BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 46–50 (1997)
ARTICLE NO. RC977421

Stationary Phase-Specific mRNAs in *Escherichia coli* Are Polyadenylated

Gong-jie Cao and Nilima Sarkar[1]

Boston Biomedical Research Institute, 20 Staniford Street, Boston, Massachusetts 02114, and Department of Molecular Pharmacology and Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

Received September 2, 1997

Polyadenylation of *Escherichia coli* specific mRNAs has so far been studied primarily during the exponential phase of growth. As part of an investigation of the polyadenylation of *E. coli* mRNAs in different physiological contexts, we studied mRNA polyadenylation in stationary phase by preparing a cDNA library from stationary phase RNA using oligodeoxythymidylate primers and analyzing the nucleotide sequence of cDNA clones corresponding to the stationary phase-specific genes, *rpoS, bolA,* and *dps.* The sites of polyadenylation were found to be primarily in the 3′-untranslated region, either at the putative rho-independent transcription termination site (*dps*) or at several different sites upstream of the putative rho-independent terminator. A few examples of polyadenylation within the coding regions were also found, suggesting that nucleolytic degradation often preceded polyadenylation. In contrast to the poly(A) tracts characteristic of exponentially growing cells, many of the uncoded poly(A) tracts associated with stationary phase mRNA were interspersed with other nucleotide residues. The observation of post-transcriptional polyadenylation of specific stationary phase mRNAs in *E. coli,* some of which are transcribed by the RNA polymerase associated with σ^s, demonstrates that mRNA polyadenylation is not confined to the exponential phase of growth.

Prokaryotic mRNA is generally polyadenylated at the 3′-ends like eukaryotic mRNA except that the poly(A) tracts of prokaryotic mRNAs are somewhat shorter, ranging from 15-60 adenylate residues (1,2). The first molecular characterization of a bacterial poly(A) RNA was achieved by the cloning of cDNA complementary to *lpp* mRNA (3). About the same time, the major poly(A) polymerase of *Escherichia coli* (PAPI) was cloned and shown to be encoded by *pcnB* locus (4). A second *E. coli* poly(A) polymerase (PAPII) has also been cloned from a PAPI-deleted strain of *E. coli* (5). These findings have laid the ground for investigations of the biological function of poly(A) tracts in bacterial mRNA. A fundamental question that needs to be addressed by such investigations is whether all types of mRNA are polyadenylated or only certain functionally related subclasses.

All studies on the polyadenylation of *E. coli* mRNA to date have been on growing cultures, when RNA synthesis is carried out by RNA polymerase programmed by σ^{70} (6), except for one report on the transition from growth to early stationary phase (7). Another sigma factor, σ^s, controls the expression of more than 30 operons that are expressed specifically during stationary phase of *E. coli* growth. The transition from exponential growth to stationary phase therefore offers a unique opportunity for determining whether polyadenylation of mRNAs occurs under conditions when the metabolic rate is exceedingly slow compared to that in exponential phase (8,9). To this end, we have examined the polyadenylation of mRNA encoded by stationary phase genes whose transcription involves σ^s. The results described in this paper show that these are indeed polyadenylated during stationary phase, but that the non-coded poly(A) tracts differ from those seen in mRNA derived from exponentially growing cells in that they are interspersed with other nucleotides.

MATERIALS AND METHODS

Materials. Radiochemicals were obtained from New England Nuclear. Restriction endonucleases and T4 DNA ligase were from New England Biolabs; M-MLV Superscript II reverse transcriptase from GIBCO/BRL; RNasin from Promega; Taq DNA polymerase from Perkin Elmer/Cetus; guanidinium thiocyanate from Fluka. Oligonucleotides were synthesized by the phosphoramidite method with a MilliGen Expedite DNA synthesizer.

