Roles of polyadenylation and nucleolytic cleavage in the filamentous phage mRNA processing and decay pathways in *Escherichia coli*

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ABSTRACT

To define basic features of mRNA processing and decay in *Escherichia coli*, we have examined a set of mRNAs encoded by the filamentous phage f1 that have structures typical of bacterial mRNAs. They bear a stable hairpin stem-loop on the 3' end left from rho-independent termination and are known to undergo processing by RNase E. A small percentage of the f1 mRNAs were found to bear poly(A) tails that were attached to heterogeneous positions near the common 3' end. In a poly(A) polymerase-deficient host, the later-appearing processed mRNAs were stabilized, and a novel small RNA accumulated. This ~125-nt RNA proved to arise via RNase E cleavage from the 3'-terminal region of the mRNAs bearing the terminator. Normally ribosomes translating gene VIII appear to protect this cleavage site from RNase E, so that release of the fragment from the mRNAs occurs very slowly. The data presented define additional steps in the f1 mRNA processing and decay pathways and clarify how features of the pathways are used in establishing and maintaining the persistent filamentous phage infection. Although the primary mode of decay is endonucleolytic cleavage generating a characteristic 5' → 3' wave of products, polyadenylation is involved in part in degradation of the processed mRNAs and is required for turnover of the 125-nt mRNA fragment. The results place polyadenylation at a later rather than an initiating step of decay. They also provide a clear illustration of how stably structured RNA 3' ends act as barriers to 3' → 5' exonucleolytic mRNA decay.

Keywords: 3' → 5' exonucleolytic mRNA decay; RNA secondary structure; RNase E cleavage

INTRODUCTION

Messenger RNA in the bacterial cell is continuously subject to nucleolytic cleavage in processing and turnover reactions that serve as an important parameter in determining levels of gene expression. Although these processes were poorly understood for many years, recent studies have identified the principal activities involved and certain features of nucleolytic cleavage pathways. Processing reactions appear to be mediated largely by endonucleases, whereas decay is initiated by one or more endonucleolytic cleavages, followed by 3' → 5' exonucleolytic digestion of the 3' ends produced (Kushner, 1996; Coburn & Mackie, 1999). Of the ~20 RNases identified in *Escherichia coli* (Deutscher, 1993), only five are known to act on mRNA molecules. Two are site-specific endonucleases. RNase III has sub-

strates in double-stranded regions of phage, plasmid, and bacterial mRNAs, but is dispensable and probably does not have a global role in cleavage of mRNA (Babitzke et al., 1993). RNase E is an essential 5' end-dependent endonuclease (Mackie, 1998). Mutations abolishing its activity confer temperature sensitivity and lead to the accumulation of mRNA fragments (Kuwano et al., 1977; Ghora & Apirion, 1978; Arraiano et al., 1988). Cleavage sites for RNase E occur in single-stranded regions of mRNAs, often adjacent to a stable secondary structure (Ehresmann et al., 1992; Mackie, 1992). Two other RNases, 3' → 5' exonucleases, are the hydrolytic enzyme RNase II and the phosphorylytic enzyme polynucleotide phosphorylase (PNPase). These RNases are functionally redundant but required in an essential process as elimination of both results in inviability (Donovan & Kushner, 1986). Both degrade RNA processively to mononucleotides, but have difficulty establishing and maintaining a processive cleavage mode on structured 3' ends with ≤6–10 unpaired nucleotides (Littauer & Soreq, 1982; Pepe et al., 1994; Coburn &

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Mackie, 1996b). A fifth RNase, oligoribonuclease, is required to complete mRNA breakdown to mononucleotides and is essential for cell viability (Ghosh & Deutscher, 1999). RNase E and PNPase are associated in a high molecular weight complex termed the degradosome (Miczak et al., 1996; Py et al., 1996; Blum et al., 1997; Vanzo et al., 1998) that contains the DEAD-box RNA helicase RhlB, polyphosphate kinase, the glycolytic enzyme enolase, and possibly other proteins, including the chaperone DnaK. The degradosome's components suggest a means for coordinating endo- and exonucleolytic cleavage, unwinding structured RNA, and maintaining ATP levels required for degradation, but leave unresolved whether enolase has a role in mRNA decay.

The extent to which PNPase and RNase II are impeded by secondary structure has been puzzling in view of the RNA 3' ends they frequently encounter. Many transcripts have stem-loop structures at or near their 3' ends. These include the structures of rho-independent terminators (Mott et al., 1985), REP sequences (Gilson et al., 1984; Stern et al., 1984), and stem-loops left from RNase III processing (reviewed in Court, 1993). The RhlB RNA helicase in the degradosome has been shown to facilitate degradation of a REP-containing RNA by PNPase (Py et al., 1996) and may provide one means of eliminating structure, but what range of structures RhlB is capable of unwinding is not yet clear. A second and perhaps more general means of circumventing the problem of degrading tightly folded RNAs has emerged recently from studies implicating poly(A) polymerase I in mRNA decay. Poly(A) tails of 10–40 nt have been found at the 3' ends of the antisense regulator of plasmid colE1 replication (RNA I) and a variety of mRNAs (reviewed in Cohen, 1995; Sarkar, 1997). They are also detected on the 3' ends of the upstream products of endonucleolytic cleavage (Haugel-Nielsen et al., 1996). In poly(A) polymerase-deficient strains, RNA I (Xu et al., 1993), the mRNAs for ribosomal proteins S15 (Hajnsdorf et al., 1995) and S20 (Coburn & Mackie, 1998), and mRNA in general are stabilized (O'Hara et al., 1995). Consistent with this finding, polyadenylation of RNA I and S20 mRNA stimulates their degradation in vitro (Xu & Cohen, 1995; Coburn & Mackie, 1998). These results have suggested that the poly(A) tract functions as a single-stranded extension to facilitate digestion of structured ends by the 3' → 5' exonucleases.

