-activating variant of *stp* isolated from a T4-related phage (Figure 1C). Such variants will be discussed later.

A

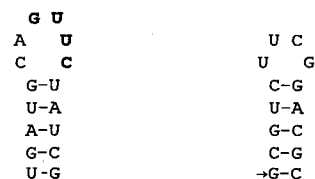
M1 S2 N3 F4 H5 N6 E7 H8 V9 M10 Q11 F12 Y13 R14 N15 N16 L17 K18 T19 K20 G21 V22 F23 G24 R25 Q26 Ter
 ATG AGT AAT TTC CAT AAC GAA CAC GTG ATG CAG TTC TAT CGT AAC AAT CTT AAA ACT AAA **GGC** GTC TTC GGA CGC CAG TGA
 ↑↑

B



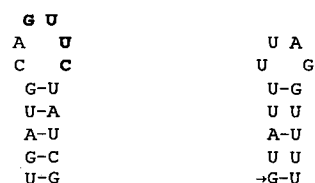
C

T4



AAUGAGUAAUUCCAUAAC**GAAC**ACG-UAACAAUCUAAAACUAAAG-CAGUGA
 ↑

Baker



AAUGAGUAAUUCCAUAAC**GAAC**AG-UAACAAUCUAAAACUUUG AAAAAUAGUGA
 ↑

Figure 1. The *Stp* orf and predicted secondary structure of *stp* mRNA. A, Corrected nucleotide and deduced amino acid sequence of the T4 *stp* orf. The concealed G bases are marked in all panels by arrows. B, A portion of *stp* DNA sequencing gel showing the "compressed" region. The correct sequence can be deciphered only from the sequencing reactions containing dITP. C, Predicted secondary mRNA structure of *stp* encoded by phage T4 and Baker. Bases participating in first stem of the pseudoknot are shown in bold type.

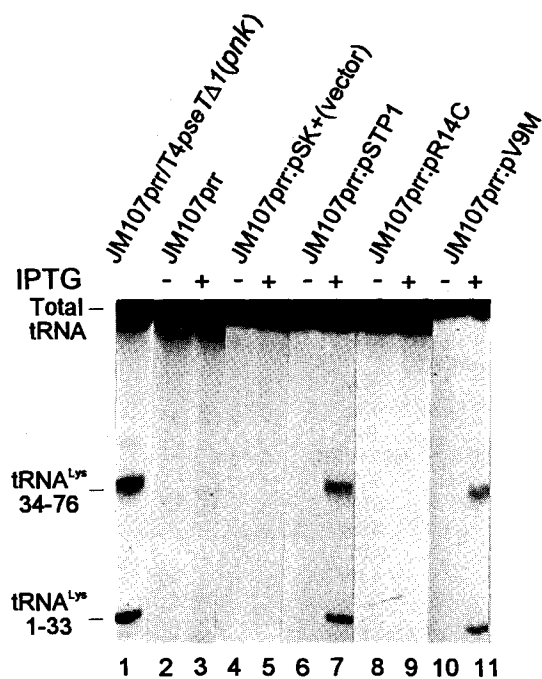


Figure 2. Activation of latent ACNase by overexpression of *stp* from a plasmid. Cleavage of tRNA^{Lys} was monitored in *E. coli* JM107pr^r infected for 20 minutes by the T4 *pnk* mutant (lane 1) or uninfected JM107pr^r (lanes 2 and 3) or JM107pr^r transformed by the indicated plasmids (lanes 4 to 11) in the absence (lanes 2, 4, 6, 8, 10) or presence of IPTG (lanes 3, 5, 7, 9, 11), as described in Materials and Methods. Plasmids R14C and V9M encode the corresponding *stp* mutants.

The sequence correction replaces an out-of-frame nonapeptide downstream of K20 with a hexapeptide. We have reported that latent ACNase can be activated by synthetic polypeptides fashioned according to the mistaken sequence (Amitsur *et al.*, 1989, 1992) but found later that a polypeptide containing the correct residues 2 to 26 activates ACNase more effectively (I. Morad, M. Penner & G. Kaufmann, unpublished results). Mutational and phylogenetic data described in subsequent sections reinforce the dispensable nature of the C-proximal portion of *Stp*, as regards its known activities.

Induction of *stp* from a plasmid elicits ACNase activity in uninfected *E. coli prr*⁺

E. coli JM107pr^r (*prr*⁺) and the isogenic strain JM107 (*prr*⁰) were transformed by the wild-type *stp*

plasmid pSTP1 (see Materials and Methods) or by the vector. ACNase activity was monitored in the transformants by following the appearance of the cleavage products, tRNA^{Lys} fragments 1 to 33 and 34 to 76. These products appear transiently during infection of *E. coli prr*⁺ by wild-type T4 phage but accumulate during infections with *pnk*⁻ or *rli*⁻ mutants (Amitsur *et al.*, 1987; Figure 2, lane 1). ACNase reaction products were detected neither in the parental *prr*⁺ cells (Figure 2, lanes 2 and 3) nor in transformants containing the vector plasmid (lanes 4 and 5). However, *prr*⁺ cells transformed by pSTP1 manifested ACNase activity when *stp* expression was induced with IPTG (compare Figure 2, lanes 6 and 7). The level of tRNA^{Lys} fragments 1 to 33 and 34 to 76 seen in the induced cells resembled that found late in T4 *pnk* infection (compare Figure 2, lanes 1 and 7). The cleavage of tRNA^{Lys} in the uninfected cells was not lethal, probably due to replenishment of tRNA^{Lys} by continued synthesis. As expected, pSTP1 elicited no ACNase activity in *prr*⁰ cells (data not shown).