Bacterial strain, growth and processing. E. coli ZK765 (W3110 *tna2* Δ*lacU169*/*pJE100*) was obtained from Roberto Kolter; the plasmid pJE100 is a pBR322 derivative containing the entire *dps* gene

[1] To whom correspondence should be addressed. Fax: (617) 523-6649. E-mail: sarkar@bbri.harvard.edu.

0006-291X/97 $25.00

(10). *E. coli* was grown in LB at 37°C on a rotary shaker from a 1.5% inoculum and cells were harvested after 27 h. For the isolation of RNA, the cultures were supplemented with 25 mM sodium azide and 160 μg/ml chloramphenicol and mixed with an equal volume of frozen medium to achieve rapid cooling, just before harvesting by centrifugation.

RNA isolation. Cells from 150-ml cultures were suspended in 1 ml of 10 mM Tris · HCl, pH 7.5, 1 mM EDTA and 10 mM NaCl with 1 mg lysozyme and frozen at −70°C and thawed at room temperature three times to lyse the cells. Total RNA was isolated from the freshly harvested and lysed stationary phase cells using a single-step method involving acid guanidinium thiocyanate-phenol-chloroform extraction as described (11).

cDNA synthesis. Total RNA was used as template for M-MLV Superscript II reverse transcriptase with 5′-CTGCCTGCAGGATCCCCGGG$(T)_{20}$, which contains a *Bam*H1 restriction site (underlined), as the primer and buffer supplied by GIBCO/BRL. Incubation for cDNA synthesis was done sequentially for 10 min at 25°C, 10 min at 42°C, and 60 min at 50°C. Kinetics of cDNA synthesis were measured by the incorporation of [α-^{35}S]dATP into acid-insoluble radioactivity. cDNA was isolated from the incubation mixture by phenol/chloroform extraction, followed by ethanol precipitation.

Amplification and cloning of cDNAs for the stationary phase specific genes rpoS, bolA and dps. Aliquots of cDNA (10 pmole nucleotide) were used separately as PCR templates, using as amplification primers the common downstream primer, 5′-CTGCCTGCAGGATCCCCGG, corresponding to the primer used for cDNA synthesis, which contains a *Bam*HI site but with different upstream amplification primers selected from specific gene sequences in the coding regions of interest. For *rpoS*, the gene for the stationary phase sigma factor (12), the upstream primer was 5′-GATGAATTCAGCCGTATGCTTCGT, corresponding to the sequence near residue 654 of the 1086-bp gene, except that two bases were changed to generate an *Eco*RI site (underlined). For amplifying the cDNA for *dps*, the gene for the DNA-binding protein from starved cells (10), the upstream primer was 5′-CGCTGAATTCATTGCCGT, corresponding to the sequence near residue 107 of the 504-bp gene, again with two bases changed. For the morphogene *bolA*, which is induced during the transition from growth to stationary phase (13), the upstream primer was 5′-GGCGGAATTCCAACCCGTATTCCTCGA, near the amino terminal coding region of the gene with two bases changed starting at residue 69 of the 348-bp gene. Amplification involved 25 cycles, with denaturation for 1 min at 95°C, annealing for 2 min at 55°C, and extension for 3 min at 72°C. After the polymerase chain reaction, the amplification mixture was treated for 30 min with proteinase K (50 μg/ml) at 37°C, extracted with phenol/$CHCl_3$ in the presence of ethidium bromide and 5 M ammonium acetate, and precipitated with ethanol. The precipitated DNA was digested with *Eco*RI and *Bam*HI, fractionated by electrophoresis in 1% low-melting-temperature agarose gel, and the bands of interest were excised and ligated to *Eco*RI and *Bam*HI-digested pUC18 or pBlueScript KS(+) for transformation of *E. coli* DH-5α or *E. coli* XL-1 (Stratagene), respectively.

