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► **To cite this version:**

Sylvain Durand, Graziella Richard, François Bontems, Marc Uzan. Bacteriophage T4 polynucleotide kinase triggers degradation of mRNAs. *Proceedings of the National Academy of Sciences of the United States of America*, 2012, 109, pp.7073 - 7078. 10.1073/pnas.1119802109 . hal-04678851

HAL Id: hal-04678851

<https://hal.science/hal-04678851>

Submitted on 27 Aug 2024

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1 **BACTERIOPHAGE T4 POLYNUCLEOTIDE KINASE TRIGGERS DEGRADATION**
2 **of mRNAs**

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31 Classification : Biological Sciences/Microbiology
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ABSTRACT

The bacteriophage T4-encoded RegB endoribonuclease is produced during the early stage of phage development and targets mostly, but not exclusively, the Shine-Dalgarno sequences of early genes. In this work, we show that the degradation of RegB-cleaved mRNAs depends on a functional T4 polynucleotide kinase/phosphatase (PNK). The 5'-OH produced by RegB cleavage is phosphorylated by the kinase activity of PNK. This modification allows host RNases G and E, whose activity is strongly stimulated by 5'-monophosphate termini, to attack mRNAs from the 5' end, causing their destabilization. The PNK dependent pathway of degradation becomes effective 5 minutes post-infection, consistent with our finding that several minutes are required for PNK to accumulate after infection. Our work emphasizes the importance of the nature of the 5' terminus for mRNA stability and depicts a new pathway of mRNA degradation with 5'-3' polarity in cells devoid of 5'-3' exonucleases. It also ascribes for the first time a role for T4 PNK during normal phage development.

Key words: Bacteriophage T4/Endoribonuclease RegB/mRNA degradation/Polynucleotide kinase/RNases E and G.

1 INTRODUCTION

2

3 Although significant progress has been made in deciphering the different pathways of
4 mRNA degradation in its host *Escherichia coli*, the way bacteriophage T4 degrades its
5 mRNAs and the contribution of host and phage factors, is far less understood (1).
6 Immediately following T4 infection, the early mRNAs are synthesized in a soup of host
7 RNases. Among them, the 3'-5' exoribonucleases, polynucleotide phosphorylase (PNPase),
8 RNase R and RNase II, together with an oligoribonuclease, are responsible for the swift
9 degradation of mRNAs into mononucleotides. Endonucleolytic cuts greatly accelerate mRNA
10 decay by creating new entry sites for 3'-5' exoribonucleases that bypass the structural
11 barriers. The endoribonuclease RNase E plays a major role here. RNase G, a paralogue of
12 RNase E, is also involved in mRNA decay, although to a much lesser extent than RNase E (2,
13 3).

14 RNases E and G share the remarkable property of sensing the nature of the 5' terminus
15 of their substrates. These endonucleases are significantly more active on RNA substrates
16 bearing a 5'-monophosphate than a 5'-triphosphate or hydroxyl (4, 5). This property accounts
17 for a mechanism of mRNA degradation from the 5' terminus. Indeed, the 5'-
18 monophosphorylated RNA fragment produced by the first RNase E/G cleavage can be rapidly
19 attacked again by RNase E or G, leading to a cascade of endonucleolytic cuts, often of 5' to 3'
20 polarity (6). Many *E. coli* primary transcripts enter this pathway of degradation upon
21 pyrophosphate removal from their 5'-terminal triphosphate group by the RNA
22 pyrophosphohydrolase, RppH (7).