stp alleviates *EcoprrI* DNA restriction

As mentioned, the primary target of *Stp* may be the *EcoprrI* restriction enzyme that masks *PrrC* activity. Therefore, we asked whether expression of *stp* from a plasmid would also prevent restriction by *EcoprrI* of phages not modified by the cognate methylase. To investigate this possibility we compared the plating efficiencies of the unmodified lambdoid phage HK022 on host strains carrying *EcoprrI* and transformed either by the wild-type *stp* plasmid pSTP1 or the cloning vector. Phage HK022 was preferred over lambda because it turned out to be restricted more stringently, probably due to the greater abundance of the restriction sites (CCAN₇RTGC and CCTN₇ATGC: Tyndall *et al.*, 1994) in HK022 DNA (R. A. Weisberg, personal communication). The results of the plating experiment are shown in Table 1. The plating efficiency of HK022 was lower by more than three orders of magnitude on *E. coli prr*⁺ with just the cloning vector. However, the presence of pSTP1 almost completely alleviated the restriction, restoring the efficiency of plating almost to that seen on strain JM107, devoid of *EcoprrI*. As expected, HK022 phage stocks passaged on a *prr*⁺ host were not restricted when plated on the same host. Interestingly, phage grown on *E. coli prr*⁺:*stp* are also not restricted on subsequent infection of *prr*⁺ cells. Taken together, these results

Table 1. Alleviation of *EcoprrI* restriction of phage HK022 by *Stp*

Host	Efficiency of plating		
	HK022	HK022. <i>EcoprrI</i>	HK022 <i>EcoprrI</i> : <i>stp</i>
JM107	1.0	1.0	1.0
JM107pr ^r :pSK ⁺	5 × 10 ⁻⁴	0.7	1.0
JM107pr ^r :pSTP1	0.4	0.3	—
JM107pr ^r ΔC11	1 × 10 ⁻³	1.0	—
JM107pr ^r ΔC11:pSTP1	0.7	0.4	—

indicate that the Stp polypeptide inhibits the restriction but not the modification activity of *EcoprrI*. The expression of *stp* also alleviated *EcoprrI* restriction in strain *E. coli* JM107*prc* Δ C11 in which *prc* had been inactivated by a large deletion. Therefore, interaction of Stp with PrrC is not required to inhibit *EcoprrI* restriction. A lower level of *stp* expression was needed to effect the anti-DNA restriction activity, compared to ACNase activation. Removal of IPTG reduced the plating efficiency of HK022 on *EcoprrI:stp* only twofold. Addition of glucose further inhibited it by a similar degree. In contrast, activation of *stp* from the same plasmid was detected only with IPTG.

Mis-sense mutations selected by loss of *prc*-restriction cluster in the N-terminal half of Stp

T4 *stp* point mutants that prevent activation of ACNase can be selected as apparent revertants of *pnk* or *rli* mutants of T4 on *E. coli prc*⁺. An unusually high frequency of *stp* mutations is observed when the deletion mutant *pseT Δ 1* is plated due to the *cd*⁻ mutator activity of some deletions in this region. The frequency of *stp* mutations was further augmented by hydroxylamine mutagenesis tenfold above background (1×10^{-3} as against 1×10^{-4}). DNA fragments containing different *stp* alleles were amplified from parental and revertant plaques by PCR (see Materials and Methods). Out of 24 revertants tested all but one yielded the expected PCR fragment. The exceptional clone probably suffered a deletion encompassing *stp*. An *stp*-containing subfragment from each PCR product was cloned in a plasmid as described for pSTP1 and sequenced. Of these clones, 20 (listed in Table 2A) had a single base change. The majority were mis-sense mutations that clustered between codons 4 and 14 and several of them reiterated. Due to a change in the initiation codon or an amber mutation, three mutants were impaired in *stp* translation.

Selected *stp* mutations inhibit ACNase activation

The *stp* mutant alleles described above were expressed under control of *Plac* in uninfected *E. coli prc*⁺ and ACNase assays were performed as before. The results are shown for representative clones: a

Table 2. Effect of *stp* mutations on ACNase activation

Mutation	Replacement	Incidence	ACNase activity
A. <i>Point, selected</i>			
G3A	M1I	1	-
T11C	F4S	1	-
G19A	E7K	1	-
C22T	H8Y	1	-
G25A	V9M	3	+
A28G	M10V	6	+
A28T	M10L	2	+
C31T	Q11am ^a	2	-
C40T	R14C	2	-
G41A	R14H	1	-
B. <i>Point, synthetic</i>			
G5T	S2I		+
T10C	F4L		+
T10G	F4V		±
C13T, G64C	H5Y, V22L		+
A20T	E7V		-
T50A	L17H		+
A53C	K18T		+
A54T	K18N		+
C56T	T19I		+
G61A	G2IS		+
T65G	V22G		+
C73A	R25S		+
C. <i>C-prox. deletions</i>			
16mer			-
18mer			±
22mer			+

The indicated *stp* mutants were assayed for ACNase activation by expression from a plasmid (see Materials and Methods). ± indicates less than 0.5 extent of tRNA^{Lys} fragments compared with wild-type *stp* but above detection level (~0.05). The amber mutant was assayed in the context of T4 infection.

^a Suppressed by *su*⁺.

null mutant (R14C, Figure 2, lanes 8 and 9) and a seemingly harmless one (V9M, lanes 10 and 11). Table 2A summarises the data for all tested clones. The null mutations included replacements of charged residues by uncharged (H8Y, R14C), lesser charged (R14H) or oppositely charged (E7K), as well as the hydrophobic-hydrophilic replacement F4S. In contrast, the hydrophobic exchanges V9M, M10V and M10L which likewise suppressed *prc* restriction did not inhibit ACNase activation in the plasmid assay. Conceivably, over-expression of these mutant Stp peptides compensated for their reduced activity or stability. To test this assumption we assayed the ACNase activation potential of some of the selected *stp* mutants during phage T4 infection. To this end the *stp* mutations investigated were crossed back into T4 *pseT Δ 1* by marker rescue. R14C was rescued more

Table 3. Marker rescue of *stp* mutations

Rescued mutation	T4 insert size (bp)	Phage yield on strains		Frequency of wild-type recombinant
		K10	CTr5X	%CTr5X/K10
R14C	301	2×10^8	2×10^6	1.0
V9M	301	2.8×10^9	6.5×10^6	0.23
E7V	81	3×10^8	6×10^5	0.20
K18T	81	1.1×10^9	5.5×10^5	0.05
None	—	1.1×10^{10}	4×10^6	0.04

Phage T4 *pseT Δ 1* was grown on *E. coli* XL1-blue (*prc*⁰) containing plasmids encoding the indicated *stp* mutation. Progeny phage were plated on strains K10 (*prc*⁰) and CTr5X (*prc*⁺).