DNA sequence analysis. The nucleotide sequence of the cDNA clones was determined by the dideoxy chain-termination method with Sequenase Version 2.0 from U.S. Biochemicals using the conditions specified by the manufacturer. Double-stranded templates were first denatured with alkali. For cDNA clones in pUC 18 primers, GTTGTAAAACGACGGCCAGT and AGGGTTTTCCCAGTCACGAC were used as sequencing primers. For cDNA clones in pBlueScript KS(+), the sequencing primers AATACGACTCACTATAG and ATTAACCCTCACTAAAG were used.

RESULTS

Oligo(dT)-Dependent cDNA Synthesis from Stationary-Phase RNA

Although the rate of RNA synthesis in stationary-phase cells is significantly reduced (7), the relative rates of synthesis of total mRNA and of poly(A) RNA, measured by labeling with [α-^{35}S]ATP in permeabilized cells, were similar in exponential growth and 27 h after reaching stationary phase (G. Cao and N. Sarkar, unpublished data). It should therefore be possible to identify specific polyadenylated mRNAs in stationary phase *E. coli* by oligo(dT)-dependent cDNA synthesis. Total RNA was isolated from a stationary-phase culture using a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture (11) to avoid potential degradation of poly(A) tracts that might accompany more lengthy multi-step procedures (3). The yield was about 11 μg of total RNA per ml of bacterial culture, which was used directly as template for cDNA synthesis (14) with an oligo(dT) primer-adapter combination as described in Materials and Methods. The amount of cDNA synthesis was 392 pmole of SS-DNA per 80 μg of total RNA, which was an appreciable yield considering that the mRNA content in total RNA is probably less than 4%.

Cloning and Sequence Analysis of DNA Complementary to Specific Stationary-Phase Genes

To analyze the pattern of polyadenylation of stationary phase-specific genes, we selected three well-characterized genes functionally related to stationary phase growth of *E. coli.* One of these was *rpoS*, the gene coding for the stationary phase sigma factor, σ^s, which controls many genes whose expression is induced during entry into stationary phase (8). *rpoS* is the promoter-distal gene in an operon together with *nlpD;* the two *nlpD* promoters contribute to *rpoS* expression only during exponential growth but the major *rpoS* transcripts start at a position within the *nlpD* gene during entry into stationary phase (15). A *rho*-independent terminator sequence immediately downstream of *rpoS* indicates the possibility of polyadenylation at the 3′ end of mRNA. The other two stationary phase-specific genes selected were *dps* (10) and *bolA* (13) which are expressed by σ^s, the *rpoS* gene product. The *dps* gene appears to be transcribed as a monocistronic mRNA, whose product is the stationary phase-specific DNA-binding protein, Dps, which protects DNA against oxidative damage (10). The *bolA* gene which is also monocistronic and yields an mRNA with a 3′-terminal stem-loop structure, is involved in the stationary-phase morphogenic pathways of *E. coli* (13). To facilitate the cloning of DNA complementary to polyadenylated *rpoS, dps,* and *bolA* mRNAs, the cDNAs were amplified by the polymerase chain reaction using appropriate primers. These consisted of gene-specific primers corresponding to regions well within the coding sequence as the upstream primers and a common downstream primer, corresponding to the extension of the oligo(dT) primer used for cDNA synthesis. The amplified products were purified and cleaved with *EcoRI* at a site

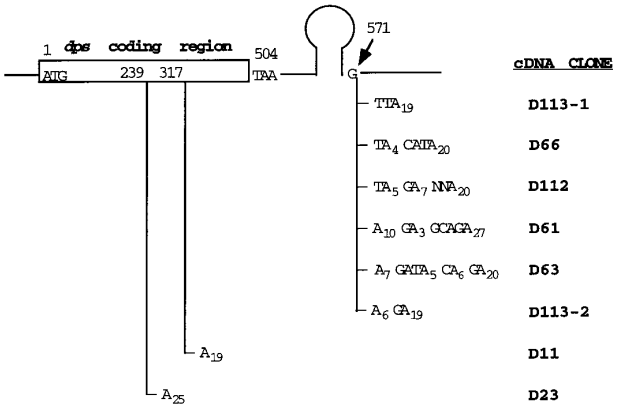

FIG. 1. Sites of polyadenylation in *dps* mRNA. cDNA clones were obtained and their nucleotide sequence was determined as described under Materials and Methods. The coding sequence is identified by the box and the stem-loop of the putative Rho-independent terminator is shown schematically. The sites of polyadenylation in the *dps* poly(A) RNAs are indicated by numbers and the uncoded post-transcriptionally added nucleotides at the 3′-end are shown.