The abundant mRNAs observed in *Escherichia coli* hosts infected with the filamentous phage f1 have attractive features for studies of mRNA processing and decay (Fig. 1). They arise from a region of the genome encoding DNA replication proteins (pII, pX, and pV), minor capsid proteins (pVII and pIX), and the major coat protein (pVIII). Of these, pV and pVIII are needed in the largest amounts, pV to sequester progeny single-stranded DNA away from the other replication proteins,

FIGURE 1. Bacteriophage f1 genetic map and major in vivo mRNAs. Gene names are indicated; corresponding proteins use the same numbers, for example, pVIII. The positions of the strong constitutive promoters (arrowheads) from which the gene II–VIII region is transcribed and the rho-independent terminator (T) are shown. The mRNA diagram is based on the work of many authors (reviewed in Model & Russel, 1988). The RNAs range in size from 370 to 2,000 nt. Coding regions for phage genes are demarcated by vertical lines, with a dashed line marking the beginning of the smaller gene of an in-frame overlapping pair (genes II and X). The known or probable (parentheses) phosphorylation status is indicated for the primary transcripts and processed mRNAs. Half-lives for an RNase E⁺ host are based on analysis of pulse-labeled RNA (Cashman et al., 1980). The region of the f1 sequence contained in the standard single-stranded DNA probe used for Northern blot analysis is indicated (shaded box).

and pVIII to coat the sides of the long, thin virion. The primary transcripts (RNAs A, B, and H) have structures typical of many bacterial RNAs, with a stem-loop at the common 3' end generated at a strong rho-independent terminator (reviewed in Model & Russel, 1988). Earlier work (Kokoska et al., 1990; Stump & Steege, 1996) established that the mRNAs are subject to processing by RNase E-independent (RNAs C, C*) and RNase E-dependent (RNAs D–G) cleavages. The cleavages produce upstream products that are rapidly degraded and downstream products that all present a very stable structure at their 3' ends to exonucleases. The RNase E-dependent cleavages appear to occur in a 5' → 3' sequence (Cashman et al., 1980; Blumer & Steege, 1984), making it possible to follow the 3' ends on the RNAs over time after their initial synthesis. As shown in Figure 1, half-lives are short for the larger f1 mRNAs, but much longer for the smaller mRNAs (Cashman et al., 1980). Here we use *E. coli* strains deficient for poly(A) polymerase and nucleases to define roles for

polyadenylation and nucleolytic cleavage in the processing and decay pathways the f1 mRNAs follow. We discuss the biological functions the pathways have during the course of the filamentous phage life cycle and features of mRNA decay that emerge from these studies.

RESULTS

Detection of f1 mRNAs with poly(A) tails

To determine if the major phage mRNAs observed in f1-infected hosts are subject to polyadenylation, we examined unfractionated RNA from a PNPase-deficient host (*pnp-7*) infected with phage f1 for species bearing poly(A) tails. The RNA was annealed to an excess of biotinylated oligo(dT₂₅) and applied to streptavidin magnetic beads. The recovery of poly(A) RNA was followed by including in the sample as an internal standard a 250-nt single-stranded DNA that contained 232 nt near the common 3' end of the f1 mRNAs followed by 18 noncoded A residues. After washing the beads extensively, RNA remaining on the beads was eluted. The fractions were analyzed on Northern blots using a 445-nt DNA strand from the gene IX–VIII region as a hybridization probe (Fig. 1). This probe visualizes only those RNAs that contain the gene IX–VIII region, that is, the primary transcripts and the downstream products of endonucleolytic cleavage. Quantitation of the blot shown in Figure 2 revealed equivalent amounts of radioactivity in RNAs A–H in the input sample and the RNA recovered from the supernatant, an initial indication that polyadenylated RNA, if present, was at a low level. An additional 1.3% of the input RNA was recovered in the first wash, and 0.03% in the second. Recovery of the oligoadenylated internal standard in the blot shown was 70%, but ranged from 70 to 93%. In the RNA sample eluted from the beads, f1-specific mRNA was detectable, and accounted for 0.011% of the input f1 mRNA. Although it was not clear if RNA A was present, RNAs with mobilities corresponding to RNAs B and D–H were visible. The smaller RNAs were also more heterogeneous in length, as would be expected if they had acquired poly(A) tails. The results provide evidence that in the steady state, a small percentage of the f1 RNAs bear poly(A) tails.

The 3' end region present in all of the f1 mRNAs is polyadenylated at multiple sites

Because transcription and 5' → 3' endonucleolytic processing of the mRNAs from the gene II–VIII region generates an overlapping series of RNAs bearing a structured 3' end, a key question was how mRNAs of this structure are ultimately degraded. Determining the pattern of polyadenylation in the 3'-terminal region of

FIGURE 2. Purification of polyadenylated f1 mRNA. Unfractionated RNA (720 μ g) prepared using Catrimox-14 as described (O'Hara et al., 1995) from phage-infected DS214 (*pnp-7*) was annealed to an excess of biotinylated oligo(dT₂₅) and applied to streptavidin magnetic beads as described in Materials and Methods. The RNA sample contained 0.1 ng of an internal standard (M) that included 232 nt near the common 3' end of the f1 mRNAs and 18 additional A residues at its 3' end. Northern blot analysis of samples employed 2% agarose/2% formaldehyde gels. The hybridization probe was a strand of a 445-nt DNA fragment from the gene IX–VIII region that had been labeled in a PCR reaction containing one [α -³²P]dNTP. The positions of the f1 mRNAs are indicated; a smear of smaller degradation products is also visible.

the f1 RNAs was thus of immediate interest. To increase the number and size of the poly(A) tails (Hajnsdorf et al., 1995; O'Hara et al., 1995), RNA was isolated from an f1-infected host deficient for both PNPase and RNase II (*pnp-7 rnb-500^{ts}*). The RNA was used as a template for reverse transcription with an oligo(dT₁₈) primer. The sites of poly(A) addition were localized by amplifying the f1 mRNA-poly(A) junctions of the cDNAs by PCR for cloning and sequence analysis. More than one PCR primer containing f1 sequence was used (nucleotide positions 1336–1362 and 1416–1444) so that true poly(A) addition sites could be distinguished from PCR artifacts. Sequencing revealed that the clones contained poly(A) tracts ranging in length from 17 to 77 nt.