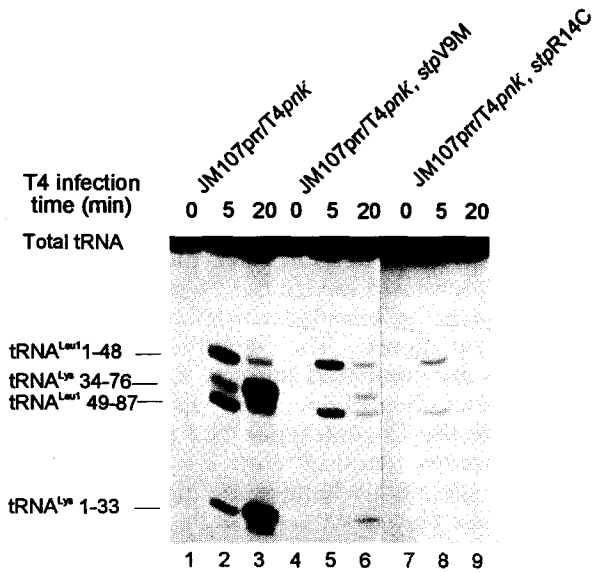


Figure 3. Effect of *stp* mutations on ACNase activation during phage T4-infection. Activation of ACNase in *E. coli* JM107^{prr} infected by T4 *pseTΔ1* (*pnk*, lanes 1 to 3) or T4 *pseTΔ1*, *stp* double mutants carrying the V9M (lanes 4 to 6) or R14C (lanes 7 to 9) mutations were monitored as described in Materials and Methods.

efficiently than V9M (Table 3), in keeping with the stronger inhibition of ACNase activation and, hence, *prr* restriction. Indeed, the V9M mutation permitted partial activation of ACNase during infection (Figure 3, compare lanes 1 to 3 with 4 to 6) whereas R14C abolished the activity (Figure 3, lanes 7 to 9). The efficiency of infection with T4 *stp*R14C was confirmed by the phage-induced cleavage of *E. coli* tRNA^{Leu1} (Kano-Sueoka & Sueoka, 1968) which does not depend on *prr* (David *et al.*, 1982).

C-Proximal *stp* mutations do not prevent ACNase activation

Clustering of selected *stp* mutations in the N-proximal region (NPR) could reflect the importance of the NPR for ACNase activation or its different propensity to DNA damage and/or repair. To distinguish between these possibilities we investigated the ACNase phenotype of nonselected *stp* mutants. They were obtained either by degenerate gene synthesis or by C-terminal deletions (see Materials and Methods). Clones of the first kind chosen for further characterization had in general a single mis-sense mutation or an additional silent mutation (Table 2B). Seven clones carried C-proximal mis-sense mutations between residues 17 and 25 and two of these entailed radical changes. Nonetheless, all seven yielded wild-type levels of ACNase activity in the plasmid assay. In contrast, two out of five non-selected N-proximal mutations, F4V and E7V, markedly reduced or abolished the activity, respectively. Surprisingly, the non-selected F4L mutation elicited weak ACNase activation even without IPTG

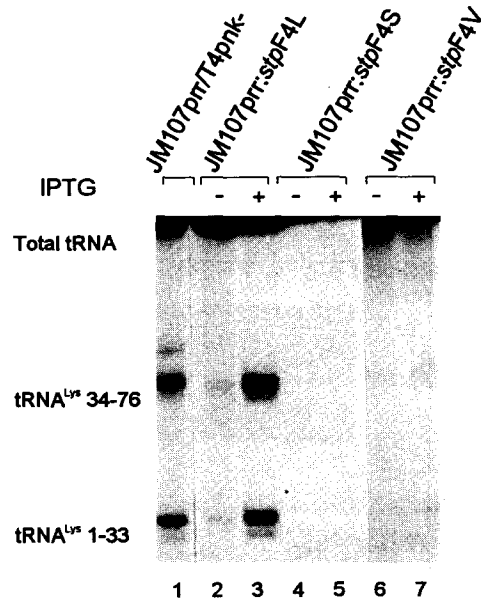


Figure 4. Effect of different substitutions of *Stp* Phe4 on ACNase activation. ACNase activation was assayed in *E. coli* JM107^{prr} cells transformed by plasmids encoding the indicated replacement of *stp* F4, as described in Materials and Methods.

(Figure 4, lanes 2 and 3). F4L differed in this regard not only from the null F4S (lanes 4 and 5) and leaky F4V mutants (lanes 6 and 7), but also from wild-type *stp* (Figure 2). Another two mutants, S2I and the double mutant H5Y, V22L, seemed as active as wild-type *stp*. The H5Y mutation is likely to be harmless itself, rather than compensating a V22L lesion, because the entire C-proximal region of *Stp* seems non-essential for ACNase activation (see below).

Attempts to rescue C-proximal mis-sense mutations by recombining them into the T4 *pnk* strand *pseTΔ1* (shown in Table 3 for K18T) yielded a low level of *prr*-resistant recombinants, barely above the spontaneous background. In contrast, the E7V null mutation residing in a T4 sequence of identical size yielded five times more recombinants (Table 3) despite its less central position compared to K18T (20 versus 29 bases from the end of the T4 fragment). Presumably, the poor rescue of the C-proximal mutation reflects weak or no inhibition of ACNase activation and, hence, weaker suppression of *prr* restriction.

A clear-cut demonstration that the C-proximal region of *Stp* is not essential for ACNase activation was provided by progressive deletions. Removal of four, eight or ten C-proximal codons reduced ACNase activation twofold (Figure 5, compare lanes 3 and 5), fourfold (lanes 3 and 7) or below detection (lanes 3 and 9), respectively.

