Scarce adenylation in bacteriophage T4 mRNAs

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The degradation of mRNA is crucial for the rapid shift of bacteriophage T4 gene expression from early to late. The present study was conducted to investigate whether T4 mRNA is polyadenylated or not, because polyadenylation is known to facilitate the degradation of mRNA in Escherichia coli cells. Total RNA extracted from T4-infected cells was subjected to self-circularization or intermolecular ligation by T4 RNA ligase, and a region containing the 3'-5' junction was amplified by RT-PCR. Cloning and sequencing as well as the length distribution of amplified DNA fragments revealed no adenines at the 3'-ends of uvsY and soc RNAs. The present result suggests that T4 mRNA is not significantly adenylated.

INTRODUCTION

Bacteriophage T4 expresses its genes in a highly ordered manner after infection to Escherichia coli cells. Many scores of T4 genes are expressed sequentially at early, middle, or late stages of infection (O'Farrell and Gold, 1973; Christensen and Young, 1984; Kutter et al., 1994b). Differential transcription from stage-specific promoters (Mossig and Hall, 1994) primarily controls the sequential expression. The shifts of T4 gene expression from early to late are, however, achieved in a period as short as 20 min (O'Farrell and Gold, 1973; Christensen and Young, 1984; Kai et al., 1996). Such rapid shifts could be sustained by degradation of mRNAs at the following stages in addition to the differential transcription. The role of mRNA degradation in a control of T4 gene expression is emphasized by the recent discovery of dmd gene, which specifies mRNAs for degradation in a stage-dependent manner (Kai et al., 1996; Ueno and Yonesaki, 2001). Mudd et al. (1990) reported that degradation of T4 mRNA is considerably slowed down by a mutation in E. coli rne gene, which encodes RNAse E that is considered to be a major determinant of host mRNA degradation. When host mRNA degradation is impaired, the pool of ribonucleotides in the cells would be smaller than in normal cells. Then, upon infection to RNAse E-deficient cells, the transcription of T4 genes might be affected by a limitation of ribonucleotide pool. Since the degradation of T4 mRNA in a stage-dependent manner would require timely and quantitative expression of genes at former stages (Kai et al., 1996; Ueno and Yonesaki, 2001), it may be possible that an rne mutation affects T4 mRNA degradation via gene expression. Furthermore, RNAse E also takes a part in processing of various functional RNAs such as ribosomal RNAs (Ghora and Apirion, 1978; Li et al., 1999; Wachi et al., 1999), tRNAs (Li and Deutscher, 2002; Ow and Kushner, 2002), the RNA subunit of RNase P (Lundberg and Altman, 1995) and ssrA RNA (Lin-Chao et al., 1999), all of which are essential for normal activity of protein synthesis and its quality control. Therefore, it is not clear whether the effect of RNAse E on T4 mRNA turnover is direct or indirect.

When a dmd mutant of T4 phage infects at low temperatures, many late genes are silenced because of rapid degradation of mRNA (Kai et al., 1996). Our recent analysis suggests that a host cell encodes the activity responsible for the dmd mutant-specific degradation of late-gene mRNA. However, E. coli endoribonucleases, RNases III, E, G, and P, all of which can initiate the degradation of mRNA in the host (Kushner, 1996; Umitsuki et al., 2001), are unlikely to cause the T4 late-gene silencing (Y. Otsuka, H. Ueno and T. Yonesaki, unpublished). Thus, the determinant of T4 mRNA degradation in a dmd mutant still remains uncovered.

Polyadenylation at the 3' end of RNA facilitates degradation of mRNAs (O'Hara et al., 1995; Hajnsdorf et al., 1995; Ingle and Kushner, 1996; Mohanty and Kushner, 1999) as well as RNA fragments (Xu and Cohen, 1995; Hajnsdorf and Regnier, 1999) in E. coli. Interestingly, Sarker and his colleagues reported that bacteriophage T7 mRNA was polyadenylated (Johnson et al., 1998). T4 phage is known to utilize host machines, such as RNA polymerase and ribosome, with or without modification for the benefit of its own gene expression (Kutter et al., 1994b; Mossig and Hall, 1994). These facts raise a possibility that T4 mRNA might be degraded by a mechanism via polyadenylation. However, knowledge about polyadenylation of T4 mRNA has been entirely lacked. In the present study, I attempted to settle this issue.
MATERIALS AND METHODS