23 RegB is a sequence-specific endoribonuclease encoded by T4 that is expressed shortly
24 after infection (8). It targets mostly, but not uniquely, Shine-Dalgarno (SD) sequences of
25 early genes (9). The direct consequence of cleavages in SD sequences is the functional
26 inactivation of mRNAs (10). In addition, RegB cleavages expedite the degradation of T4
27 early but not middle or late bulk mRNA, consistent with the quasi absence of RegB consensus
28 sequences in these two classes of mRNAs (11). By providing a mechanism that frees the
29 translation apparatus from abundant early mRNAs, this endonuclease is thought to facilitate
30 the transition between early and later stages of T4 development. The RegB nuclease produces
31 5'-OH and 2',3'-cyclic PO₄ termini (8, 12) like two *E. coli* ribotoxins, YoeB and RelE, with
32 which it shows structural and functional similarities (13). The mechanism by which RegB
33 facilitates mRNA degradation is still unclear. One likely model is that RegB accelerates
34 mRNA decay by increasing the number of entry sites for 3'-5' exoribonucleases within long

1 polycistronic mRNAs. Another mechanism was suggested by the finding that some early
2 transcripts undergo cleavages by RNases G and E that depend on prior cleavage by RegB a
3 few nucleotides upstream (10, 14, 15). These cuts, called secondary cuts, were detected in
4 seven different early transcripts. In most cases, multiple adjacent cleavages were observed. In
5 all seven cases, the primary RegB cut does not lie in a SD sequence, but at some distance
6 upstream. We interpreted these observations as meaning that RegB triggers a cascade of
7 endonucleolytic cuts that lead to degradation of the transcript with 5'-3' polarity (10).
8 However, the dependence of RNase G and E cleavages on a RegB primary cut appeared
9 paradoxical in view of the strong preference of both RNases G and E for 5'-
10 monophosphorylated RNA substrates.

11 Here, we show that formation of secondary cleavages and other RNase E/G cleavages
12 farther downstream in transcripts depends on prior phosphorylation of the 5'-OH terminus left
13 by RegB, by T4 polynucleotide kinase/phosphatase (PNK). We thus describe a new pathway
14 of degradation of T4 early transcripts with 5'-3' polarity, which depends on the phage RegB
15 and PNK enzymes and the host RNases G and E. We also ascribe for the first time a role for
16 T4 PNK during normal phage development other than in tRNA repair (16, 17).

17

18 RESULTS

19

20 T4 polynucleotide kinase allows RNase G and RNase E to act at secondary sites.

21

22 How can RNases G and E be responsible for secondary cleavages when RegB leaves a
23 5'-OH moiety after cleavage? Bacteriophage T4 codes for a 5' polynucleotide kinase-3'
24 phosphatase (PNK), which, a priori, could phosphorylate RNA termini during the phage
25 cycle. PNK, encoded by the *pseT* gene, has three activities: 5'-polynucleotide kinase, 3'-
26 phosphomonoesterase and 2',3'-cyclic phosphodiesterase. Both DNA and RNA are substrates
27 of PNK (18, 19).

28 To test whether PNK was involved in the generation of secondary cuts, we analyzed
29 the 5' region of the *motB* and *cef* mRNAs by primer extension at various times after infection
30 of strain CSH26 with T4 wild-type or a phage carrying an almost total deletion of the *pseT*
31 gene (T4 Δ *pseT*; see Materials & Methods). A similar analysis performed after infection of a
32 host deficient in RNase G (CSH26*rng::cat*) with T4 wild-type was included for comparison.
33 The full-length *motB* mRNA (denoted by P in Fig. 1A) was converted by RegB into shorter
34 transcripts (arrow 1) at the same rate in all three conditions of infection analyzed. Moreover,

1 secondary cleavages, which appeared typically 4 to 5 minutes post-infection (arrow 2) (10),
2 were significantly reduced in the *rng* mutant and almost suppressed in the absence of a
3 functional PNK (Fig. 1A & S1B). Concomitantly, the RegB processed transcripts
4 accumulated to levels above those observed in infection of wild-type cells with T4 wild-type
5 (Fig. 1A). A very similar pattern was observed upstream of *cef* (Fig. 1B). In this case, RNase
6 G contributes to only one, and RNase E to the other, of the two secondary cleavages (Fig.
7 S1A) (15). Both cleavages disappeared in the absence of a functional PNK (Fig. 1B). Similar
8 analyses showed that the multiple secondary cleavages observed upstream of genes 39 and 43,
9 contributed mostly by RNase G, are dramatically reduced in the T4 Δ *pseT* infection (Fig. S2).
10 In an infection by a phage carrying the *pseT1* mutation, where the 3'-phosphatase activity is
11 abolished while the kinase activity is unaffected (20), secondary cleavages upstream of genes
12 39 and 43 occur normally (Fig. S3). In conclusion, cleavages at secondary sites depend on
13 prior phosphorylation of the RegB-generated 5'-OH RNA termini by the kinase activity of
14 PNK.