ACNase activation depends on *stp* translation

A revertant of T4 *pseTΔ1* carrying the *stp* Q11am mutation did not activate ACNase during infection of

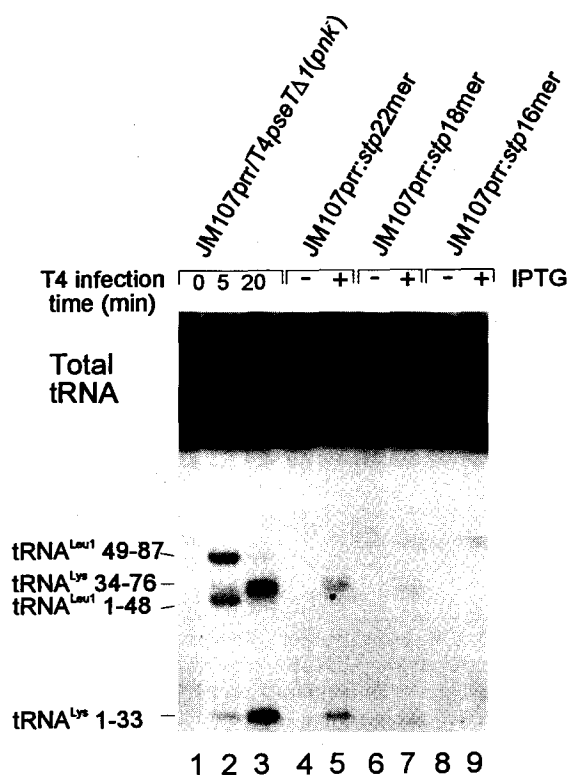


Figure 5. Effect of C-terminal truncations of *Stp* on ACNase activation. ACNase activation was followed in *E. coli* JM107*prf* cells transformed by the plasmids encoding the indicated *stp* deletion mutants as described in Materials and Methods. The additional fragments in lanes 1 and 9 (marked by arrows) are non-specific products arising occasionally during isolation of the RNA. The level of the ACNase cleavage relative to total tRNA was determined by counting corresponding gel slices in scintillation fluid.

the *sup⁻* strain B834*prf* (Figure 6, lanes 2 to 4). However, weak activation was noticed during infection of the isogenic *sup⁺* host (compare lanes 4 and 7). A higher level of ACNase activation, albeit less than wild-type, was observed with *E. coli* JM107*prf* (*sup^E*) encoding the tRNA^{Cln} suppressor (compare Figure 6, lanes 1, 7 and 10). The improved suppression by JM107*prf* is attributed to restoration of the correct amino acid by *sup^E* (the

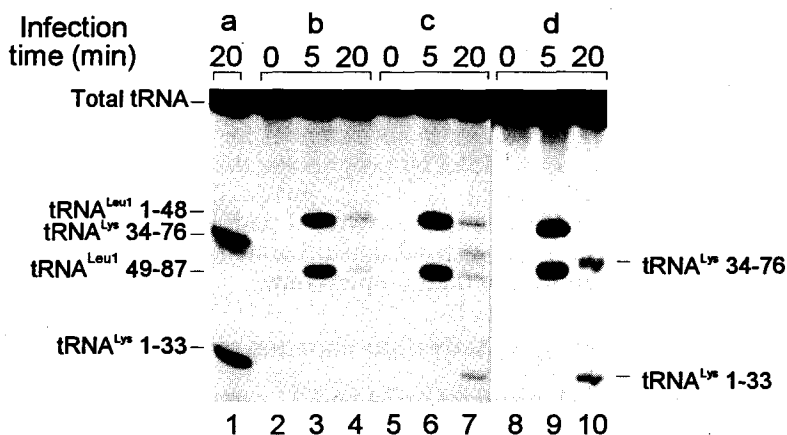


Figure 6. Suppression of an *stp* nonsense mutation by *sup^E*. ACNase activation during phage infection was followed as described in Materials and Methods. a, *E. coli* B834*prf*/T4 *pseTΔ1*. b, *E. coli* B834*prf*/T4 *pseTΔ1*, *stpQ11am*. c, *E. coli* B834*prf,sup⁺*/T4 *pseTΔ1*, *stpQ11am*. d, *E. coli* JM107*prf*/T4 *pseTΔ1*, *stpQ11am*.

suppressor of the B834*prf* derivative has not been characterized).

The N-proximal region is conserved in *stp* variants encoded by T-even related phages

Naturally occurring *stp* variants were isolated by PCR from various hydroxymethyl cytosine-containing phages related to the T-even phages. The PCR products resembled in size the T4 counterpart, but DNA sequencing revealed variations between the resident *stp* genes. The deduced amino acid sequences are compared in Table 4. One group of phages, including LZ3, LZ5, LZ8, and Tu1B (henceforth the T4 prototype), had deduced amino acid sequences identical with T4 but varied by two-three silent base changes, in different positions. Each of these phages activated ACNase in *E. coli prf⁺* (data not shown). A closely related *stp* variant encoded by phage Tu1A featured a H8Y change, identical with a selected T4 null mutant (Table 3A). In accordance, Tu1A did not activate ACNase, i.e. the fragments of tRNA^{Lys} that appear transiently during wild-type T4 infection (David *et al.*, 1982) could not be detected with Tu1A (Figure 7). A second class of phages, termed the Baker prototype, encoded *stp* variants containing at least 17/18 of the N-proximal codons of T4 *stp* (Ox2, Baker, SCI). In contrast, the sequences and lengths of the downstream portions of these *Stp* variants differed from that of the T4 prototype. However, residues G21 and G24 persisted and several other C-proximal positions featured only conservative changes. The difference between the two prototypes extends to the RNA level: the Baker prototype lacks the CUUCGG hairpin found in the T4 transcript (Figure 1C). Despite the different C-proximal regions (CPRs), the phages belonging to the Baker prototype activated ACNase efficiently, as did the cloned Baker *stp* gene. Phage Ox2 was unique in that it failed to induce the cleavage of tRNA^{Leu1} (data not shown).