The vast majority contained 18 to 26 residues, however, suggesting that the oligo(dT₁₈) primer was usually annealed very close to the junction with the f1 sequence when it was extended by reverse transcriptase. Figure 3 summarizes the locations of poly(A) addition sites in independent clones. One cluster mapped in the vicinity of the 3' end generated by rho-independent termination. Of these, two sites correspond to the 3' terminated ends observed for pulse-labeled f1 mRNA preparations (Cashman et al., 1980), and two reflect limited "nibbling" that presumably is hindered by the hairpin structure. The site 86 nt downstream from the

FIGURE 3. Poly(A) addition sites in the 3'-terminal region of the f1 mRNAs. The template for cDNA synthesis was RNA from f1-infected DS215 (*pnp-7 rnb-500^{ts}*) that bears a nonsense allele in *pnp* and a temperature-sensitive allele in *rnb* (Arraiano et al., 1988). The culture was grown at 30 °C to a density of 5×10^7 cells/mL and shifted to 37 °C to permit synthesis of F pili. At a density of 2×10^8 cells/mL, the culture was infected, and 15 min later, shifted to 42 °C. RNA was isolated 30 min after infection. cDNA synthesis reactions contained Bamoligo(dT₁₈) as the primer and 2 or 18 U of RAV-2 reverse transcriptase. After PCR amplification with primers containing f1 sequence (f1 1336 and f1 1416, Table 2) and cloning, the locations of polyadenylation sites were determined by dideoxy sequencing (Tabor & Richardson, 1987). Nucleotide positions in the f1 sequence (Hill & Petersen, 1982) are given at the left, and the position of the gene VIII stop codon is shown. Nucleotides changed in the primers to generate a *Kpn*I site are in italics. The stem-loop structure corresponds to the rho-independent terminator beyond gene VIII (T); the 3' ends observed for pulse-labeled RNA (Cashman et al., 1980) are shown (arrowheads). The mRNA-poly(A) junctions (arrows) are shown for independent clones, with the number given if more than one clone was isolated. The positions of the novel RNase E-dependent endonucleolytic cleavage at 1438 and 1439 described below are indicated (■).

termination site appears to represent a rare transcript extended beyond the efficient terminator (Edens et al., 1975). These cDNA data very likely underestimate the number of RNAs with poly(A) addition sites beyond the terminator stem-loop, as this same structure presumably also impedes reverse transcriptase. All of the remaining sites reflect polyadenylation on heterogeneous RNA 3' ends shortened upstream of the hairpin stem-loop by residual exonucleolytic digestion.

Effects on the phage f1 mRNAs in hosts deficient for poly(A) polymerase and RNase E

Northern blot analysis was employed to examine the effects on the steady-state levels of the f1 mRNAs of a deletion in the gene encoding poly(A) polymerase I ($\Delta pcnB$) and the temperature-sensitive *rne-1* allele of the RNase E gene. Figure 4 shows data in triplicate for infected hosts lacking the mutations (WT), harboring one mutation ($\Delta pcnB$ or *rne-1*), or carrying both (*rne-1* $\Delta pcnB$). Data from several blots were quantified using a PhosphorImager and are reported in Table 1 as RNA levels relative to the radioactivity in long-lived RNA H (Cashman et al., 1980). The pattern of mRNAs observed for the WT strain was identical to that seen in many *E. coli* hosts. In the $\Delta pcnB$ mutant host, overall radioactivity in f1 mRNA remained essentially the same,

FIGURE 4. Northern blot analysis of the f1 mRNAs in *E. coli* strains deficient for poly(A) polymerase and/or RNase E. The strains used for f1 infections are labeled as follows: DS213 (WT), DS216 ($\Delta pcnB$), DS217 (*rne-1*), DS218 (*rne-1* $\Delta pcnB$). Samples of the cultures not bearing the temperature-sensitive *rne-1* mutation were withdrawn 60 min after phage infection. Samples of the *rne-1* hosts were withdrawn 60 min after shifting the phage-infected culture to 42 °C. RNA samples (1 μ g) were analyzed in triplicate on Northern blots as in Figure 2. The positions of the principal RNA species observed are indicated, with the novel RNA appearing in the $\Delta pcnB$ strain denoted RNA T. The 250-nt internal standard (M) was included as a size marker.

TABLE 1. f1 mRNA levels in wild-type, poly(A) polymerase- and RNase E-deficient hosts.

RNA	Bacterial strain			
	Wild-type	$\Delta pcnB$	<i>rne-1</i>	<i>rne-1 \Delta pcnB</i>
A	8.0 ^a	4.6	65.6	34.8
B	10.9	12.7	47.1	24.2
CC*	5.4	5.2	33.0	21.4
D	13.0	15.6	8.4	8.2
E	13.1	18.9	11.6	10.4
F	11.2	14.8	15.3	12.6
G	39.2	48.0	17.1	19.9
H	100	100	100	100
T	4.8	18.2		
Total ^b	3.3×10^6	3.2×10^6	5.8×10^6	7.3×10^6

^aPercent of radioactivity in RNA H.^bTotal radioactivity in PhosphorImager counts in the labeled bands of the blot shown in Figure 4.

but the amounts of individual species changed. No increase or slight decreases (e.g., RNA A) occurred in the levels of the large primary transcripts and the RNase E-independent cleavage products C and C*, suggesting that they are processed before being affected by the absence of polyadenylation. RNA H levels also remained virtually the same. By contrast, the products of RNase E-dependent processing appeared to have been stabilized in the absence of polyadenylation. RNA G, the species with the longest-lived 3' end remaining from transcription, showed the largest increase. Most important, a small RNA (T) with a mobility of 135 ± 10 nt not previously seen in other hosts accumulated in the $\Delta pcnB$ strain. Its discrete size, considered with the location of the most distal cluster of poly(A) addition sites, made RNA T a candidate for the downstream product of an endonucleolytic cleavage within gene VIII, the gene for the major coat protein of the phage.

In the strains harboring the temperature-sensitive *rne-1* allele, a more pronounced defect in processing/decay was observed. After the shift to the nonpermissive temperature, radioactivity in the f1 mRNA species in the single mutant increased 1.8- to 2.0-fold, and in the double mutant, 2.2- to 2.6-fold. These are minimum estimates for the increases in the amount of f1-specific message, as a significant amount of cleavage by other nucleases appeared to occur in the absence of RNase E and give a higher nonspecific background. The Northern blot data for the *rne-1* strain confirm earlier S1 nuclease mapping results indicating that inactivation of RNase E differentially stabilizes the large primary transcripts (Kokoska & Steege, 1998). The level here of RNA A increased eightfold, whereas that of RNA B, only fourfold. With the RNase E-dependent pathway blocked, the levels of RNase E-dependent cleavage products (D, E, F, and G) began to disappear, but the levels of the processed RNAs generated by RNase

E-independent cleavage (C and C*) increased. The fact that RNA T was not present suggested strongly that this RNA species represents the product of an RNase E-dependent cleavage. In the *rne-1 \Delta pcnB* double mutant, the pattern of f1 mRNAs was qualitatively similar to that for the *rne-1* single mutant. However, the increases in the levels of RNAs D–G, the nonspecific background, and the overall radioactivity relative to the single mutant indicate that turnover of f1-specific mRNA was down substantially.

