Effects of 3′ Terminus Modifications on mRNA Functional Decay during in Vitro Protein Synthesis*

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The penB gene, which encodes the principal poly(A) polymerase of Escherichia coli, promotes 3′-polyadenylation and chemical decay of mRNA. However, there is no evidence that penB-mediated mRNA destabilization decreases protein synthesis, suggesting that polyadenylation may enhance translational efficiency. Using in vitro translation by E. coli cell extracts and toeprinting analysis of transcripts encoded by the chloramphenicol acetyltransferase (CAT) and β-galactosidase genes to investigate this notion, we found no effect of poly(A) tails on protein synthesis. However, we observed that 3′-polyguanylation delayed the chemical decay of CAT mRNA and, even more dramatically, increased the ability of CAT mRNA to produce enzymatically active full-length protein in 30 S E. coli cell fractions. This resulted from interference with the primary mechanism for inactivation of CAT transcript function in cell extracts, which occurred by 3′-exonucleolytic degradation rather than endonucleolytic fragmentation by RNase E. Using bacteriophage T7 RNA polymerase to install poly(G) tails on mRNAs transcribed from polymerase chain reaction-generated DNA templates, we observed sharply increased synthesis of active proteins in vitro in coupled transcription/translation reactions. The ability of poly(G) tails to functionally stabilize transcripts from polymerase chain reaction-generated templates allows proteins encoded by translational open reading frames on genomic DNA or cDNA to be synthesized directly and efficiently in vitro.

Polyadenylation of RNA at the 3′-end is now known to occur in prokaryotic organisms as in eukaryotes (for reviews, see Refs. 1 and 2). The addition of poly(A) tails to bacterial RNA leads to accelerated RNA degradation by polynucleotide phosphorylase (PNPase)† (3–6) and possibly other 3′- to 5′-exonucleases. However, the only biological consequence of slowing RNA decay by impeding polyadenylation demonstrated thus far is altered control of plasmid DNA replication (6). Escherichia coli penB mutants that are deficient in poly(A) polymerase I (7) show stabilization of RNAI (the antisense repressor of replication for ColE1-type plasmids), inhibition of plasmid DNA replication, and sharply decreased plasmid copy number. Although the failure to add poly(A) tails can also stabilize a variety of mRNA species in E. coli (8–10), enhanced synthesis of proteins encoded by these RNAs has not been reported in penB mutant bacteria, raising the possibility that poly(A) tails may, while accelerating the decay of mRNAs, also lead to a compensatory increase in translational efficiency.

During experiments aimed at testing the above idea by analyzing the effects of poly(A) tails and other types of 3′-transcript ends on RNA translation in E. coli cell extracts, we observed surprisingly that short tracts of G nucleotides at 3′-termini interfere with the ribonucleolytic step responsible for inactivation of the ability of mRNA to function in translation in vitro, enabling the use of poly(G) tails as a tool to investigate mechanisms of mRNA functional decay. Our results indicate that poly(G) tails prolong mRNA functional half-life in E. coli extracts by interfering with 3′-5′-degradation, implying that inactivation of mRNA function occurred by this process rather than by endonucleolytic fragmentation during in vitro translation. The ability of poly(G) tails to functionally stabilize mRNA, together with our ancillary discovery of a method for efficiently installing poly(G) tails on transcripts synthesized by T7 RNA polymerase from polymerase chain reaction (PCR)-amplified DNA templates, has yielded an approach for the in vitro synthesis of proteins encoded by translational open reading frame sequences on genomic DNA or uncloned cDNA.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—**E. coli B strain BL21 (F−, hsdS, gal, OmgT−) was initially used for S-30 preparation when mRNAs encoding chloramphenicol acetyltransferase (CAT) and LacZ were used in in vitro translation. SL119 (11), an recD (12) derivative of BL21, was used to prepare S-30 for a coupled transcription/translation system. For the preparation of 30 S ribosomal subunits, E. coli K12 strain CA244 (lacZ, trp, relA, spoT) (13) was used. All plasmids were maintained in E. coli DH5α (supE44, hsdR17, recA1, endA1, gyrA96, thi-1, F−, ΔlacY1ΔargF1U169, deoR [Δ800lacA (lac25M15)], p synaptic) and cloning into the BamHI site of pET3a (Novagen). pET3a-CAT was constructed by amplifying the DNA sequence encoding the LacZ fragment (amino acids 1–94) from chromosomal DNA of E. coli strain N443 (lacZ43, relA, spoT, thi-1) (15) using oligonucleotides 5′-5′-ACAGATGATGATGGTGAGAAAATCACTGGA) and cloning them into the BamHI site of pET3a. Plasmid pLAC-RNE2 is a derivative of pPP30 (17) that directs conditional synthesis of a full-length carboxyl-terminal-tagged form of LacZ mediated by a UV5 promoter. A hexa-histidine-affinity tag was inserted right before the stop codon of the RNase E gene, and a stronger ribosome binding sequence was incorporated upstream of the RNase E coding sequence (5′-CGGCCCGCAGAGGTTACAGTG, the ribosome binding sequence is underlined and the start codon is in bold type). Plasmid pGL3Basic was purchased from Promega.
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from Promega, Madison, WI, and pSE371 was a gift from Dr. George Jones (18). Plasmid pTH90 was a gift from Dr. Alexander von Gabain (19).

