Bacteriophage T4 polynucleotide kinase triggers degradation of mRNAs

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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, with activity that 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 min postinfection, 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 pathway of mRNA degradation with 5'- to 3'-polarity in cells devoid of 5'-3' exonucleases. It also ascribes a role for T4 PNK during normal phage development.

mRNA turnover | viral cycle

Although significant progress has been made in deciphering the different pathways of mRNA degradation in its host *Escherichia coli*, the way that bacteriophage T4 degrades its mRNAs and the contribution of host and phage factors are far less understood (1). Immediately after T4 infection, the early mRNAs are synthesized in a soup of host RNases. Among them, the 3'-5' exoribonucleases, polynucleotide phosphorylase, RNase R, and RNase II together with an oligoribonuclease are responsible for the swift degradation of mRNAs into mononucleotides. Endonucleolytic cuts greatly accelerate mRNA decay by creating new entry sites for 3'-5' exoribonucleases that bypass the structural barriers. The endoribonuclease RNase E plays a major role here. Indeed, examination of the decay of T4 gene 32 mRNA was instrumental in the discovery of the role of RNase E in mRNA decay (1). RNase G, a paralogue of RNase E, is also involved in mRNA decay, although to a much lesser extent than RNase E (2, 3).

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

RegB is a sequence-specific endoribonuclease encoded by T4 that is expressed shortly after infection (8). It targets mostly but not uniquely Shine–Dalgarno sequences of early genes (9). The direct consequence of cleavages in Shine–Dalgarno sequences is the functional inactivation of mRNAs (10). In addition, RegB cleavages expedite the degradation of T4 early (but not middle or late)

bulk mRNA, consistent with the quasiabsence of RegB consensus sequences in these two classes of mRNAs (11). By providing a mechanism that frees the translation apparatus from abundant early mRNAs, this endonuclease is thought to facilitate the transition between early and later stages of T4 development. The RegB nuclease produces 5'-OH and 2',3'-cyclic PO₄ termini (8, 12) like two *E. coli* ribotoxins, YoeB and RelE, with which it shows structural and functional similarities (13). The mechanism by which RegB facilitates mRNA degradation is still unclear. One likely model is that RegB accelerates mRNA decay by increasing the number of entry sites for 3'-5' exoribonucleases within long polycistronic mRNAs. Another mechanism was suggested by the finding that some early transcripts undergo cleavages by RNases G and E that depend on prior cleavage by RegB a few nucleotides upstream (10, 14, 15). These cuts, called secondary cuts, were detected in seven different early transcripts. In most cases, multiple adjacent cleavages were observed. In all seven cases, the primary RegB cut does not lie in a Shine–Dalgarno sequence but at some distance upstream. We interpreted these observations as meaning that RegB triggers a cascade of endonucleolytic cuts that leads to degradation of the transcript with 5'- to 3'-polarity (10). However, the dependence of RNase G and E cleavages on an RegB primary cut seemed paradoxical in view of the strong preference of both RNases G and E for 5'-monophosphorylated RNA substrates.

Here, we show that formation of secondary cleavages and other RNase E/G cleavages farther downstream in transcripts depends on prior phosphorylation of the 5'-OH terminus left by RegB by T4 polynucleotide kinase/phosphatase (PNK). We, thus, describe a way of entering the host 5'-phosphate-dependent degradation pathway of RNases E and G using the RegB and PNK enzymes of T4. We also ascribe a role for T4 PNK during normal phage development other than in tRNA repair (16, 17).

Results

T4 PNK Allows RNases G and E to Act at Secondary Sites. How can RNases G and E be responsible for secondary cleavages when RegB leaves a 5'-OH moiety after cleavage? Bacteriophage T4 codes for a 5'-PNK-3' phosphatase that, a priori, could phosphorylate RNA termini during the phage cycle. PNK, encoded by the *pseT* gene, has three activities: 5'-PNK, 3'-phosphomonoesterase, and 2',3'-cyclic phosphodiesterase. Both DNA and RNA are substrates of PNK (18, 19).

To test whether PNK was involved in the generation of secondary cuts, we analyzed the 5'-region of the *motB* and *cef* mRNAs