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17 **PNK controls endonucleolytic cuts at some distance from RegB sites**

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19 Given that PNK controls the accessibility of RNases G and E to secondary sites, via
20 phosphorylation of the 5' terminus, we asked whether it could also control the accessibility of
21 these RNases to sites located further downstream. We therefore performed primer extension
22 assays on wild-type T4 mRNAs using primers located either 200 nucleotides (primer #4, Fig
23 2A) or 390 nucleotides (primer #5, Fig. S4) downstream of the initial RegB cleavage site in
24 the *cef* transcript. These assays revealed many additional RT stops (locations given in Fig.
25 2B), all of which were weaker than those seen at the RegB site or the secondary site. The
26 same analysis performed on RNA isolated after infection of host strains defective in RNase E
27 (*rne-1*) or RNase G (*rng::cat*) permitted attribution of most of these RT stops to cleavages by
28 RNase E; only a few were RNase G-dependent (Fig. S5A & B). After infection with
29 T4 Δ *pseT*, the cleavages located in the first 200 nucleotides downstream of the RegB site
30 disappeared or were dramatically weakened (Fig. 2A). The cuts located farther away
31 (numbered 11 to 18 on Fig. S4) were also affected by the lack of PNK, but to a lesser extent.

32 Since a RegB cut is the initial event that triggers all others, a *regB* mutation should
33 have the same effect on the RNase G and E cleavages as the Δ *pseT* mutation. Primer
34 extension carried out with primer #4 showed that the pattern of cleavage in T4*regB* mutant

1 infection is strikingly similar to that observed in the $\Delta pseT$ mutant (Fig. 2A, right panel). The
2 effect of the *regB* mutation on the cleavages located farther downstream in the *cef* mRNA is
3 much less visible (Fig. S4, right panel), similar to what was seen with T4 $\Delta pseT$.

4 In conclusion, our results show that when the 5'-OH RNA end generated by RegB is
5 not phosphorylated by PNK, the attack by RNases E and G is blocked or decreased over a
6 distance of about 300 nucleotides from the RegB site. However, once PNK has modified the
7 5' terminus and RNase G (or E) has cut at secondary sites, the new 5'-monophosphorylated
8 RNA ends can presumably activate RNases E and G in cascade.

10 **PNK controls the decay of early transcripts predominantly from their 5' termini**

11
12 We asked what were the consequences of PNK activity on the rate of degradation of
13 RegB-processed transcripts. The primer extension experiments shown above already indicated
14 that in a T4 $\Delta pseT$ infection, the RegB-processed RNA species accumulated to levels much
15 higher than in the wild-type infection, suggesting mRNA stabilization (Fig. 1 and S2). Since
16 the early promoters are abruptly turned off 2 to 3 minutes after infection (10, 21), any
17 variation in the accumulation of early transcripts observed after this time in different genetic
18 backgrounds should reflect modifications in their rate of degradation.

19 **The *cef* mRNA.** The RNA fragment generated by RegB cuts upstream of the SD
20 sequence of *cef* and downstream of *orf39.1* (Fig. 3A) was chosen for half-life studies because
21 the RegB cleavage on its 5' side gives rise to secondary cuts (see above). This transcript bears
22 a hairpin structure immediately upstream of the distal RegB site, with the stabilizing UUCG
23 tetraloop, showing enough stability to block the progression of AMV reverse transcriptase in
24 a primer extension assay (10). Northern blot analysis of RNA extracted after T4 infection
25 using a probe for the *cef* mRNA (probe #6; Fig. 3A) revealed a unique band of the anticipated
26 size (Fig. 3C). This 1.2 kb RNA species is hereafter designated the "*cef* mRNA".