RNA isolation. E. coli MH1 (Kai et al., 1996) cells were grown to a density of 5 × 10^8 cells/ml in M9 minimal medium supplemented with 0.3% casaminoacids, 1 µg/ml thiamine and 20 µg/ml tryptophan, and infected at 30°C at a m.o.i. (multiplicity of infection) of 10 with T4 phage. Because a middle gene ussY and a late gene soc are expressed during different periods, RNA was extracted from cells at 10 min after infection for the preparation of ussY RNA or at 20 min for soc RNA. Total RNA was prepared as described previously (Kai et al., 1996).

RNA ligation. Couttet et al. (1997) reported that self-circularization of an RNA molecule was useful to analyze both the 3′- and 5′-terminal structures of eukaryotic mRNA simultaneously. In the present study, this method was modified according to the following two aspects: First, prokaryotic mRNA is cap-less and has a triphosphate at its 5′-terminus. In order to convert the 5′-termini to be ligatable, the triphosphate was removed by phosphatase and then monophosphate was added to the 5′-OH. Second, for analysis of ussY RNA (see Results), total RNA was subjected to intermolecular ligation instead of self-circularization. Briefly, the 5′-end of mRNA was dephosphorylated by incubating total RNA with calf intestine alkaline phosphatase (Toyobo co., ltd.) and phosphorylated by T4 polynucleotide kinase (RNase block II, Stratagene) with calf intestine alkaline phosphatase (Toyobo co., ltd) corresponding to the nucleotide position from 15335 to 15354 of T4 DNA, for soc and 5′-ggcttgctgataattcag (ussY3′), corresponding to 114553-144572 of T4 DNA, for ussY. Before use, all primers were phosphorylated by T4 polynucleotide kinase. Amplified DNA was cloned into a vector pBlue-script II KS+ (Stratagene) by blunt-end-ligation.

RESULTS

Polyadenylation of mRNA can be assessed by reverse transcription with oligo(dT) as a primer, depending on annealing of oligo(dT) to 3′-poly(A) tail of mRNA, and subsequent PCR with synthesized cDNA as a template. Although this method is convenient, it was rather avoided in this study. T4 DNA is rich in A and T residues, comprising 66% of total residues, and has many A-clusters; for example, more than 6 consecutive runs of A exist at 140 sites in 169-kb T4 DNA. Such A-clusters would allow annealing of oligo(dT) to an endogenous region of transcript as well as 3′-poly(A) tail, making analysis complicated. Furthermore, a comprehensive method is needed to show, if required, the absence of polyadenylation. RNA self-circularization method (circularization-RT PCR) developed to analyze terminal structures of eukaryotic mRNA (Couttet et al., 1997) is quite adequate to this demand. Briefly, the 3′ and 5′ ends of a linear RNA molecule are joined by T4 RNA ligase, and a region containing the 3′-5′ junction is converted to cDNA by reverse transcriptase. Successively, the cDNA is amplified by PCR with one primer close to the 3′-end and the other to the 5′-end. Cloning and sequencing of amplified DNA fragments definitively demonstrate the number and species of nucleotides between the 3′- and 5′-ends of a given transcript.

For circularization, two termini of an RNA molecule should be close enough to be ligated. Since a short species would be eligible for the reaction, I chose a late gene soc mRNA, which is only 300 nucleotides long (Kutter et al., 1994a). After ligation, total RNA was subjected to reverse transcription and synthesized cDNA was utilized as a template for PCR. Polyacrylamide gel electrophoresis in Fig. 1A shows an example of thus amplified DNA. The amplified DNA containing the 3′-5′ junction of circularized soc mRNA was expected to be 218 bp, assuming that transcription of soc gene was started from its own late promoter and ceased after 6 Us following the stem-loop of transcription terminator (Kai and Yonesaki, 2002).
and that its 3'-end was not adenylated. In addition, a 159-bp DNA was also expected. The soc gene can be transcribed even at early and middle stages from 0.8- and 1.2-kb upstream early and middle promoters. These transcripts, however, suffer from a truncation of 59 nucleotides at the 5'-terminus of soc mRNA, and the truncated soc RNA is so stable as to remain abundant even at late stages (Kai and Yonesaki, 2002). Two major DNA bands indicated by letters F and T in Fig. 1A corresponded to the expected two species, and we called the longer DNA soc DNA. Other fragments longer than 400 bp would be artifacts, presumably resulting from mismatched annealing of primers.