within the upstream primer of each gene and with *Bam* HI at a site within the downstream primer and ligated into the corresponding sites of pBlueScript KS(+) or pUC18. Numerous colonies were obtained after transformation of the appropriate *E. coli* host, of which representatives were checked for plasmids with inserts of appropriate size for each gene in preparation for DNA sequencing.

Sequences of cDNA clones derived from *dps* gene mRNA are shown in Fig. 1. The site of polyadenylation in six of the eight clones was at nucleotide 571 in the 3′-untranslated region just after the 3′-terminal stem-loop structure, a putative Rho-independent terminator. The other two clones were polyadenylated at nucleotides 239 and 371 within the dps coding region. The length of the uncoded A-rich sequence ranged from 19 to 45 nucleotides. While two of the clones, D11 and D23, had pure poly(A) tracts, the others had A-rich tracts interspersed with 4-11% of other nucleotides.

The 11 cDNA clones corresponding to the *rpoS* gene are shown in Fig. 2. In 9 of these clones, the site of polyadenylation was in the 3′-untranslated region but before the putative stem-loop structure beginning at nucleotide #1088, with noncoded A-rich tracts ranging from 18 to 44 nucleotides. In the two other clones, the site of polyadenylation was within the C-terminal portion of the rpoS coding region. While some of the clones had pure poly(A) tracts, more than half had up to 17% other nucleotides interspersed among the adenylate residues.

The five cDNA clones corresponding to *bolA* mRNA are presented in Fig. 3. The sites of polyadenylation were rather diverse, ranging from within the bolA coding region to either before or after the putative stem-loop structure in the 3′-untranslated region. Three clones had pure poly(A) tracts, while the others had uncoded A-rich sequences interspersed with other nucleotides.

DISCUSSION

Although an impressive body of information on the physiology and regulation of gene expression during stationary phase of *E. coli* is rapidly accumulating (8,16), comparatively little is known about mRNA metabolism during this stage of growth. The results presented here show that mRNAs specifically synthesized in stationary phase are polyadenylated. The conclusion that mRNA polyadenylation plays a role in all phases of bacterial growth is consistent with the earlier observation in the gram-positive bacterium, *B. subtilis*, that polyadenylation of mRNA persists during sporulation, albeit at slightly lower levels than in vegetative cells (17). Comparison of the yield of cDNA obtained from stationary phase *E. coli* cells (1.6 ng per μg total RNA) with that obtained earlier from exponentially growing cells (2.6 ng per μg total RNA) (14), suggests that the extent of mRNA polyadenylation is reduced at most 2-fold during transition from growth to stationary phase.

Nucleotide sequence analysis of the cDNA clones corresponding to the three stationary phase-specific genes, *rpoS*, *dps*, and *bolA*, revealed several different types of polyadenylation sites, all of which also occur in mRNA from exponentially growing cells (2,3). The predominant site of polyadenylation of *dps* mRNA was just downstream of the terminal stem-loop structure. This type of poly(A) RNA has been termed Class I and probably reflects the polyadenylation of the primary transcript at the site of rho-independent termination (3). In contrast, the polyadenylation sites of *rpoS* mRNA were primarily just upstream of the terminal stem-loop structure. This pattern of polyadenylation, termed Class II, occurs most frequently in the exponential phase poly(A) RNAs studied and presumably represent polyadenylation near the sites of cleavage of endonucleases such as RNase E (3). *bolA* mRNA was polyadenylated either considerably downstream of the terminal stem-loop structure, perhaps near the site of rho-dependent termination, characteristic of Class III poly(A) RNA (2), or in the 3′-untranslated region upstream of the terminal stem-loop [Class II poly(A) RNA]. In addition, some of the cDNAs for all three stationary phase genes were polyadenylated within the coding sequences. Polyadenylation of truncated mRNA is also observed during growth [Class V poly(A) RNA; ref. (2,18)] and may be a reflection of indiscriminate polyadenylation of mRNA degradation products. The observation that similar types of mRNA polyadenylation patterns are observed in growing and in stationary phase *E. coli* cells suggest that the general features of 3′-end processing of mRNA are maintained during the transition from growth to stationary phase.