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by primer extension at various times after infection of strain CSH26 with WT T4 or a phage carrying an almost total deletion of the *pseT* gene (T4 Δ *pseT*; *Materials and Methods*). A similar analysis performed after infection of a host deficient in RNase G (CSH26*rng::cat*) with WT T4 was included for comparison. The full-length *motB* mRNA (denoted by P in Fig. 1A) was converted by RegB into shorter transcripts (Fig. 1A, arrow 1) at the same rate in all three conditions of infection analyzed. Moreover, secondary cleavages (Fig. 1A, arrow 2), which appeared typically 4–5 min postinfection (10), were significantly reduced in the *rng* mutant and almost suppressed in the absence of a functional PNK (Fig. 1A and Fig. S1A). Concomitantly, the RegB processed transcripts accumulated to levels above those levels observed in infection of WT cells with WT T4 (Fig. 1A). A very similar pattern was observed for the *cef* mRNA (Fig. 1B). In this case, RNase G contributes to only one and RNase E contributes to the other of the two secondary cleavages (Fig. S1B) (15). Both cleavages disappeared in the absence of PNK (Fig. 1B). Similar analyses showed that the multiple secondary cleavages observed upstream of genes 39 and 43, contributed mostly by RNase G, are dramatically reduced in the T4 Δ *pseT* infection (Fig. S2). Thus, RNase G and E cleavages at secondary sites depend on a functional PNK. In an infection by a phage carrying the *pseT1* mutation, where the 3'-phosphatase activity is abolished but the kinase activity is unaffected (20), secondary cleavages upstream of genes 39 and 43 occur normally (Fig. S3). Taken together, these results show that cleavages at secondary sites depend on prior phosphorylation of the RegB-generated 5'-OH RNA termini by the kinase activity of PNK.

PNK Controls Endonucleolytic Cuts at Some Distance from RegB Sites. Given that PNK controls the accessibility of RNases G and E to secondary sites through phosphorylation of the 5' terminus, we

asked whether it could also control the accessibility of these RNases to sites located farther downstream. We, therefore, performed primer extension assays on WT T4 mRNAs using primers located either 200 (Fig. 2A) or 390 (Fig. S4) nt downstream of the initial RegB cleavage site in the *cef* transcript. These assays revealed many additional reverse transcriptase (RT) stops (locations given in Fig. 2B), all of which were weaker than those stops seen at the RegB or secondary site. The same analysis performed on RNA isolated after infection of host strains defective in RNase E (*rne-1*) or G (*rng::cat*) permitted attribution of most of these RT stops to cleavages by RNase E; only a few were RNase G-dependent (Fig. S5A and B). After infection with T4 Δ *pseT*, the cleavages located in the first 200 nt downstream of the RegB site disappeared or were dramatically weakened (Fig. 2A). The cuts located farther away (numbered 11–18 in Fig. S4) were also affected by the lack of PNK but to a lesser extent.

Because a RegB cut is the initial event that triggers all others, an *regB* mutation should have the same effect on the RNase G and E cleavages as the Δ *pseT* mutation. Primer extension carried out with primer 4 showed that the pattern of cleavage in T4*regB* mutant infection is strikingly similar to the pattern observed in the Δ *pseT* mutant (Fig. 2A, Right). The effect of the *regB* mutation on the cleavages located farther downstream in the *cef* mRNA is much less visible (Fig. S4, Right), similar to what was seen with T4 Δ *pseT*.

Fig. 1. Effect of PNK or RNase G inactivation on the generation of secondary cleavages. Primer extension assays of the intergenic regions upstream of the *motB* (A) and *cef* (B) genes using primers 3 and 6 (Fig. 3A and Table S1), respectively, were performed on RNA extracted at the times indicated above the gel images after infection of CSH26 or CSH26 *rng::cat* by T4 WT or CSH26 by T4 Δ *pseT*. Arrows on the left show RT stops corresponding to RegB cleavages (labeled 1), secondary cleavages (collectively labeled 2), and transcription starts (P). The dots in the sequences replace nucleotides not represented. Their number is shown below the sequence. RegB cleavage sites and start codons are boxed. Bent arrows indicate transcriptional starts. Vertical arrows show the positions of RegB, RNase G (G), and RNase E (E) cleavage sites. The intensity of the RT stops is indicated by the shading of the arrows; the darkest arrows represent the strongest stops.

Fig. 2. Effect of PNK or RegB inactivation on the occurrence of RNase E and G cleavages in the *cef* transcript. (A) Primer 4 (Fig. 3A and Table S1), located 200 nt from the RegB site, was used in primer extensions of RNAs extracted at the times indicated above the gel image after infection of CSH26 by T4 WT, T4 Δ *pseT*, or T4*regB* R52L. Sequences of RNA extracted 4 min after infection of CSH26 by T4 *regB* R52L obtained with the same primers were run along the cDNAs (lanes labeled CTAG). RNase E and G cleavages are indicated by arrows; numbering starts with the RegB cleavage. *New degradation product appearing upstream of the RegB cleavage site in *regB* mutant strains. (B) The positions of RegB, RNase E (arrows below the sequence), and RNase G (arrows above the sequence) cleavages mapped in A and Fig. S5 in the *cef* sequence. The *cef* translation initiation and termination codons are underlined.