Redundant clones carrying a DNA fragment derived from an identical soc RNA molecule could be avoided as follows. After reverse transcription, cDNAs were divided into portions and each portion was utilized for amplification by PCR as described in Materials and Methods. Amplified DNAs were electrophoresed through a 5% polyacrylamide gel. The figure shows representative results. An arrow with a letter F or T in (A) indicates the DNA expected for self-circularized soc mRNA or 5'-truncated soc RNA, respectively. An arrow in (B) indicates the DNA expected for ligated RNA of uvsY RNA and 16S rRNA. HaeIII-digest of pBluescript II KS+ DNA was run in the right lane as size markers.

The primary transcript from uvsY gene is 0.9 kb long. After RNA ligation and reverse transcription, PCR with primers close to the 3' and 5' ends of uvsY mRNA did not amplify any significant DNA fragment. This result suggests that two termini of uvsY mRNA are not close enough for ligation (see Discussion). Therefore, intermolecular ligation instead of self-circularization was intended to analyze the 3' terminal sequence of uvsY mRNA. The 3'-end of uvsY mRNA was ligated to the 5'-end of a distinct RNA species, 16S rRNA, and a sequence at their junction was cloned for analysis. Ribosomal 16S RNA was chosen from two reasons; first, it is very abundant and second, its 5'-end is considered to be unpaired (Gutell, 1994). In order to stimulate intermolecular ligation, the concentration of total RNA in the reaction was raised by 20-fold in comparison to that for self-circularization. cDNA generated from ligated uvsY mRNA-16S rRNA was then amplified by PCR with one primer close to the 3'-end of uvsY mRNA and the other to the 5'-end of 16S rRNA. Fig. 1B shows thus amplified DNA (Y16S DNA) as indicated by an arrow. Assuming that transcription of uvsY gene is terminated with 6 Us after a stem-loop and that the 3'-end is not adenylated, a 254-bp DNA fragment was expected after amplification (Gutell, 1994; Kutter et al., 1994a). The Y16S DNA had a mobility of such DNA. To avoid redundant clones carrying Y16S DNA originated from an identical uvsY mRNA molecule in the same manner as described above, cDNAs were divided into portions, each of which was utilized as a template for PCR, and only one clone carrying Y16S DNA was selected for each amplification. Seven clones were obtained and their plasmids, pY-EC16S#1 ~ #7, were analyzed for sequence at the 3'-5' junction of circular soc mRNA (Fig. 2A). Five out of these plasmids exhibited 5 Us at the 3'-end, suggesting the major transcription termination site. All of seven plasmids demonstrated the absence of A residues between the 5'-terminal nucleotide and the 3'-U cluster of soc mRNA. Similarly, four plasmids selected for the 5'-truncated soc mRNA (band T in Fig. 1A) also showed the absence of adenine residues downstream of 3'-U cluster (data not shown).

The circularization-RT PCR method was extended to soc mRNA of the dmd mutant. Sequencing of cloned csocDNA without redundancy revealed unexpected feature of soc mRNA from the mutant (Fig. 2B). Among six clones (pCdSOC#1 ~ #6), five had an extra sequence (see Discussion). Interestingly, all extra sequences were derived from the sequence upstream of the late transcription start site. This result strongly suggested that these clones were derived from soc RNA transcribed from upstream early and middle promoters and processed. All six clones showed the absence of adenine residues downstream of 3'-U cluster.

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Us at the 3'-end of uvsY mRNA, suggesting the major transcription termination site. All seven clones showed no A residues between the 5'-terminal nucleotide of 16S rRNA and the 3'-U cluster of uvsY mRNA.