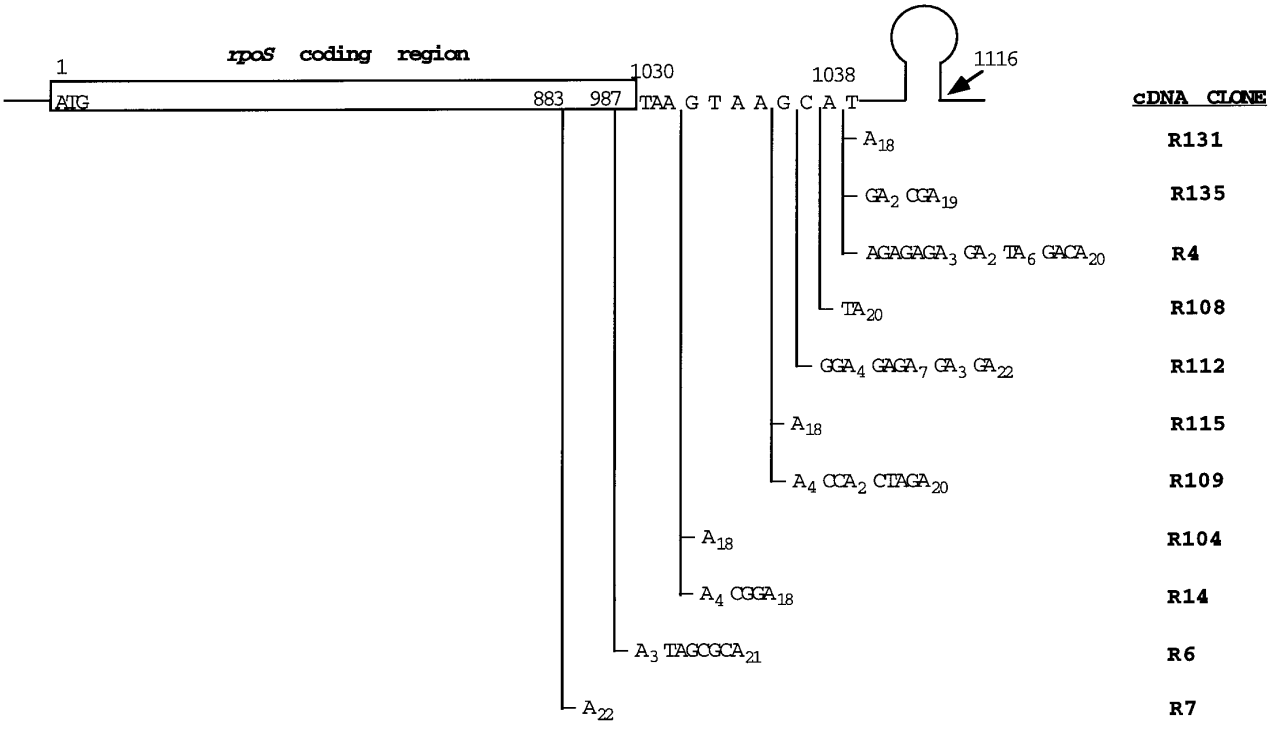

FIG. 2. Sites of polyadenylation in *rpoS* mRNA. The symbols and numbers are the same as those in Fig. 1.