Mutations of *stp* affect ACNase activation and anti-*Ecopr1* restriction similarly

Since the *Stp* peptide can manifest both ACNase

Table 4. Deduced amino acid sequences of Stp variants

Phage	Deduced Stp amino acid sequence	ACNase
T4, LZ8, LZ3, Tu1B, LZ5	<u>MSNFHNEHV</u> <u>MQFYRN</u> <u>NLKT</u> KGVFGRQ*	+
Tu1A	Y	-
Baker, SCI	NFGILGFKK*	+
Ox2	D NS*	+

The conserved NPR is underlined, variations in it are in bold type.

activation and alleviation of *EcoprrI* restriction, it was important to determine if the same amino acid residues are needed for both functions. Accordingly, we expressed mutant forms of Stp from plasmids and determined their ability to alleviate the DNA restriction. It is apparent from the results that in most cases the same replacements had similar effects on the two functions of Stp (Table 5). For example, even though the CPR of Baker *stp* differs substantially from that of the T4 counterpart (Table 4), both variants activated ACNase and alleviated *EcoprrI* restriction similarly. Moreover, mutations that abolished or weakened the ACNase activation potential of T4 Stp had in general a similar effect on the anti-DNA restriction activity (Table 5). Thus, most null ACNase mutations did not inhibit *EcoprrI* restriction while mutations that inhibited ACNase activation in part or not at all had little effect on the anti-*EcoprrI* restriction activity. F4S was exceptional, in that it abolished ACNase activation but hardly affected the anti-DNA restriction activity. Perhaps this difference is related to the observation that more Stp is required for ACNase activation.

Discussion

The Stp polypeptide is necessary and sufficient for ACNase activation

ACNase activation is abolished by *stp* mutations (Kaufmann *et al.*, 1986) and synthetic, Stp-like polypeptides activate ACNase *in vitro* (Amitsur *et al.*, 1989, 1992). These data have implicated the Stp polypeptide, despite its small size, as the only T4-encoded function necessary for activation of ACNase during T4 infection of *E. coli prr⁺*. The results reported here support this notion and provide new information about *stp* functions and mechanism of action.

Transcriptional induction of cloned *stp* elicited ACNase activity in uninfected *E. coli prr⁺*, confirming that no other T4 gene product is required for this function. The *stp* Q11am mutant failed to activate ACNase but activation was effectively restored by a suitable suppressor tRNA (Figure 6). ACNase activation was also abolished by a mutation in the *stp* initiation codon (Table 2A). Other selected *stp* point mutations that prevented ACNase activation were all mis-sense mutations. Silent changes in third codon positions were not scored among the selected *stp* mutants but such changes abounded in naturally occurring variants of *stp* that do activate ACNase. Taken together with the previous *in vitro* data these facts suggest that the Stp polypeptide is the only T4 product needed for ACNase activation. However,

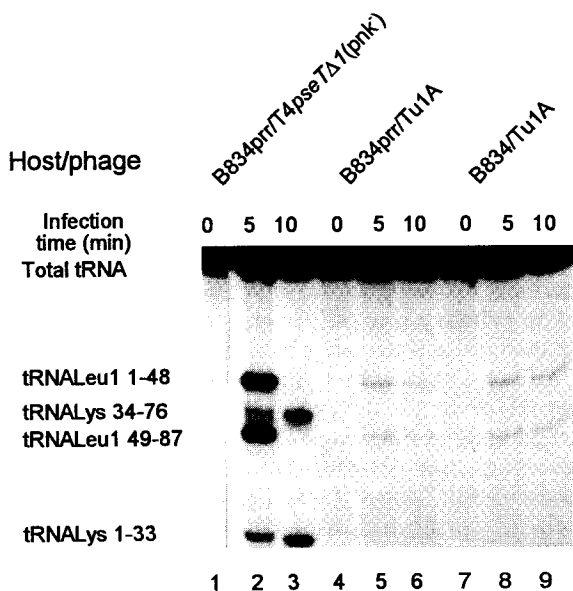


Figure 7. Phage Tu1A does not activate ACNase. The fate of host tRNAs during infection of the indicated *E. coli* strains by phages T4 *pseTΔ1* and Tu1A was followed essentially as described for Figure 3.

Table 5. Effect of *stp* mutations on the anti-*EcoprrI* DNA restriction and ACNase activation functions

Host	HK022 e.o.p. ^a	Active ACNase
<i>prr</i>	5×10^{-4}	-
<i>prr:stp</i>	0.4	+
<i>prr:stpF4L</i>	0.7	+
<i>prr:stpF4V</i>	0.5	±
<i>prr:stpF4S</i>	0.2	-
<i>prr:stpE7V</i>	10^{-3}	-
<i>prr:stpE7K</i>	2×10^{-4}	-
<i>prr:stpH8Y</i>	1.5×10^{-4}	-
<i>prr:stpV9M</i>	0.7	±
<i>prr:stpM10L</i>	0.6	±
<i>prr:stpM10V</i>	0.8	±
<i>prr:stpR14H</i>	8×10^{-4}	-
<i>prr:stpR14C</i>	5×10^{-4}	-
<i>prr:stpG21V</i>	1.0	+
<i>prr:stpR25S</i>	1.0	+
<i>prr:stp16mer</i>	5×10^{-4}	-
<i>prr:stp18mer</i>	2.5×10^{-2}	±
<i>prr:stp22mer</i>	0.1	+
<i>prr:stp(Baker)</i>	0.8	+

^a Efficiency of plating.

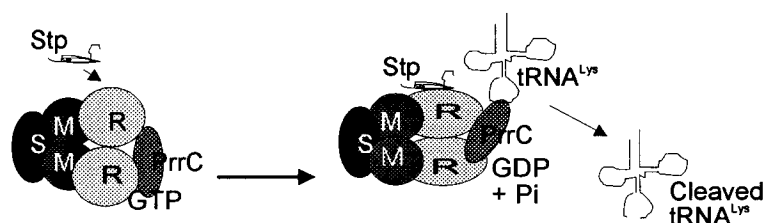


Figure 8. Activation of latent ACNase by *Stp*. In this model *Stp* binding to an Hsd subunit causes a conformational change that is propagated to the Hsd-PrrC interface, allowing cleavage of tRNA^{Lys}. GTP may be utilized in the process. S, M and R denote the corresponding Hsd subunits.