27 Quantification of the signals of Fig. 3C showed that the *cef* mRNA accumulated until
28 the fifth minute of a wild-type T4 infection and declined thereafter (Fig. 3D). In a T4 $\Delta pseT$
29 infection, the *cef* mRNA started to accumulate at a rate similar to that observed with wild-type
30 phage; however, past the fourth minute, it continued to accumulate to reach levels twice those
31 obtained with the wild-type and started to decay after the seventh minute, but more slowly
32 than in the wild-type infection (Fig. 3D). Thus, the elimination of PNK leads to a significant
33 stabilization of the *cef* mRNA, such that 12 minutes post-infection with the *pseT* deficient

1 phage, the *cef* mRNA was 4-fold more abundant than with the wild-type phage.

2 To determine the respective contribution of the kinase and phosphatase activities of
3 PNK in *cef* mRNA degradation, we carried out a similar Northern analysis with RNA isolated
4 after infection with the T4*pseT1* mutant deficient for just the phosphatase activity (Fig. 4A).
5 Quantification of the T4 wild-type versus *pseT1* Northern blot (Fig. 4B) showed that
6 eliminating the phosphatase activity of PNK led to a pattern of *cef* mRNA accumulation
7 similar to that observed during wild-type infection, with a slight shift to earlier times. Thus,
8 the lack of kinase activity is primarily responsible for the slower degradation rates.

9 **The *orf30.8* mRNA.** In the cases examined above (*motB*, *cef*, genes 39 and 43), the
10 PNK-dependent secondary cleavages were observed following a primary RegB cut in a non-
11 SD sequence. Therefore, we asked whether the stability of a transcript processed in a SD
12 sequence, such as that of *orf30.8*, which does not undergo secondary cleavages, could also be
13 influenced by PNK. RegB also cuts the early T4 *orf30.8* mRNA 59 nt downstream of the end
14 of the orf, producing a 333 nucleotide RNA fragment visible in Northern blots using probe #7
15 (Fig. 3B and C). This mRNA also differs from the *cef* mRNA in that no secondary structure
16 can be formed on its 3' side. In infection of *E. coli* with wild-type T4, accumulation of the
17 *orf30.8* mRNA reached a maximum three minutes post-infection and declined abruptly
18 thereafter, indicative of a very short-lived mRNA (Fig. 3E). In infection with T4 Δ *pseT*, the
19 *orf30.8* mRNA started to accumulate in a manner very similar to that observed in the wild-
20 type infection. However, after the third minute, the transcript decayed in a biphasic mode:
21 first rather rapidly, but less abruptly than in the wild-type infection, until the sixth minute, and
22 then, extremely slowly. Between the sixth and 12th minute, the *orf30.8* mRNA level was
23 consistently about 4-fold higher than that in the wild-type infection (Fig. 3E). Preventing the
24 removal of the 2',3'-phosphate led to a very similar accumulation curve for this transcript in
25 the T4*pseT1* infection compared to wild-type (Fig. 4A & C), but with a one minute-shift
26 towards earlier times, as observed with the *cef* transcript. Thus, here also, the kinase activity
27 of PNK plays the main role. The biphasic mode of decay (Fig. 3E), in the *pseT* deletion
28 mutant, suggests that this small transcript is degraded *via* two pathways. The first is
29 independent of PNK and is effective as soon as the promoter is shut down three minutes post-
30 infection. The second pathway depends on the kinase activity of PNK and takes place after
31 the fifth minute of infection, presumably after sufficient PNK has accumulated. This
32 demonstrates that, even in the absence of detectable secondary cuts, PNK plays a role in the
33 degradation of RegB-processed transcripts from their 5'- termini. It is noteworthy that with
34 both *cef* and *orf30.8* mRNAs, the destabilizing effect of PNK becomes effective after 5

1 minutes of phage development, around the time secondary cleavages (by RNases G and E)
2 start to be detectable ((10, 14); this work).