RNase III implicated in cleavage at the C and C* sites

Because RNAs C and C* are only visible at low levels in wild-type and most nuclease-deficient hosts, it has not been possible to survey other host endonucleases for this cleavage activity. We thus exploited the increased levels in *rne-1* hosts to search for the activity. We examined the f1 mRNAs made in an available strain harboring mutations affecting RNase E, RNase III, and the tRNA processing enzyme RNase P. The base change affecting RNase E was *rne-3071* (Apirion & Lasser, 1978), which, although only 6 bp from the *rne-1* mutation (McDowall et al., 1993), is less deleterious to activity at the nonpermissive temperature than *rne-1*. The mutation abolishing RNase III activity was the classical *rnc-105* missense allele (Kindler et al., 1973). The RNase P mutation was the strong temperature-sensitive A49 allele affecting the protein subunit (Schedl & Primakoff, 1973). Earlier tests of this allele showed no effects on the pattern of f1 mRNA processing (Cashman et al., 1980). A Northern blot of RNA from the triple mutant that was probed with sequence from the 9S precursor to 5S rRNA confirmed that processing by RNase E was reduced after a temperature shift, and ethidium bromide-stained gels showed that the 17S precursor to 16S generated by RNase III cleavage was not detectable. Figure 5 compares the pattern of f1 mRNAs present in the wild-type host and *rne-1* single mutant to that for the triple mutant. The f1 hybridization probe visualized the *rne-1* defect developed in the single mutant after the temperature shift to 37 °C or 43 °C: decreased levels of RNAs D–G and increased levels of RNAs C and C*. When the triple mutant was shifted to 43 °C, the cumulative effects of the mutations appeared to abort the phage infection, as very little phage RNA was detectable. At 37 °C, some RNase E-dependent processing continued after the temperature shift, as was found earlier for *rne-3071* (Stump & Steege, 1996). By contrast, the products of cleavage at the C and C* sites were undetectable. The result raises the possibility that cleavage at these sites depends on RNase III. It concurs with the finding that a C site-containing substrate was cleaved in vitro using extracts from a wild-type but not an RNase III[−] strain (S.H. Alberts and D.A. Steege, unpubl. results).

FIGURE 5. Northern blot analysis of the f1 mRNAs in *rne*⁺, *rne*⁻, and *rne*⁻ *rnc*⁻ *rnpA* hosts. The strains were DS213 (*rne*⁺), DS217 (*rne*⁻), and DS219 (*rne*⁻3071 *rnc*⁻105 *rnpA*49). The temperature sensitivities exhibited by the mutant alleles, in increasing order of severity, are *rne*⁻3071, *rne*⁻1, and *rnpA*49. At 15 min after infection, cultures were placed at the indicated temperatures, and samples were removed at the times shown. The *rne*⁺ strain was maintained at 37°C for 60 min after infection.

RNA T contains the common 3' end from the f1 mRNAs

The region of the f1 sequence present in RNA T was determined by Northern blot analysis using smaller probes from the gene IX–VIII region. The probe used for Figure 6A included gene IX and the first third of gene VIII. That used for Figure 6B contained the last third of gene VIII and sequence up to the terminator stem-loop. RNA T was clearly visualized by only the downstream probe and was present at much higher levels in the $\Delta pcnB$ mutant than in the WT strain. By contrast, the band slightly smaller than the internal standard (M) was visualized by both probes, suggesting that it represents a mixture of degradation products from the gene IX–VIII region. The 5' end(s) of RNA T were localized by primer extension using a primer ~95 nt upstream from the 3' end of the f1 mRNAs. Figure 7A reveals that RNA T has two major 5' end-

FIGURE 6. Localization of RNA T sequences. RNA from f1-infected DS213 (WT) and DS216 ($\Delta pcnB$) was fractionated on a 2% agarose/2% formaldehyde gel and analyzed on Northern blots using double-stranded probes containing f1 nucleotide positions 1198–1378 (A) or 1442–1542 (B).

points spaced 1 nt apart (R1 lanes). The overexposure to visualize the sequencing lanes reveals additional stops to reverse transcriptase. These are at minimum 13- to 18-fold weaker than the major stops and are not detectable in appropriately exposed reactions (Fig. 7A, R2 lanes). These major endpoints map to nucleotide positions 1438 and 1439 (see Fig. 3). As shown in Figure 7B, the region of the f1 sequence containing these endpoints shows similarities to consensus sequences for RNase E-dependent cleavage sites (Ehresmann et al., 1992; Mackie, 1992) and to the other RNase E-dependent processing sites on the f1 mRNAs (Stump et al., 1997). Altogether, the results establish that RNA T represents the ~125-nt downstream product of an endonucleolytic cleavage dependent on RNase E (see Fig. 4). The RNA fragment is somewhat heterogeneous in length, depending on precisely where RNA polymerase terminates, the extent to which the nascent 3' end is digested by exonucleases, and where endonuclease cleavage occurs to generate the 5' end. In view of where the 5' ends of the RNA T map within gene VIII, however, the certain consequence of the endonucleolytic cleavage generating RNA T is functional inactivation of the coding region for the major phage coat protein.

FIGURE 7. Primer extension analysis of RNA T and comparison of nucleotide sequences implicated in RNase E-dependent cleavage. **A:** Primer extension (R1 and R2 lanes) and sequencing reactions (A, C, G, T lanes) were carried out as described (Mierendorf & Pfeffer, 1987) with primer f1 1492. Unfractionated RNA (5 μ g) isolated from the phage-infected $\Delta pcnB$ strain (DS216) served as the template. Reactions in the left six lanes contained 5 U RAV-2 reverse transcriptase (Amersham) and [α - 35 S]dATP (1,250 Ci/mmol, New England Nuclear). Reactions in the right two lanes employed the same primer labeled at its 5' end with [γ - 32 P]dATP. The data were quantitated using a photometric grade, cooled CCD camera (Photometrics, Inc). Arrows mark the positions of the two major 5' endpoints. **B:** Sequences are aligned with respect to the 5' endpoints (underlined) determined experimentally (Blumer & Steege, 1989, and this work). Shading denotes nucleotides that match consensus sequences proposed for the region of cleavage in RNase E processing sites and in addition, those identical in at least three of the five sequences. R = A or G, W = A or U; a: Ehresmann et al., 1992; b: Mackie, 1992.