Fig. 4. Effect of inactivation of the phosphatase activity of PNK on *cef* and *orf30.8* mRNA accumulation during T4 development. (A) Northern blot analysis using probe 6 (*cef*) or 7 (*orf30.8*) of RNA extracted after infection of CSH26 with either T4 WT or T4*pseT1*. Quantification of the *cef* (B) and *orf30.8* (C) signals obtained in Northern blots. Two independent experiments were performed, one of which is shown in A, as indicated in Fig. 3. SD is indicated by error bars.

the kinase activity of PNK plays the main role. The biphasic mode of decay (Fig. 3E) in the *pseT* deletion mutant suggests that this small transcript is degraded through two pathways. The first pathway is independent of PNK and effective as soon as the promoter is shut down 3 min postinfection. The second pathway depends on the kinase activity of PNK and takes place after the fifth minute of infection, presumably after sufficient PNK has accumulated. This finding shows that, even in the absence of detectable secondary cuts, PNK plays a role in the degradation of RegB-processed transcripts from their 5' termini. It is noteworthy that, with both *cef* and *orf30.8* mRNAs, the destabilizing effect of PNK becomes effective after 5 min of phage development, around the time that secondary cleavages (by RNases G and E) start to be detectable (10, 14).

PNK Accumulates Slowly After Infection. The above data shows that the effect of PNK on mRNA stability is revealed only after 5 min of infection. We, therefore, checked whether this finding reflected the timing of PNK biosynthesis during the phage cycle. This process was done by pulse-labeling of cells with [³⁵S]-

methionine for 1 min at different time intervals after infection to identify newly synthesized phage proteins. The band corresponding to PNK was identified on protein gels as a band comigrating with purified T4 PNK and present after WT but not T4*ΔpseT* infection. PNK was barely visible before the fourth minute of infection and accumulated thereafter (Fig. 5). This delay in expression likely explains the timing of the effect of PNK on *cef* and 30.8 mRNA stability.

Discussion

We showed in this work that bacteriophage T4 PNK plays a key role in the degradation of phage mRNAs that are processed by the T4 RegB endonuclease. On cleavage, RegB leaves 5'-OH and 2',3'-cyclic phosphate termini (8, 12). Phosphorylation of the 5'-OH terminus by the kinase activity of PNK permits attack by the host RNases G and E, two endoribonucleases that have strong preference for 5'-monophosphorylated RNA termini (4–6, 22). RNases E and G cut first in the vicinity of the initial RegB cleavage site, generating the so-called secondary cleavages (10, 14, 15). Because the cleavage products of RNases E and G bear 5'-PO₄ groups, they are themselves substrates for additional cleavage by these endonucleases, causing a cascade of endonucleolytic cuts with 5'- to 3'-polarity that leads to mRNA destabilization. Supporting this view is the fact that the RNase G and E cleavages that normally occur in the *cef* transcript over a distance of about 300 nt downstream of the RegB cut are abolished or significantly reduced in infections with a T4 PNK deletion mutant. An important observation of this paper is that the two RegB-generated transcripts, *cef* and *orf30.8*, are stabilized after 5 min of phage development in the absence of PNK. Although primary cuts by RegB can be observed as early as the first minute of infection (8), the secondary cleavages (by RNases G and E) start to be detectable only 3–4 min later (10, 14), concomitantly with the observed destabilizing effect of PNK. Here, we showed that the PNK protein accumulates slowly after infection, suggesting that the delay between primary and secondary cuts reflects the time necessary for PNK to accumulate in sufficient quantities after infection. The work by Zajackauskaite et al. (15) proposed that a phage factor can modify RNases E and G, permitting them to attack the RegB-processed transcripts with a 5'-OH terminus. We showed here that a phage factor indeed plays a key role, but it modifies the 5'-end of mRNAs rather than the host RNases. Thus, RegB and PNK cooperate to create new entry sites for RNases G and E, leading to mRNA degradation in a stage-dependent manner. In an earlier work, we reported that RegB destabilizes (threefold) bulk early mRNA synthesized during the first 3 min of infection (11). Clearly, the RegB/PNK-dependent degradation pathway, which is effective after the fifth minute of infection, cannot account for this early wave of degradation. Other pathways may play a role in this phenomenon, such as the exonucleolytic attack of RegB-cleaved fragments from their 3'-end or in cases where translation is blocked after RegB cleavage, the greater access to RNase E in a 5'-independent mechanism.

Fig. 5. Rate of PNK synthesis during T4 development. Phage proteins synthesized after infection of CSH26 with T4 WT or *ΔpseT* (30 °C) were pulse-labeled with [³⁵S]-Methionine for 1 min at each of the times indicated after infection as described in *Materials and Methods*. The T4 proteins were run on SDS/PAGE along with purified T4 PNK (Biolabs). Aliquots of T4 WT or *ΔpseT*-infected cells were loaded alternatively for each pulse. The arrowheads indicate the position of the PNK band.

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