3

4 **PNK accumulates slowly after infection**

5

6 The above data shows that the effect of PNK on mRNA stability is revealed only after
7 5 minutes of infection. We therefore checked whether this reflected the timing of PNK
8 biosynthesis during the phage cycle. This was done by pulse-labeling of cells with [³⁵S]-
9 methionine for one minute at different time intervals after infection to identify newly
10 synthesized phage proteins. The band corresponding to PNK was identified on protein gels as
11 a band co-migrating with purified T4 PNK and present after wild-type but not T4 Δ *pseT*
12 infection. PNK was barely visible before the 4th minute of infection and accumulated
13 thereafter (Fig. 5). This delay in expression likely explains the timing of the effect of PNK on
14 *cef* and *30.8* mRNA stability.

15

16 **DISCUSSION**

17

18 We showed in this work that bacteriophage T4 polynucleotide kinase plays a key role
19 in the degradation of phage mRNAs that are processed by the T4 RegB endonuclease. Upon
20 cleavage, RegB leaves 5'-OH and 2',3'-cyclic phosphate termini (8, 12). Phosphorylation of
21 the 5'-OH terminus by the kinase activity of PNK permits attack by the host RNases G and E,
22 two endoribonucleases that have strong preference for 5'- monophosphorylated RNA termini
23 (4-6, 22). RNases E and G cut first in the vicinity of the initial RegB cleavage site, generating
24 the so-called "secondary cleavages" (10, 14, 15). Since the cleavage products of RNases E
25 and G bear 5'-PO₄ groups, they are themselves substrates for further cleavage by these
26 endonucleases, causing a cascade of endonucleolytic cuts with 5' to 3' polarity that leads to
27 mRNA destabilization. Supporting this view is the fact that the RNase G and E cleavages that
28 normally occur in the *cef* transcript over a distance of about 300 nucleotides downstream of
29 the RegB cut are abolished or are significantly reduced in infections with a T4 PNK-deletion
30 mutant. An important observation of this paper is that the two RegB-generated transcripts, *cef*
31 and *orf30.8*, are stabilized after 5 minutes of phage development in the absence of PNK.
32 While primary cuts by RegB can be observed as early as the first minute of infection (8), the
33 secondary cleavages (by RNases G and E) start to be detectable only three to four minutes
34 later ((10, 14); this work), concomitantly with the observed destabilizing effect of PNK. Here,

1 we showed that the PNK protein accumulates slowly after infection, suggesting that the delay
2 between primary and secondary cuts reflects the time necessary for PNK to accumulate in
3 sufficient quantities after infection. Nivinskas and colleagues (15) proposed that a phage
4 factor can modify RNases E and G, permitting them to attack the RegB-processed transcripts
5 with a 5'-OH terminus. We showed here that a phage factor indeed plays a key role, but by
6 modifying the 5' end of mRNAs rather than the host RNases. Thus, RegB and PNK cooperate
7 to create new entry sites for RNases G and E, leading to mRNA degradation in a stage-
8 dependent manner. In earlier work, we reported that RegB destabilizes (3-fold) bulk early
9 mRNA synthesized during the first three minutes of infection (11). Clearly, the RegB/PNK-
10 dependent degradation pathway, which is effective after the fifth minute of infection, cannot
11 account for this early wave of degradation. Other pathways may play a role in this
12 phenomenon, such as the exonucleolytic attack of RegB-cleaved fragments from their 3' end
13 or, in cases where translation is blocked following RegB cleavage, greater access to RNase E
14 in a 5' independent mechanism.