further mutational analysis is required to rule out involvement of *stp* mRNA in ACNase activation, above its function as a template. This possibility is raised by the extensive secondary structure predicted for *stp*-mRNA, including a central pseudoknot and a C-proximal CUUCGG hairpin (Figure 1C). The predicted pseudoknot could be destabilized by the inactivating mis-sense mutations E7K and E7V. Therefore, it remains to be determined whether mutations that change this structure but not the protein sequence affect *Stp*'s functions. CUUCGG hairpins are found in intergenic regions of T4 and *E. coli* and have been implicated with regulatory functions such as transcriptional termination (Turek *et al.*, 1988). The CUUCGG hairpin of T4 *stp* is exceptional in that it occurs within the gene, albeit close to the C terminus (Figure 1C). The only other CUUCGG sequence known to occupy such a position exists in T4 *pseT* but is not flanked by stem-forming bases (Turek *et al.*, 1988). This coincident occurrence may portend a common function, although unrelated to ACNase activation, anti-Hsd restriction or even *Stp* expression, because fully active *stp* variants of the Baker prototype lack a CUUCGG hairpin (Figure 1C). Moreover, C-proximal mutations in T4 *stp* (Table 2B), expected to destabilize this structure, had no effect on *Stp* functions. Nor does the CUUCGG hairpin prevent translation of T4 *Stp*'s C terminus, as judged by comparing the *in vivo* translation products of wild-type *stp* and the truncated 18mer (M. Penner, unpublished results).

***Stp* as an alleviator of DNA restriction**

Triggering tRNA^{Lys} cleavage by *Stp* is potentially harmful to T4 and must be countered by an elaborate RNA repair mechanism. Conceivably, if *stp* had no useful function for the phage it would have succumbed to selection pressure. Yet *stp* variants occur in different hydroxymethyl cytosine-containing phages, including phage Tu1A which fails to activate ACNase (Table 4 and Figure 7), reinforcing the notion that *Stp* and its variants benefit their phages in some other fashion. Our finding that *Stp* inactivates restriction nucleases of type IC including *EcoprrI* (Table 1) and *EcoR124I* (our unpublished results) confirms this expectation and suggests how *Stp* could benefit the phage. Moreover, the fact that in general the same amino acid residues seem to be needed for both functions (Table 5) argues for a common underlying mechanism. The failure of Tu1A

to activate ACNase also hints that the polynucleotide kinase and RNA ligase likely to be encoded by Tu1A (L. Snyder, unpublished results) play roles additional to restoring tRNA^{Lys}. Alternatively, Tu1A lost its ACNase activation potential and anti-DNA restriction activity fairly recently.

Phage-encoded alleviators of Type I DNA restriction have been described (Bickle & Krüger, 1993). Well-documented cases include the polypeptide product of phage T7 gene 0.3 (*Ocr*) and the *Ral* protein of phage λ . *Ocr* counteracts *EcoKI* and *EcoBI* by preventing the binding of the enzyme to target DNA or inhibiting the modification and restriction activities of the enzyme once bound to DNA (Mark & Studier, 1981; Bandyopadhyay *et al.*, 1985). *Ral* offsets *EcoKI* and *EcoBI* restriction by enhancing the modification of target DNA (Zabeau *et al.*, 1980; Loenen & Murray, 1986). That *Stp* is a novel alleviator of DNA restriction is indicated by its ability to abolish the restriction activity of *EcoprrI* but not the cognate modification activity (Table 1). *Stp* differs from *Ocr* and *Ral* in its specificity: it inhibits *EcoR124I*, another type IC enzyme, but not the type Ia enzymes *EcoKI* and *EcoBI* (our unpublished results). The anti-*EcoprrI* restriction activity of *Stp* did not depend on the presence of PrrC. This fact and the inhibition of *EcoR124I* which has no appended ACNase activity support the conclusion that the R-M enzyme rather than PrrC is the immediate target of *Stp*. Because *Stp* inhibits the restriction but not modification activity of the R-M enzyme, the HsdR subunit, which specifies restriction, seems the most likely partner for the interaction. One possibility is that *Stp* distorts the interaction of HsdR with the other Hsd subunits, inhibiting the restriction without affecting the modification activity. The conformational change may be relayed to the *EcoprrI*-PrrC interface, allowing ACNase activation (Figure 8).

The primary role of *Stp* was most likely to offset the antiviral activity of its Hsd target. At a later stage, in a host cell in which PrrC became appended to *EcoprrI* or to an ancestral R-M enzyme, *Stp* was turned against the phage as an activator of ACNase. However, this model does not explain why "present-day" phage T4 needs *Stp*. After all, T4 DNA is already protected from *EcoprrI*-restriction by the modifications of its cytosine bases and *stp* can be deleted without apparent adverse effect on T4 growth in the presence of *EcoprrI* (Abdul-Jabbar & Snyder, 1984). Moreover, *stp* is expressed at a delayed-early schedule and, therefore, is unlikely to be included in the phage particle and able to protect

the incoming phage DNA. In fact, *stp*⁻, cytosine-containing T4 phage is equally restricted by *EcoprrI*, whether or not propagated on *E. coli* expressing the *Stp* polypeptide (L. Snyder, unpublished observation). These facts portray *Stp* as a back-up measure for protecting the nascent DNA of T4 (or an ancestral phage) from the restriction activity of R-M enzymes related to *EcoprrI*. However, *Stp* may benefit the phage in additional, unexpected ways. Clearly, more has to be learned about the molecular basis of the known functions of *Stp* to understand its origin and biological significance.

Stp structure-function relationships

The phylogenetic survey (Table 4) defined in *Stp* a conserved NPR of 18 codons and a CPR of variable sequence and length. The significance of this division is underscored by the retention of ACNase activation and alleviation of *EcoprrI* restriction by a deletion mutant encoding only the NPR. Further deletion and certain NPR point mutations abolished both activities (Table 5). A subregion of NPR ranging between residues 4 and 14 may be of particular importance for these *Stp* functions. Mis-sense mutations selected by loss of *prrr*-restriction and, hence, impairment of ACNase activation, clustered in this subregion (Table 2A). In contrast, changes in flanking positions appeared to be tolerated. The ACNase activating phage Ox2 encodes D instead of N in position 15 (Table 4) and T4 *Stp* residues S2, L17 and K18 could be mutated without apparent effect on ACNase activation (Tables 2B and 3). Within the active subregion, the charged residues E7, H8 and R14 and the hydrophobic residue F4 seem critical for ACNase activation and, with the exception of F4, also for anti-*EcoprrI* restriction. Most of the investigated mutations in these positions elicited a null phenotype even when the mutants were over-expressed (Figure 2, Table 2A and Table 5). However, in the case of F4, different replacements elicited a wide spectrum of phenotypes, complete (F4S), partial (F4V) and no loss (F4L) of ACNase activation (Figure 4). The latter replacement even augmented the ACNase activation potential compared with wild-type *stp*. These facts suggest that the hydrophobicity and bulk of the aromatic ring of F4 are more important for ACNase activation than its specific shape or electronic properties. However, phenylalanine may be preferred in this position because it confers optimal interaction with the Hsd target. The ability to modulate the ACNase activation potential by different F4 mutations and the differential effect of the F4S mutations on the two activities of *Stp* suggest that the interactions of this residue with its target site in Hsd may provide important mechanistic clues. Other residues within the active subregion, H5, V9 and M10, could be replaced with no loss of ACNase activation (Table 2 and Figure 2) or anti-*EcoprrI* restriction activities, at least when overexpressed (Table 5). If the active subregion of *Stp* is folded into an α -helix, as predicted by the GOR