On the other hand, our results did reveal a significant difference between poly(A) RNAs from growing and stationary phase cells. This concerned the composition of the non-coded poly(A) tract, which in growing cells consists solely of adenylate residues (2,3,19), whereas in stationary phase mRNAs, the poly(A) tracts were often interspersed with other nucleotides, to an extent which ranged from 0 to 17 %, with all three ribonucleotides being represented (Figs. 1-3). It is known that PAPI can incorporate CMP and UMP residues at less than 5% of the level of AMP incorporation but not GMP residues to any significant extent (20). The enzymatic properties of PAPII with respect to low level incorporation of other nucleotides have not been studied. It is possible that an alteration in the sizes of the riboncleoside triphosphate pools relative to ATP in stationary phase could lead to nucleotide misincorporation. On the other hand, the reduced nucleotide specificity of polyadenylation could be the result of modification of host poly(A) polymerases. The modification of RNA polymerases during stationary phase is not unprecedented, the phosphorylation of RNA polymerase core being one example (21). A third possibility is that there may be specific poly(A) polymerase for stationary phase genes with reduced nucleotide specificity. Our recent analysis of T7 mRNAs by cDNA cloning and sequencing of the poly(A) tracts has also revealed this type of poly(A) sequences interspersed with other

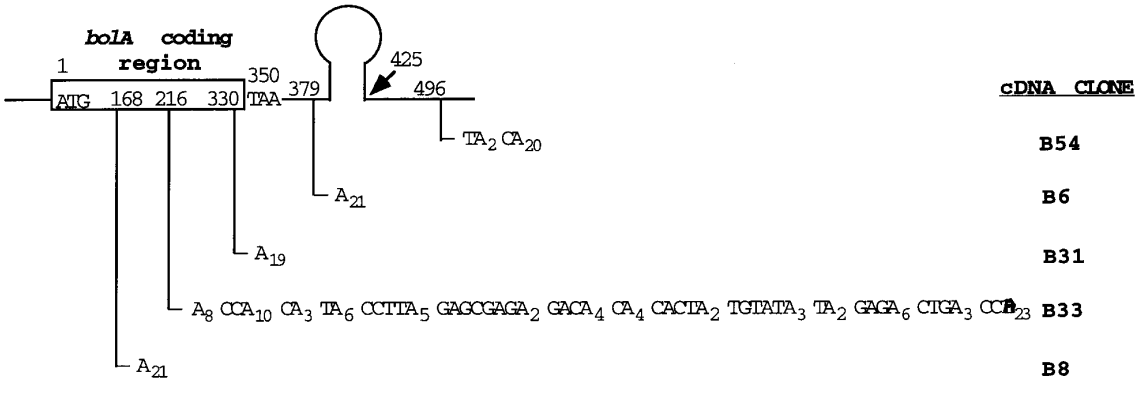

FIG. 3. Sites of polyadenylation in *bolA* mRNA. The symbols and numbers are the same as those in Fig. 1.

nucleotides (unpublished observation). In addition, poly(A) tracts interspersed with other nucleotides have been found in chloroplast *pbsA* mRNA (22). The elucidation of the cause of this interesting difference in the poly(A) tracts associated with mRNA in growing and in stationary phase cells, which is also observed under certain other circumstances, may give insights into changes in ribonucleotide and mRNA metabolism that occur during the transition from growth to stationary phase. Considering the economics of stationary phase cultures (16) when efficient energy generation is at a premium, the fact that 20-40 adenylates are added to mRNA molecules at the expense of considerable amounts of ATP would be unexpected unless the 3′-terminal poly(A) tracts had an important biological function.

ACKNOWLEDGMENTS

This work was supported by grant GM26517 from the National Institutes of General Medical Sciences. We thank Dr. Henry Paulus for stimulating discussions and critical reading of the manuscript.

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