Gene VIII message is inactivated very slowly

Given the biological consequence of the cleavage that generates RNA T, it was of interest to determine when RNA T becomes detectable. Figure 8A shows the appearance of the f1 mRNAs over time after phage infection. As found previously (Blumer & Steege, 1984), the primary transcripts appear quickly. RNAs B and H are detectable by 4 min, and RNA A, 2 min later. The processed RNAs appear later than the primary transcripts in sequence, with RNAs D and E visible before RNA F, and RNA G not present until 10 min. RNA T, detectable only at 12 min after infection, clearly appears slowly over time.

Given when RNA G appears, cleavage of RNA G at a later time to release RNA T was not surprising. However, a delay in cleavage of RNA H to remove RNA T was not expected, as the smallest primary transcript appears rapidly after infection. A plausible explanation for delayed cleavage was that ribosomes translating gene VIII at the normal high level mask the cleavage site. If this is the case, premature termination of translation at an amber mutation upstream from the cleavage site would be predicted to accelerate cleavage and perhaps destabilize RNA H. To test this, the sequences corresponding to RNA H of wild-type f1 and an M13 amber mutant causing termination 62 nt upstream from the cleavage site were cloned in a pBR322 derivative

under control of the IPTG-inducible Trp-Lac (Trc) promoter. The plasmid also carried the *lacI^Q* repressor. The segments were assembled with minimal disruption of mRNA sequence and structure to avoid altering mRNA stability. However, it was anticipated that even if the 5' leader region contributed by the Trc sequence did affect half-lives, any change in the stability of the amber mutant RNA (AmH') relative to the wild-type RNA (H') would be apparent.

Figure 8B shows the fate of the wild-type and amber mutant RNAs following IPTG induction and addition of rifampicin 3 min later. Although the presence of a new 5' leader did decrease the stability of the wild-type mRNA approximately fourfold and make the RNAs more accessible generally to the decay machinery, distinctions are apparent between the two samples. First, quantitations showed that decay of AmH' commenced immediately, with a half-life 20% lower than that for H', whereas wild-type RNA H' showed a 1-min lag in decay following rifampicin addition. Second, release of RNA T from the amber mutant was more evident throughout the time course and appeared to be accelerated, with maximal radioactivity in RNA T appearing at least 2–3 min before the small peak of RNA T released from the wild-type RNA H'. The data for these plasmid-borne transcription units provide evidence that in the natural context of the phage mRNAs, ribosomes translating gene VIII mask the final RNase E cleavage

FIGURE 8. Appearance of RNA T. **A:** Following infection of the $\Delta pcnB$ strain DS216, samples were removed at the indicated times in minutes for isolation of RNA. The RNA indicated by the arrow is of appropriate size to represent a transcript initiated upstream from gene IV, extended beyond the rho-dependent terminator in the intergenic region, and terminated at the rho-independent terminator following gene VIII (Model, 1988). **B:** Plasmids with IPTG-inducible transcription units containing sequences corresponding to wild-type RNA H and an amber mutant early in gene VIII were introduced by electroporation (Dower et al., 1988) into the $\Delta pcnB$ strain SK7988. Cultures of the strains in LB broth containing 50 $\mu\text{g}/\text{mL}$ thymine were grown to a density of 2×10^8 cells/mL. Immediately after removal of the first sample, IPTG was added to 0.2 mM, and 3 min later, rifampicin was added to 200 $\mu\text{g}/\text{mL}$. Samples were removed at the indicated times after IPTG induction and the RNAs analyzed in parallel on the same 2% agarose/2% formaldehyde gel. The hybridization probe for both **A** and **B** was as in Figure 2. The positions of the RNA H equivalents (H' and AmH') and RNA T are indicated. It was noted in the complementation experiments that the presence of wild-type pVIII was toxic to the host. The ~ 3 -fold higher level of transcription from the amber mutant construct relative to the wild-type presumably reflects a higher plasmid copy number tolerated by the host in the absence of toxic pVIII.

site and delay inactivation of the coding region for the major coat protein. This finding supports other results suggesting that ribosomes generally stabilize mRNA (reviewed in Petersen, 1993) and that untranslated *lacZ* mRNA is rendered unstable (lost & Dreyfus, 1995). In the specific case of ribosomal protein S15 mRNA, Régnier and colleagues (Braun et al., 1998) have recently shown that cleavage at the 3'-most RNase E cleavage site just downstream from the stop codon is also inhibited by ribosomes.

DISCUSSION

As part of defining the steps of bacterial processing and decay pathways illustrated by the major phage f1 mRNAs, the roles of polyadenylation and nucleolytic cleavage have been explored. In the steady state, a small percentage of the mRNAs were found to bear poly(A) tails that were attached to heterogeneous ends near the common 3' end generated by rho-independent termination. Deletion of the *pcnB* gene encoding poly(A) polymerase I resulted in stabilization of the later-appearing processed mRNAs, indicating that polyadenylation is involved in part in their degradation. Importantly, polyadenylation appears to be required for

turnover of RNA T, a ~ 125 -nt RNA bearing the structured 3' end that is removed from the f1 mRNAs by RNase E-dependent cleavage. Under normal conditions ribosomes translating gene VIII protect this cleavage site from RNase E, such that release of the fragment bearing the terminator occurs very slowly. Inactivation of RNase E resulted in marked defects in processing, stabilization of the large primary transcripts and RNase E-independent cleavage products, and persistence of the structured 3' end on the f1 mRNAs. Taken together, the results of this and earlier studies (Kokoska et al., 1990; Kokoska & Steege, 1998) indicate that RNase E, poly(A) polymerase, and translational activity in gene VIII are the primary factors responsible for the overlapping pattern of mRNA species observed in the steady state in phage-infected hosts.