The above results strongly suggested the tendency of soc and uvsY mRNAs to lack adenylation. A small number of molecules analyzed would be, however, inappropriate to deduce an accurate proportion of adenylated to non-adenylated mRNA. In addition, the collection of only one clone from each DNA preparation by PCR must have tended to exclude a minor population and/or the result might be influenced by feasibility for cloning of a DNA fragment among those in different lengths. In order to display even a minor population, i.e. adenylated soc mRNA, PCR was performed as in Fig. 1A and amplified DNAs ranging from 210 to 280 nucleotides in length, corresponding to soc DNA and longer DNA, were eluted from polyacrylamide gel and analyzed for their length distribution. Eluted DNA was heat-denatured and used as a template for DNA synthesis primed by a $^{32}$P-labeled primer. Then, labeled and extended strands were electrophoresed through a sequencing gel (Fig. 3A). As a reference, $^{32}$P-labeled strands synthesized with pCSOC#5 as a template were run in parallel. The reference strands were almost homogeneous in length and longer by one nucleotide than the cloned soc sequence in pCSOC#5, because of addition of an adenine at the 3'-terminus of synthesized strand by Taq DNA polymerase (Hemsley et al., 1989). On the other hand, a majority of $^{32}$P-labeled strands derived from csoc DNA and longer DNA had a length shorter by one nucleotide than the reference strand. This result was reconciled with the result in Fig. 2A; among cloned csoc DNAs, pCSOC#5 DNA was the only one that had 6 Ts at the 3'-5' junction of circularized soc mRNA, while other five clones had 5 Ts and another one 4 Ts. Adenylation at the 3'-end of soc mRNA should give rise a ladder above the bands originated from non-adenylated csoc DNA. As seen in Fig. 3A, no such a ladder was found and only less than 5% of radioactivity associated with non-adenylated DNA was detected for putative adenylated species.

The 3'-terminal length distribution of uvsY mRNA was also estimated in the same manner (Fig. 3B). After PCR, 250 to 320-nucleotides long DNAs including Y16S DNA were eluted from polyacrylamide gel and used as a template for DNA synthesis primed by a $^{32}$P-labeled primer.
In this study, cDNAs were run to see an accurate distribution of products in lane 3, and 5-fold amounts of cDNAs were run to detect any adenylated species (lane 1). A majority of $^{32}$P-labeled strands synthesized with the eluted DNA as a template migrated at the same rate as a reference strand, which was synthesized with pYC16#4- or 16S5' primer for soc RNA or (B) ligated uvsY RNA-16S rRNA. After reverse transcription of total RNA from T4-infected cells, cDNA was utilized for amplification by PCR as described in Materials and Methods. After amplified DNAs were electrophoresed through a 5% polyacrylamide gel, DNA fragments in a size range of 210–280 bp for soc and of 250–320 bp for uvsY were eluted from gel, heat-denatured and utilized for the length analysis. The length analysis was performed as follows. The soc-up primer or 16S5' primer for uvsY RNA was labeled at its 5'-end with $^{32}$P using T4 polynucleotide kinase, annealed to heat-denatured DNA and subjected to chain-elongation by Taq DNA polymerase at 72°C for 160 sec. The chain-elongation reaction was carried out in a mixture containing 50 mM Tris-HCl (pH 8.3), 3 mM MgCl$_2$, 250 µg/ml bovine serum albumin, 2% sucrose and 0.2 mM each of four deoxyribonucleotides. The products were heat-denatured and analyzed through a 5%-polyacrylamide gel containing 6 M urea (lanes 1 and 3). Preparation of reference strands was as follows: pCSOC#5 and pY-EC16#4 DNA was used as a template for PCR with a respective set of primers (soc-dw and soc-up for soc, and uvsY3' and 16S5' for uvsY). The amplified fragments were heat-denatured and used as a template for strand elongation in the same manner as above. The reference strands were run in parallel (lanes 2 and 4). Sequence ladders obtained by dideoxynucleotide method (Innis et al., 1988) with either a combination of pCSOC#5 and soc-up primer or a combination of pY-EC16#4 and 16S5' primer were also run in each left four lanes (G, A, T, C) of figure A or B, respectively. In the left margins, sequences at the border of cloned DNA and vector are shown.