15 The RegB/PNK degradation pathway described here unveils new layers of regulation
16 during T4 development. In the case of the *cef* mRNA and of the 6 other transcripts showing
17 secondary cleavages, RegB does not cut in translation initiation regions. Thus, until sufficient
18 PNK has accumulated to trigger their degradation by RNases G and E, these relatively stable
19 early transcripts should produce their encoded proteins long after early promoters have shut
20 down (second to third minute post-infection; (10, 21). In agreement with this hypothesis, we
21 have previously observed that the biosynthesis of ComC□□ one of the proteins encoded by
22 the *cef* mRNA, persists until the onset of the late period, although with a decreasing rate of
23 synthesis (23). Fig. 6 summarizes our view of the way the *cef* mRNA is degraded via the
24 RegB/PNK pathway. The *orf30.8* mRNA illustrates the most common case, where RegB
25 cleavage occurs in the SD sequence and leads to immediate translation shut down. The lack of
26 a 3' stabilizing structure in *orf30.8* makes the transcript particularly susceptible to degradation
27 from the 3' end, which probably explains the inability to detect secondary cleavages in this
28 transcript. We nonetheless saw that that turnover of this transcript was PNK dependent after
29 the fourth minute of infection. Thus, in the first three minutes post-infection, expression of *orf*
30 *30.8* is expected to be quite similar to that of other typical early genes (e.g. *motA*), with a
31 burst of protein synthesis very soon after infection followed by abrupt silencing (10).

32 A few minutes after infection, many T4 genes are transcribed from a proximal middle
33 promoter in addition to the distal early, and sometimes middle promoters (24). This is the case
34 for genes *39* and *43*, coding for a subunit of topoisomerase and for DNA polymerase,

1 respectively. Both the polycistronic early transcripts and the monocistronic middle transcripts
2 for genes 39 and 43 end at a strong terminator immediately downstream. What distinguishes
3 the two overlapping transcripts in each case is that the polycistronic species can be cut by
4 RegB in the upstream intergenic regions, whereas the monocistronic middle transcripts cannot
5 (see Fig. 3A). This leads to degradation of the polycistronic transcripts, but leaves the
6 overlapping middle mRNA intact. Thus, the RegB/PNK pathway of degradation also permits
7 uncoupling of the fate of the overlapping mRNA species. It is noteworthy that this mechanism
8 of degradation takes place 4 to 5 minutes after infection, precisely when middle transcription
9 takes over from early transcription.

10 Until now, the only role ascribed to T4 PNK was that of a healing enzyme in a process
11 of tRNA repair. ((17) and references therein). The work presented here shows for the first
12 time clear evidence that PNK can also participate in a mechanism of mRNA degradation via
13 5'-phosphorylation. The status of the 5' terminus has recently been highlighted as an
14 important factor for mRNA degradation. Indeed, the number of endo- and exoribonucleases
15 that require 5'-monophosphorylated RNA as substrate has increased significantly these last
16 years. The RNase E/G family of endonucleases in the α -proteobacteria (4, 5, 22), the 5'-3'
17 exonucleases RNases J1 and J2, and the endonuclease RNase Y in *B. subtilis* (25, 26), the 5'-
18 3' exonucleases Xrn1, Rat1 and to a lesser extent, Rrp17, in yeast (27-30), are among them.
19 In *E. coli* and *B. subtilis*, 5'-monophosphorylated RNAs are generated by cleavages by most
20 endoribonucleases or derive from 5'-triphosphorylated primary transcripts after removal of a
21 pyrophosphate group by the RppH pyrophosphohydrolase (7, 31). In yeast, an enzyme with
22 pyrophosphohydrolase activity towards 5' triphosphorylated RNA called Rai1 has been
23 proposed to stimulate Rat1 activity in nucleus by a modification of 5'-end of decapped RNA
24 (32). RNA kinases have been reported to influence the processing or degradation of some
25 RNAs in Eukaryotes, but their mechanism of action is not yet clear (33-35).

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1 MATERIALS AND METHODS

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3 **Bacteria and bacteriophages.** *E. coli* BE (*sup*^o) was our standard strain for growing
4 bacteriophage T4. CTr5x is restrictive for the growth of T4 *pseT* mutants (36). CSH26 (*ara*,
5 Δ (*lac-pro*), *thi*) and GC11, which is CSH26*rng::cat* (37), were described. CSH26*rne-1* and
6 the CSH26(*rng::cat*, *rne-1*) double mutant in RNases G and E, were constructed by
7 transduction with P1 grown on strain GW20 (*rne-1*) (38). Bacteriophage T4 wild-type was
8 T4D. T4*regB* R52L and T4*pseT1* were described (11, 19, 36). T4 Δ *pseT* mutant was obtained
9 using the directed insertion/substitution mutagenesis method described in ref. (39). The
10 resulting phage carries an 853 nucleotide deletion of the *pseT* gene (903 nucleotides), starting
11 at the AUG translation initiation codon.