From the data presented, pathways for processing and decay of the f1 mRNAs can be diagrammed as shown in Figure 9. Following synthesis, a small amount of the large primary transcripts may be subject to cleavage by RNase III. However, most of the mass moves through the RNase E-dependent cleavage pathway, presumably in the degradosome. In view of evidence that 5'-terminal triphosphate groups inhibit RNase E cleavage (Lin-Chao & Cohen, 1991; Mackie, 1998), the initiating RNase E cleavages probably occur some-

FIGURE 9. Model for the processing and decay pathways of the phage f1 mRNAs as described in the text. Major steps in the pathways are denoted by arrows with solid lines, and minor steps are indicated by arrows with dashed lines. Diagrams of the RNAs are as in Figure 1.

where in the 5'-proximal region of gene II/X. Although these sites have not yet been mapped precisely, our finding that RNA A is stabilized more than RNA B following inactivation of RNase E (Kokoska & Steege, 1998; this work) suggests that the first cleavages occur in sequences unique to RNA A. Apparent movement in a 5' → 3' direction then occurs slowly over a period of ~10 min to generate upstream products that are degraded quickly (Kokoska et al., 1990) and the longer-lived processed mRNAs. Over the course of the endonucleolytic pathway, as much as 30% of the processed mRNA species are degraded via the poly(A)-dependent 3' exonucleolytic decay pathway, but most persist because of their resistance to exonuclease digestion. The final cleavage removes the structured 3' end from RNAs G and H. RNA T, the downstream product bearing the terminator stem-loop, then requires polyadenylation and 3' → 5' exonuclease digestion for its turnover. By contrast, the 3' end of the upstream product from this cleavage was not found to be a site of poly(A) addition (Fig. 3). This may suggest that coordinated digestion by PNPase in the degrado-

some rapidly degrades such products before they become accessible to poly(A) polymerase.

At least three features of these processing and decay pathways are used to establish and maintain the persistent filamentous phage infection in its bacterial hosts. First, although RNAs A and B are transcribed constitutively, their relative stabilities are determined by RNase E cleavage. We have shown earlier (Kokoska & Steege, 1998) that the resulting steady-state levels of the transcripts are required to set the appropriate ratio of the proteins encoded by in-frame overlapping genes II and X, which have opposing functions in DNA replication. Cleavage of RNA A functions to limit the yield of the larger protein pII, and conditions that change the stability of RNA A lead to an aberrant distribution of phage DNA species. Second, the RNase E cleavage pathway at later times generates processed mRNAs with longer half-lives. These provide abundant templates for synthesis of the two proteins needed in highest amounts during the life cycle, pV and pVIII. Once pV reaches the proper concentration, it acts both as the switch to sequester newly synthesized single-

stranded DNA for assembly into phage particles and as a translational repressor to turn down synthesis of pII and pX. Finally, the observed delay in the RNase E cleavage that releases RNA T permits the major coat protein pVIII to be produced from an intact coding region throughout the entire 15–20-min latent period prior to the large initial burst of 1,000 phage particles that occurs during the first generation following infection (Webster, 1996).

Our observations suggest that 3' ends on primary transcripts and perhaps the upstream products of endonuclease cleavage are only slowly accessed by poly(A) polymerase following their synthesis. In the poly(A) polymerase-deficient host, neither the primary transcripts nor the products of RNase E-independent cleavage were stabilized appreciably, probably because they were processed endonucleolytically before becoming subject to polyadenylation. RNAs G and T, on the other hand, were the species stabilized most markedly. RNA G appears 5–6 min after the large primary transcripts, and RNA T becomes detectable 5 min later. Both RNAs have 3' ends identical to the ends of the other f1 mRNAs, but they are distinct in being the longest-lived remaining from transcription. These results place polyadenylation at a later rather than an initiating step of decay. The data obtained here for the f1 mRNAs concur with the conclusion drawn from studies of ribosomal protein S20 mRNA that polyadenylation does not play a general role in initiating the decay of most mRNAs (Coburn & Mackie, 1998). RNA T, however, is an exception that probably falls into a class of tightly structured RNAs like RNA-OUT (Pepe et al., 1994) that are not substrates for RNase E. Such RNAs therefore appear to depend on poly(A) polymerase and 3'-exonucleases to initiate their decay.

The accumulation of RNA T in $\Delta pcnB$ strains underscores the degree to which stably structured RNA 3' ends act as barriers to 3' $\rightarrow$ 5' exonucleolytic mRNA decay. In view of how many mRNAs have stem-loop structures at their 3' ends, the cell is likely to contain in the steady state a pool of small fragments like RNA T that are in need of exonuclease digestion for turnover. The most stable structures, such as the terminator stem-loops in RNA-OUT (Pepe et al., 1994) and ribosomal protein S20 mRNA (Coburn & Mackie, 1996a), have been shown to be substantially resistant to PNPase and completely resistant to RNase II, which accounts for 90% of the exonuclease activity in *E. coli* extracts (Deutscher & Reuven, 1991). Polyadenylation of S20 mRNA cannot overcome the resistance of the 3' end to RNase II (Coburn & Mackie, 1996a). Instead, it appears from in vitro reconstitution studies (Coburn & Mackie, 1998) that continuous rounds of hydrolysis to the base of the stem-loop, stalling of the exonuclease, and displacement by poly(A) polymerase to add a 3' extension are required to degrade S20 mRNA. This is likely to be the case as well for other mRNA fragments

with thermodynamically stable structures at their 3' ends. Our in vivo studies of the f1 mRNAs provide support for these features of mRNA decay. Over the course of RNase E-dependent processing, the cleavage products become subject to polyadenylation, but a large fraction of the mRNAs remain stable as long as the distal segment bearing the terminator stem-loop is present. The heterogeneity of the poly(A) addition sites we observe is strong evidence for reiterative events in the region of the common 3' end, with competition between poly(A) polymerase and the residual RNase II remaining at 42 °C in the *pnp-7 rnb-500^{ts}* host. The low level of polyadenylation observed in the steady state in the *pnp-7* host (Fig. 2) very likely reflects the normal dynamic process as well, because RNase II is present along with poly(A) polymerase to continuously remove newly synthesized poly(A) tails.