algorithm (Garnier *et al.*, 1978), the critical residues F4, E7, H8 and R14 would occupy one face of the polypeptide and the less critical residues the opposite. As regards *Stp*'s CPR, conservation of G21 and G24 and the conservative changes seen in neighbouring positions (Table 4) suggest that this region may also play an important role, although one which cannot be defined at present. One possibility is that the variable residues of the CPR impart specificity for interactions with individual members of a generic group of proteins whereas the constant glycine residues define a common structural frame for these interactions. Potential partners for such interactions are different type IC R-M enzymes that may correspond to different members of the family of *Stp* polypeptides. The N-constant/C-variable pattern of the latter mirrors, perhaps, a matching organisation within the different Hsd enzymes that likewise comprise highly conserved and hypervariable sequences (Wilson & Murray, 1991; Bickle & Krüger, 1993). Detailed mutational and structural analyses of *Stp* and identification of its target site in the R-M enzyme are needed to examine these notions.

The overall similarity of the mutational patterns of *Stp*'s two known activities implies a common underlying mechanism. Yet there may be subtle differences in the mode of *Stp* utilization in each reaction. This is hinted at, firstly, by the differential effect of the F4S mutation, which abolished ACNase activation but did not inhibit the anti-*EcoprrI* restriction activity, and secondly, by the fact that a lower level of *stp* expression was needed to elicit the anti-*EcoprrI* restriction activity, compared to ACNase activation. A lower requirement of *Stp* for inactivating *EcoprrI* may portend a single-hit mechanism, as opposed to continued utilization of *Stp* during ACNase activation. Noteworthy in this regard is that ACNase activation may depend on the utilization of GTP, mediated by a cognate motif in *PrrC* (Morad *et al.*, 1993). These facts support a model in which ACNase catalyses cycles of tRNA^{Lys} cleavage, alternating between latent and active forms at the expense of continued utilization of GTP (Figure 8). *Stp* may also be consumed in the process.

Materials and Methods

Materials

Synthetic oligonucleotides were custom-ordered from B.T.G. Inc. Israel. Radioactive materials were purchased from Amersham Ltd, UK and restriction endonucleases and DNA modifying enzymes from New England Biolabs Inc., USA.

Bacteria, phage and plasmids

The bacterial strains, phages and plasmids used in this study are listed in Table 6. The strain JM107*prrr* was derived from *E. coli* JM107 by transducing in the *prrr* element, selecting a nearby Tn10 transposon (Abdul Jabbar & Snyder, 1984). The strain *E. coli* JM107*prrr* Δ C11 (*EcoprrI*⁺, ACNase⁻) was derived from JM107*prrr* by a deletion in *prrrC*

Table 6. Bacteria, phase and plasmids

		Relevant genotype and source
A. <i>E. coli</i>		
B834	<i>met</i> , <i>hsdR_B</i> , <i>hsdM_B</i>	Wood (1966)
B834 <i>prrr</i> (BJMn10)	<i>tet^r</i> <i>prrr</i> ⁻ -transductant of B834	Abdul-Jabbar & Snyder (1984)
B834 <i>prrr</i> , <i>su</i> ⁺	<i>sup</i> ⁺ derivative of B834 <i>prrr</i>	This work
CTr5X	<i>prrr</i> ⁺ Hfr cross of K12 with a CT196 recipient	Depew & Cozzarelli (1974)
DH10B	Δ <i>lac-pro, endA1, gyrA, thi-1, hsdR17, supE44</i> , <i>lac, F'</i> [<i>proAB, lacI^q, lacZ</i> ΔM15, Tn10]	Grant <i>et al.</i> (1990)
JM107	<i>F</i> ⁻ , [<i>supE44, thi1, leuB6, lacY1, tonA2</i>] <i>relA1, F'</i> [<i>traD36, proAB, lacI^q, lacZ</i> ΔM15]	Yanisch-Perron <i>et al.</i> (1985)
JM107 <i>prrr</i>	<i>tet^r</i> <i>prrr</i> ⁻ -transductant of JM107	This work
JM107 <i>prrr</i> ΔC11	Derived from JM107 <i>prrr</i> by deletion of <i>prrrC</i>	S. Benjamin (unpublished work)
K10	HfrC, <i>pit</i>	Skaar & Garen (1956)
XL1-Blue	<i>supE44, hsdR17, recA1, endA1, gyrA, thi-1, relA1</i> , <i>lac</i> ⁻ , <i>F'</i> [<i>proAB, lacI^q, lacZ</i> ΔM15, Tn10]	Bullock <i>et al.</i> (1987)
B. T4 phage		
T4 <i>pseT</i> Δ1	Δ(<i>cd, pseT, nlc</i>) polynucleotide kinase deficient	Snyder <i>et al.</i> (1976)
T4 <i>pseT</i> Δ1, <i>stpR14C</i>	<i>stp</i> mutant of T4 <i>pseT</i> Δ1	This work
T4 <i>pseT</i> Δ1, <i>stpV9M</i>	<i>stp</i> mutant of T4 <i>pseT</i> Δ1	This work
C. Phages related to T4		
Baker	Cold Spring Harbor	S. Eddy
LZ3	Hyena, Denver Zoo (1989)	S. Eddy
LZ5	Snow leopard, Denver Zoo (1989)	S. Eddy
LZ8	Clouded leopard, Denver Zoo (1989)	S. Eddy
Tu1A	Tübingen sewage	Datta <i>et al.</i> (1977)
Tu1B	Tübingen sewage	Datta <i>et al.</i> (1977)
Ox2	Oxford, England	Kay & Fields (1962)
SC1		S. Benzer collection
D. Lambdoid phage		
HK022		Dhillon <i>et al.</i> (1981)
E. Plasmids		
pBluescript SK ⁺	<i>ori-colE1, bla, lacI, lacP, ori-f1</i>	Michelle <i>et al.</i> (1992)
pSTP1	<i>ori-colE1, bla, lacI, lacP ori-f1, stp</i> ⁺	This work