12
13 **Experiments with RNA.** For RNA extraction, *E. coli* cells were grown in MOPS–
14 Tricine medium supplemented with 1% casaminoacids and infected at a cell density of
15 2.5×10^8 /ml, at a multiplicity of 10 at 30°C. When the effect of RNase E inactivation had to be
16 analyzed, the temperature of the cell cultures (CSH26 *rne-1* or CSH26 *rne-1*, *rng::cat*) was
17 raised to 43°C for 30 minutes prior to phage infection. Methods for RNA extractions, primer
18 extension and RNA sequencing were described in (8-10). Routinely, 700 μ l of infected cells
19 were added to 77 μ l of (SDS 10%, Na-EDTA 20 mM) and 500 μ l water-saturated phenol
20 maintained at 70°C. RNA was then phenol-extracted three times. Northern blots were
21 performed essentially as described in (40). The membrane was scanned with a
22 PhosphorImager. Quantification of the band intensities was done with ImageJ software (41)
23 after removing the background according to the “rolling ball” method. The values were then
24 normalized to 23S rRNA, which was considered constant throughout infection. The
25 oligonucleotides used in this study are listed in Table S1.

26
27 **T4 protein labeling and electrophoresis.** *In vivo* labeling and electrophoresis of T4
28 proteins were performed as described in (10). Proteins were pulse-labeled for 1 min by
29 addition of L-[³⁵S]-methionine (Amersham, >1000 Ci/mmol, 20 μ Ci/ml) to the medium. T4
30 proteins were separated by electrophoresis on an SDS-EDTA 12% polyacrylamide gel.

31 ACKNOWLEDGEMENTS

1 We thank Drs. M. Wachi for the gift of strains, L. Snyder for phages, C. Condon for
2 discussions and critical reading of the manuscript, C. Portier and A. El Hage for fruitful
3 discussions. S. D. was supported by a fellowship from the French Ministère de
4 l'Enseignement Supérieur et de la Recherche. This work was funded by grants from CNRS,
5 UPMC and Ministère de l'Enseignement Supérieur et de la Recherche to M.U. ("Programme
6 de recherche en Microbiologie: Microbiologie fondamentale et appliquée, maladies
7 infectieuses, environnement et bioterrorisme").

9 AUTHOR CONTRIBUTION

11 S.D. designed and performed research and analyzed data. G.R. performed research. F.B.
12 analyzed data. M.U. designed research, analyzed data and wrote the paper.

14 CONFLICT OF INTEREST

16 The authors declare that they have no conflict of interest.

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36 **FIGURE LEGENDS**

37

38 Fig. 1. Effect of PNK or RNase G inactivation on the generation of secondary
 39 cleavages. Primer extension assays of the intergenic regions upstream of the *motB* (A) and *cef*
 40 (B) genes, using primers #3 and #6 (Fig. 3A and Table S1), respectively, were performed on
 41 RNA extracted at the times indicated above the gel images, after infection of CSH26 or
 42 CSH26 *rng ::cat* by T4 wild-type or CSH26 by T4 Δ *pseT*. Arrows to the left show RT stops
 43 corresponding to RegB cleavages (1), secondary cleavages (collectively labeled 2),
 44 transcription starts (P). The number of missing nucleotides represented by dots is indicated

1 below the sequences. RegB cleavage sites and start codons are boxed. Bent arrows indicate
2 transcriptional starts. Vertical arrows show the positions of RegB, RNase G (G) and RNase E
3 (E) cleavage sites. The intensity of the RT stops is indicated by the shading of the arrows, the
4 darkest arrows representing the strongest stops.