If the f1 mRNA processing/decay pathway is compared with the others defined in detail thus far, it becomes evident that the mode and overall appearance of a pathway is determined primarily by the position of the initiating endonucleolytic cleavage. With the initiating cleavages occurring in the 5'-proximal region of the large f1 primary transcripts, a characteristic 5' $\rightarrow$ 3' wave of cleavage products is generated (Fig. 9). By contrast, the initiating RNase E cleavage in the S20 mRNA occurs approximately two-thirds of the way from the 5' end. Cleavage here yields an upstream fragment that is sensitive to exonuclease digestion and a downstream product containing numerous stable structures. This is degraded to mononucleotides in vitro in a series of steps requiring poly(A) polymerase, PNPase, ATP, and phosphate (Coburn & Mackie, 1998). Finally, decay of the S15 mRNA is initiated by an RNase E cleavage removing the terminator hairpin stem-loop at the 3' end that protects the mRNA from exonuclease attack while it is being translated (Régnier & Hajnsdorf, 1991; Braun et al., 1998). Once the small fragment is removed, the primary mode of decay is exonucleolytic digestion in the 3' $\rightarrow$ 5' direction. As is the case for the f1 mRNAs and expression of the genes they encode, the S15 mRNA decay pathway probably has a significant role in appropriately regulating the synthesis of the ribosomal protein. Because S15 autoregulates its expression by translational repression at a site in the promoter-proximal region of the mRNA, rapid 3' $\rightarrow$ 5' degradation of the mRNA under conditions of S15 excess prevents further mRNA accumulation, titration of repressor, and continued gene expression (Braun et al., 1998).

MATERIALS AND METHODS

Bacterial strains

Nucleic acid manipulations were performed using standard protocols (Sambrook et al., 1989; Ausubel et al., 1994). The

principal primers used are listed in Table 2; others were generated as needed for sequencing. The principal *E. coli* strains were ribonuclease- or poly(A) polymerase-deficient strains made male for infection with the filamentous phage. Genotypes and sources were as follows: DS213: F⁺::Tn10/*thyA715* λ⁻ (MG1693, *E. coli* Genetic Stock Center, Yale University); DS214: F⁺::Tn10/*leu thr pnp-7* (SK5002, S. Kushner); DS215: F⁺::Tn10/*leu thr pnp-7 rnb-500^{ts}* pDK39 [*rnb-500^{ts}*] (SK5003 (Donovan & Kushner, 1986)); DS216: F⁺::Tn10/*thyA715 ΔpcnB* [SK7988 (O'Hara et al., 1995)]; DS217: F⁺::Tn10/*thyA715 rne-1* [SK5665 (Arraiano et al., 1988)]; DS218: F⁺::Tn10/*thyA715 rne-1 ΔpcnB* [*ΔpcnB* derivative of DS217 via P1 transduction (Liu & Parkinson, 1991)]; and DS219: F⁺::Tn10/*nadB4 rpsL rnpA49 rnc-105 rne-3071* [N3525 (Apirion & Gitelman, 1980)].

mRNA isolation

Infection of hosts with f1 strain R-111 was at a multiplicity of infection (MOI) = 50 using cultures at 2×10^8 cells/mL. Growth of overnight cultures of temperature-sensitive strains was at 30 °C in LB broth supplemented with 50 μg/mL thymine and antibiotics as necessary. Subcultures grown at 35 °C were shifted to 37 °C or 42 °C 15 min after infection, and samples (5 mL) were removed at various times after the temperature shift. Growth of other strains was at 37 °C throughout. Isolation of nucleic acids was generally by rapid cell lysis at 65 °C in 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 0.5% SDS and immediate extraction with phenol equilibrated to 65 °C (Kokoska et al., 1990). Following two additional extractions with phenol, the nucleic acids were precipitated twice with ethanol, dried, and resuspended in 10 mM Tris-HCl and 1 mM EDTA, pH 7.5. RNA extracted using Catrimox-14 as described (O'Hara et al., 1995) was used for purification of poly(A)-containing RNA. RNA bearing poly(A) tails was purified on streptavidin magnetic beads as specified by the supplier (PolyATtract Systems, Promega). After denaturing the RNA (300–800 μg) in 0.5 mL dH₂O at 65 °C for 10 min, 150 pmol biotinylated oligo(dT) probe was added and the solution was adjusted to 0.5× SSC. When cooled to room tempera-

ture, the RNA solution was applied to a 0.6-mL aliquot of streptavidin magnetic beads previously washed three times in 0.5× SSC and resuspended in 0.1 mL 0.5× SSC. After incubation at room temperature for 10 min, the beads and annealed oligo(dT) hybrids were captured and washed four times with 0.1× SSC. Elution of poly(A) RNA was in RNase-free H₂O.

Northern blot hybridization

RNA samples in 10 μL 50% formamide, 10 mM MOPS, pH 7, 2.5 mM Na acetate, 0.5 mM EDTA, 5% glycerol, and 0.01% bromophenol blue were heated at 60 °C for 3 min and quickly chilled. They were fractionated on agarose/formaldehyde gels containing 2% (w/v) agarose, 2% (v/v) formaldehyde, 20 mM MOPS, pH 7.0, 5 mM Na acetate, 1 mM EDTA, and 0.5 μg/mL ethidium bromide and run in the same buffer containing 1% (v/v) formaldehyde. Transfer to Gene ScreenPlus, hybridization with 5–6 × 10⁷ cpm-labeled probe, and wash steps were carried out as recommended by the membrane supplier (NEN Life Science Products). The 445-nt DNA used as the standard hybridization probe contained f1 nucleotide positions 1198–1542 (Hill & Petersen, 1982). It was labeled in PCR reactions containing [α -³²P]dATP or [α -³²P]dCTP (400 or 3,000 Ci/mM, 6.25 μM), the remaining dNTPs at 12.5 μM, 0.50 μM each primer, 1 ng template, and 2.5 U AmpliTaq (Perkin Elmer). After heating at 94 °C for 2 min, samples were amplified 35 cycles (1 min each at 94 °C, 50 °C, and 72 °C), with the final extension at 72 °C for 10 min. Following extraction steps and precipitation in the presence of 20 μg/mL glycogen (Boehringer Mannheim) as carrier, the DNA was heated in 0.5 × TBE (TBE is 89 mM Tris-borate, pH 8.3, and 2.5 mM EDTA), 45% formamide, and 0.01% bromophenol blue for 3 min at 95 °C and chilled quickly. The DNA strands were separated on 5% polyacrylamide gels (acrylamide:bisacrylamide ratio 37:1, w/w) containing 0.5 × TBE as described (Maniatis et al., 1982). The 250-nt internal standard (M) included f1 nucleotide positions 1198–1429 and 18 noncoded A residues at the 3' end. During PCR amplification of this DNA, the primer for the undesired strand (f1 1327) carried a

TABLE 2. Principal primers used in this study.