(S. Benjamin, unpublished results). To construct the plasmid pSTP1, a DNA fragment containing the *stp* orf and the upstream ribosome binding site was amplified from phage T4 strain *pseT*Δ1 by PCR and placed under the control of *Plac* in the expression plasmid pBluescript SK⁺ (Stratagene).

Anticodon nuclease assay

ACNase was assayed in *E. coli prrr*⁺ infected by the indicated phages or transformed by *stp* plasmids. The activity was monitored by the appearance of uniformly labeled [³²P]tRNA^{Lys} fragments 1 to 33 and 34 to 76, essentially as described (David *et al.*, 1982). When *stp* expression was induced from a plasmid, the media used for cell growth, pulse-labelling and subsequent chase contained 1% (v/v) glycerol as the only carbon source. ACNase activity was induced by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG) during the chase period.

Mutagenesis of *stp*

Random base transitions were induced by hydroxylamine mutagenesis (Davis *et al.*, 1984) of phage T4 strain *pseT*Δ1 (*pnk*⁻) (Snyder *et al.*, 1976). Resulting *stp* mutants were selected by growth on the restrictive *prrr*⁺ host *E. coli* CTr5X (Depew & Cozzarelli, 1974). Sequences containing *stp* mutations were amplified by PCR using sense and antisense primers: 5' ACCTGCAGTAATACGACT-

CACTATAGGGAGATGAAACCTCTAACGAC 3' and 5' CCCAAGCTTCACTGGCGTCCGAAGACGCCTTTAGT-TTTAAGATTGTTACGATAGA ACTGC 3', respectively. The PCR products were cleaved with *Pst*I and *Cla*I restriction nucleases and the *stp*-containing subfragment was ligated between corresponding sites of plasmid pBluescript SK⁺. Non-selected *stp* point mutations were created by saturation mutagenesis, using the degenerate and partially complementing sense and antisense oligo-nucleotides: 5' GGAATTCGTCATATGAGTAATTTCCATAACGAACACGTGATGCAGTTCTATCGTA 3' and 5' CCCAAGCTTCACTGGCGTCCGAAGACGCCTTTAGTTTAAAGATTGTTACGATAGA ACTGC 3' respectively. The syntheses were programmed such that codons 2 to 26 were mutagenised by non-wild-type nucleotides included in each step at a concentration of 1%. The oligonucleotides were annealed and converted into a DNA duplex by incubation with Klenow polymerase. The duplex was trimmed at the terminal *Eco*RI and *Hind*III sites and ligated between the corresponding sites of pBluescript SK⁺, placing *stp* under control of the vector's *Plac*. *E. coli* DH10B (*prrr*⁰) cells were transformed by the resultant *stp* mutant library. Individual clones were identified by DNA sequencing using the T3 primer (Stratagene).

C-Proximal deletions yielding *stp* 16, 18 and 22mers were performed by PCR, using the respective oligonucleotides 5' CCCAAGCTTCAGACGCCTTTAGTTTAAAGA 3', 5' CCCAAGCTTCATTTAAGATTGTTACGATAG 3' and 5' CCCAAGCTTCAATTGTTACGATAGA ACTGC 3' to create the mutant C terminus.

Marker rescue

Marker rescue (Mattson *et al.*, 1977) of selected *stp* alleles cloned in a plasmid was performed by crossing the mutations back into T4 *pseTΔ1* essentially as described (Chapman *et al.*, 1988).

DNA sequencing

DNA sequencing was performed on double-stranded plasmid DNA by the dideoxy sequencing method (Sanger *et al.*, 1977) using the Sequenase version 2.0 kit (USB) and an oligonucleotide primer of a sequence flanking *stp* 5' AACACATGCTACTATCCAAC 3' or the T3 primer. The reactions were conducted in parallel with dITP or dGTP, to overcome ambiguities due to secondary structure. Reaction mixtures containing ddATP and ddTTP were supplemented by 2 μl of Sequence Extending Mix to facilitate analysis of AT-rich sequences. The use of Mn²⁺ facilitated reading the sequence close to the primer 3' end.

Restriction-modification tests

The effect of *stp* on the restriction and modification activities of *EcoprrI* towards phage HK022 was tested with phage stocks grown on *E. coli* strains containing *EcoprrI* either with or without *prcC*, with or without a plasmid encoding a cloned T4 wild-type or mutant *stp* gene. The phage stocks were grown either on strain JM107 or JM107*prc*. Those grown on JM107*prc* were expected to be modified by the *EcoprrI* methylase. Dilutions of phage stocks were plated on strains JM107, JM107*prc* and JM107*prcΔC11* or transformants containing the indicated *stp* allele. Before using them, the indicator bacteria were grown overnight at 37°C in LB medium without shaking and then were induced for one hour with 1 mM IPTG with shaking. IPTG was added at the same concentration to the top agar and the medium was supplemented with 10 mM MgSO₄. Antibiotics were added when needed.

Other methods and accession numbers

PCR protocols were adapted from Innis *et al.* (1990) and recombinant DNA methods from Sambrook *et al.* (1989). RNA secondary structure was predicted by the foldRNA algorithm of Zuker & Stiegler (1981) and *Stp* structure by the method of Garnier *et al.* (1978). The accession numbers of the DNA sequences of the *stp* orfs of phages T4, LZ3 /8, LZ5, Tu1A, Baker, SCL, OX2 and Tu1B are in respective order, Z46874 to Z46880 and Z46884.

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