5

6 Fig. 2. Effect of PNK or RegB inactivation on the occurrence of RNase E and RNase
7 G cleavages in the *cef* transcript. Primer #4 (Fig. 3A and Table S1), located 200 nucleotides
8 from the RegB site was used in primer extensions of RNAs extracted at the times indicated
9 above the gel image, after infection of CSH26 by T4 wild-type, T4 Δ *pseT* or T4*regB* R52L.
10 Sequences of RNA extracted 4 minutes after infection of CSH26 by T4 *regB* R52L, obtained
11 with the same primers, were run alongside the cDNAs (lanes labeled CTAG). RNase E and G
12 cleavages are indicated by arrows on the figure; numbering starts with the RegB cleavage. (*)
13 indicates a new degradation product appearing upstream of the RegB cleavage site in *regB*
14 mutant strains. (B) The positions of RegB, RNase E (arrows below the sequence) and RNase
15 G (arrows above the sequence) cleavages mapped in panel (A) and figure S4 in the *cef*
16 sequence. The *cef* translation initiation and termination codons are underlined.

17

18 Fig. 3. Effect of the *pseT* deletion on *cef* and *orf30.8* mRNA accumulation during T4
19 development. (A) Topology of the *motB-cef-39* region. Gray rectangles represent ORFs. Gene
20 *39* is truncated. The vertical lines below the figure show RegB cleavage sites. Hairpin
21 structures are symbolized by a vertical line topped by a circle. (B) Topology of the *orf30.8*
22 region. Symbols are as in (A). (C) Northern blot analysis of RNA extracted at times
23 (minutes) indicated above the blot after infection of CSH26 with either T4 wild-type or
24 T4 Δ *pseT*. Equal amounts of RNA were loaded in each lane of the agarose gel. The 1.2 kb *cef*
25 and *orf30.8* RNA species were probed with ³²P-labeled oligonucleotides #6 and #7,
26 respectively. The 23S rRNA was probed with the “23S” oligonucleotide (Table S1). (D) and
27 (E) Quantification of the *cef* and *orf30.8* signals obtained in Northern blots from two
28 independent experiments, one of which is shown in (C) using ImageJ software, after
29 normalization to 23S rRNA. Standard deviation is indicated by error bars.

30

31 Fig. 4. Effect of inactivation of the phosphatase activity of PNK on *cef* and *orf30.8*
32 mRNA accumulation during T4 development. (A) Northern blot analysis, using probe #6 (*cef*)
33 or probe #7 (*orf30.8*), of RNA extracted after infection of CSH26 with either T4 wild-type or
34 T4*pseT1*. (B) Quantification of the *cef* and *orf30.8* signals obtained in Northern blots. Two

1 independent experiments were performed, one of which is shown in (A), as indicated in the
2 legend to Fig. 3. Standard deviation is indicated by error bars.

3

4 Fig. 5. Rate of PNK synthesis during T4 development. Phage proteins synthesized
5 after infection of CSH26 with T4 wild-type or $\Delta pseT$ (30°C) were pulse labeled with [³⁵S]-
6 Methionine for 1 minute at each of the times indicated after infection, as described in
7 Materials & Methods. The T4 proteins were run on SDS-PAGE along with purified T4
8 polynucleotide kinase (Biolabs). Aliquots of T4 wild-type (left) or $\Delta pseT$ (right) infected cells
9 were loaded alternatively for each pulse. The arrowhead indicates the position of the PNK
10 band.

11

12 Fig. 6. Schematic representation of the pathways of *cef* mRNA degradation during the
13 first 6 minutes of infection. The vertical gray arrow on left represents time (in minutes) after
14 T4 infection. The horizontal lines represent the *cef* transcripts with triphosphate (PPP),
15 monophosphate (P) and hydroxyl (OH) 5' extremities. RegB cleavage sites (vertical white
16 arrows) and the stem-loop structure downstream of the *cef* transcription unit are shown. 3'-5'
17 exonucleases are represented by a white 'PacMan' symbol. The two endoribonucleases,
18 RNases E and G, are collectively symbolized by a black hexagon. PNK is represented as grey
19 'PacMan' symbol. See text for further explanations.

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