Name	Sequence ^a	Restriction site
f1 1198	5'-GGTCACAtATGAGTGTTTATGTC	<i>NdeI</i>
f1 1327	5'-pTTTTTTTTTTTTTTTTTTTCGGTCGCTGAGGCTTGC	
f1 1542	5'-CCTTTAATTcTATCcGTTTATCAGC	
Bamoligo(dT ₁₈)	5'-CGGGATCCCGTTTTTTTTTTTTTTTTTTT	<i>Bam</i> HI
f1 1336	5'-CGTTGgTACCCTCGTTCCGATGCTGTC	<i>KpnI</i>
f1 1416	5'-CCTCAGgtACCGAATATATCGTTATGCG	<i>KpnI</i>
5' 9S	5'-GCAGAAGCGGTCTGATAAAACAG	
3' 9S	5'-GTCTTTTCTGACTGAGCCTTTTCG	
f1 1368	5'-GCGAAAGACAGCATCGGAACG	
f1 1442	5'-GCGTGGGCGATGGTTGTTGTC	
f1 1492	5'-CAGCTTGATACCGATAGTTGCGCC	
f1 1189	5'-ccATCGaTGGGGGTCAAAGATGAGTG	<i>Clal</i>
f1 1533	5'-GTATCtagaTATCAGCTTGCTTTTCGAGG	<i>XbaI</i>
I ^Q	5'-CGAAGCGGCATGCATTACG	<i>Clal</i>
Trc	5'-GTGatcgaTTGTTATCCGCTCACAATTCC	

^aLower case letters indicate substitutions introduced to generate mutations or restriction sites (underlined).

5' phosphate so that the strand could be digested with lambda exonuclease (GibcoBRL). Digestions were carried out for 30 min at 37 °C in 67 mM glycine-NaOH, pH 9.4, 2.5 mM MgCl₂, 50 µg/mL BSA, and 1 U lambda exonuclease. The desired single strand was purified as described above. After labeling, double-stranded probes were purified on Elutip columns (Schleicher and Schuell).

PCR amplification and localization of polyadenylation sites

RT-PCR was used to prepare double-stranded DNA, with cDNA synthesis essentially as described (Hajnsdorf et al., 1995). Total RNA (20 µg) from f1-infected DS215 (*pnp-7 rnb-500^{ts}*) cultures was denatured in 20 µL 5 mM Tris-HCl, and 0.5 mM EDTA at 100 °C for 3 min and chilled on ice. Following addition of 50 pmol BamIgo(dT₁₈), the 25-µL sample was incubated at 25 °C for 10 min and then an additional 10 min in 80 mM KCl. The RNA-primer hybrids were extended in 50-µL reactions containing 50 mM Tris-HCl, pH 8, 10 mM MgCl₂, 4 mM dithiothreitol, 80 mM KCl, 0.5 mM each dNTP, and 2 or 18 U RAV-2 reverse transcriptase (Amersham). Reactions were incubated sequentially for 10 min at 25 °C, 10 min at 42 °C, and 40 min at 50 °C. cDNA preparations were quantitated by slot blot analysis as specified by the Gene ScreenPlus supplier (NEN Life Science Products). The hybridization probe was a 5'-³²P-labeled 29-mer (f1 1416 primer). Amplification of cDNAs employed the oligo(dT) primer containing the BamHI site and a 27-mer containing f1 nucleotide positions 1336–1362 (f1 1336) or a 29-mer containing positions 1416–1444 (f1 1416). Amplification was carried out for 30 cycles in 100 µL 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM each dNTP, and 2.5 U AmpliTaq. Amplification conditions were 92 °C/1.5 min, 36 °C/2 min, and 72 °C/2 min. After the first 5 cycles, the annealing temperature was increased to 45 °C. Following extraction with a mixture of phenol/chloroform/isoamyl alcohol (25:24:1), ethanol precipitation, and digestion with KpnI and BamHI, the amplification products were cloned into the appropriate sites in phagemid pTZ18U (Pharmacia). Single-stranded DNAs produced in JM109 (F' *traD36 lacI^Q lacZΔM15 proA⁺ B⁺/e14⁻* (McrA) *Δ(lac-proAB) recA1 thi gyrA96 endA1 hsdR17 relA1 supE44 λ⁻*) or JM101 (F' *traD36 lacI^Q lacZΔM15 proA⁺ B⁺/Δ(lac-proAB) supE thi*) (Yanisch-Perron et al., 1985) superinfected with M13KO7 (New England Biolabs) were sequenced using the M13 (–40) primer (Amersham Life Science).

Construction of inducible transcription units bearing the RNA H sequence

Plasmids bearing the RNA H sequence under inducible control were generated by assembling the necessary elements in a derivative of pSP73 (Pharmacia) that contained the f1 rho-independent terminator oriented such that inserts would be transcribed in the same direction as β-lactamase (R.J. Kokoska and D.A. Steege, unpubl. results). PCR products containing f1 nucleotide positions 1187–1529 or the M13 equivalents were cloned into plasmid digested with XbaI and ClaI. A 1,460-nt PCR product from pTrc99A (Pharmacia) bearing the *lacI^Q* repressor and the Trc promoter was then inserted

into plasmid digested with ClaI and SphI to give wild-type pTrcHTer and the amber mutant pTrcAmHTer. The structures of the final constructs were verified by dideoxy sequencing. The sequences differ from RNA H as follows: the 5' leader is longer by 17 nt from Trc and 8 nt from f1 sequence normally upstream from the RNA H startpoint, and the junction of the H insert with the terminator element has a 4-nt substitution. The functional properties of the constructs were confirmed by complementation experiments with the M13am8H1 phage using 0.01 mM IPTG to induce the transcription unit (Haigh & Webster, 1998). M13am8H1 was obtained from R. Webster and propagated in strain S26r1eλ⁻ (Hfr Cavalli *phoA4*(Am) *serU132 λ⁻*). The amber mutation in gene VIII is a G → T change at amino acid position 25 (Van Wezenbeek et al., 1980). The region of M13 including gene VIII has one G → A substitution from the f1 sequence at nucleotide position 1403; an additional change substituting proline 29 of gene VIII with serine (C → T) at position 1385 was noted. Transcription from the plasmids in the *ΔpcnB* strain (SK7988) was induced by the addition of IPTG to 0.2 mM. After a pulse of 3 min, rifampicin was added to 200 µg/mL and samples were withdrawn over time.

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