**DISCUSSION**

In this study, I analyzed three stage-specific transcripts of T4 phage; the 5'-truncated soc RNA which was transcribed from early and middle promoters, the uvsY RNA transcribed from a middle promoter, and the full-length soc mRNA which was transcribed from a late promoter. It is reported that very few E. coli mRNAs (less than 2%) have polyadenylated ends (Cao and Sarker, 1992; Mohanty and Kushner, 1999). Like E. coli mRNAs, most molecules of three T4 RNA species, if not all, are not adenylated. In fact, a mutation in pcnB gene encoding a major poly(A) polymerase in E. coli (Lopilato et al., 1986; Cao and Sarker, 1992) did not significantly affect the growth of wild-type T4 phage (data not shown). These results suggest that bacteriophage T4 does not encode its own poly(A) polymerase nor stimulate host poly(A) polymerase activity. The dmd gene is required for T4 growth especially at low temperatures such as 30°C, and many late genes of this mutant are silenced because of rapid mRNA degradation (Kai et al., 1996). Since T4 RNA was isolated after infection at 30°C for analysis in this study, the present results suggest that the rapid degradation of late-gene mRNA in a dmd mutant may not be mediated by polyadenylation of mRNA. Consistent with this notion, the pcnB mutation was not able to alleviate the growth defect of a dmd mutant (data not shown).

Circularization is a useful method to analyze both the 3' and 5'-terminal structures of RNA simultaneously. However, it should be noted that the application of this method may not be general at present. A DNA fragment was not amplified by circularization-RT PCR of uvsY RNA. This failure can be attributable to the inability of uvsY RNA to be circularized, because the primer used in this trial was proven to prime DNA synthesis efficiently.

**Fig. 3.** The length distribution of the 3'-5' junction of (A) circular soc RNA or (B) ligated uvsY RNA-16S rRNA. After reverse transcription of total RNA from T4-infected cells, cDNA was utilized for amplification by PCR as described in Materials and Methods. After amplified DNAs were electrophoresed through a 5% polyacrylamide gel, DNA fragments in a size range of 210–280 bp for soc and of 250–320 bp for uvsY were eluted from gel, heat-denatured and utilized for the length analysis. The length analysis was performed as follows. The soc-up primer for soc RNA or 16S5' primer for uvsY RNA was labeled at its 5'-end with $^{32}$P using T4 polynucleotide kinase, annealed to heat-denatured DNA and subjected to chain-elongation by Taq DNA polymerase at 72°C for 160 sec. The chain-elongation reaction was carried out in a mixture containing 50 mM Tris-HCl (pH 8.3), 3 mM MgCl$_2$, 250 µg/ml bovine serum albumin, 2% sucrose and 0.2 mM each of four deoxyribonucleotides. The products were heat-denatured and analyzed through a 5%-polyacrylamide gel containing 6 M urea (lanes 1 and 3). Preparation of reference strands was as follows: pCSOC#5 or pY-EC16#4 DNA was used as a template for PCR with a respective set of primers (soc-dw and soc-up for soc, and uvsY3' and 16S5' for uvsY). The amplified fragments were heat-denatured and used as a template for strand elongation in the same manner as above. The reference strands were run in parallel (lanes 2 and 4). Sequence ladders obtained by dideoxynucleotide method (Innis et al., 1988) with either a combination of pCSOC#5 and soc-up primer or a combination of pY-EC16#4 and 16S5' primer were also run in each left four lanes (G, A, T, C) of figure A or B, respectively. In the left margins, sequences at the border of cloned DNA and vector are shown.
in both reverse transcription and PCR. For circularization, both ends of RNA molecule should be close enough to be ligated. The two ends of wusY RNA molecule would be unsuitably separated by a high order structure. In this connection, the result of soc RNA from dmd mutant is very suggestive of circularization. Primer extension analysis as well as Northern blot analysis reveal the full-length late soc RNA as a detectable species and no species corresponding to one having an extra 5' sequence (Kai and Yonesaki, 2002). Nevertheless, most clones obtained after circularization-RT PCR had an extra 5' sequence. This striking bias would be a reflection of differential feasibility for RNA circularization.

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