Enzymes and Reagents—Avian myeloblastosis virus reverse transcriptase, T7 polynucleotide kinase, T7 RNA polymerase, and the restriction enzymes EcoRI and HindIII were from New England Biolabs (Beverly, MA). The oligonucleotides were from Life Technologies, Inc. [γ-32P]ATP (6000 Ci/mmol), [α-32P]UTP (3000 Ci/mmol), [α-32P]CTP (3000 Ci/mmol), [3H]chloramphenicol (38.9 Ci/mmol), and ECL detection kit were from PerkinElmer Life Sciences. Anti-T7-tag antibody horseradish peroxidase (HRP) conjugate and T7-tag affinity purification kit were from Novagen. M1 antibody was from Eastman Kodak Co., and anti-rabbit IgG conjugated with HRP was from Promega. Polyclonal antibodies against E. coli RNAPase were a gift from Dr. A. J. Carpousis. Other chemicals and tRNAs were purchased from Sigma.

S-30 Preparation and Reactions—An E. coli-coupled transcription/translation system (S-30) was prepared from E. coli strain BL21 essentially as described by Lesley et al. (11). mRNAs containing 20 A tails were used to determine the optimal concentration of CAT and LucZa mRNA in reactions. Optimal protein production was observed at 120 nmol mRNA for both mRNAs. Coupled transcription/translation reactions were incubated at 37 °C for 1 h in reaction mixtures containing 1 μg of agarose gel-purified DNA, unless otherwise indicated.

Synthesis of DNA and RNA—All mRNAs used in this study were synthesized using the MEGAscript™ T7 kit (Ambion, Austin, TX) and PCR DNA as a template according to the manufacturer's instructions. 9 S ribosomal RNA was synthesized from HaeIII-cut plasmid pTH90 using the MEGAscript™ T7 kit (Ambion). RNA was purified from 6% acrylamide gel containing 8 M urea. For in vitro synthesis of CAT and LucZa RNAs containing no 3′-additions or containing A, C, G, or U in vitro, PCR-generated DNAs were prepared using 5′-primer (5′-TAATAGCACTAATATAGG) and 3′-prime (5′-C, 5′-T, or 5′-AAGGCTTGTTACAGCGCCGATCC) and pET3-a or pET3a-CAT as template. PCR DNAs for purified transcription/translation reactions were prepared as follows. First, for PCR DNAs containing CAT coding region, 5′-primer that installs 3′-poly(A) additions would necessarily be re-directed to already completed transcripts. According to the course of its synthesis (28), so that any translational enhancement by 3′-poly(A) nucleotides at the 3′-end were gel-purified and added to an E. coli extract-based reaction mixture for in vitro translation. Transcripts containing twenty 3′-G nucleotides were used as controls. The mRNAs chosen for translation were relatively small (417 and 807 nucleotides for RNA, and the proteins they encode are well characterized.

Within 2 min after the addition of CAP or α-mRNA to in vitro translation reaction mixtures, poly(A) tails 20 or 40 nucleotides in length had been degraded, and decay of primary transcripts that initially had contained poly(A) tails proceeded at the same rate as decay of nonadenylated RNA (Fig. 1, A–D). Consistent with the rapidity of poly(A) tail removal in these E. coli cell extracts, we did not detect an effect of poly(A) tails on overall CAT or LacZa protein synthesis (Fig. 1, E and F). However, we observed during these experiments that poly(G) tails, which had been added to CAT-encoding transcripts as a control, dramatically enhanced the production of CAT and LacZa protein in vitro (Fig. 1, E and F).

Initiation of polypeptide chains is known to be the rate-limiting step in mRNA translation (29–31). Toeprinting as-

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Fig. 1. Effects of different 3'-tails of mRNA on mRNA decay and translation. In vitro translation reaction was carried out at 30 °C using gel-purified, uniformly labeled CAT (A) or lacZa (B) mRNA with 32P-UTP (120 nM) to measure mRNA decay in the reaction. Samples were removed at the times indicated, and mRNA was purified by phenol extraction and ethanol precipitation and analyzed in 6% polyacrylamide gel containing 8M urea. Extraction and ethanol precipitation and analyzed in 6% polyacrylamide gel containing 8M urea.

Fig. 2. Effect of poly(A) tail on the rate of 30 S initiation complex formation. CAT mRNA containing 40 A nucleotides or lacking any 3'-additions were used in primer extension inhibition (toeprinting) assays with varying concentrations of small ribosomal subunits with (30S) or without S1 (30S/S1) protein. B, the toeprinting signal was quantitated as percent toeprinting band relative to the sum of the mature product and toeprinting bands using a PhosphorImager and plotted. A, the portions of the mature products, the toeprinting signal, and the primer bands are indicated. The left four lanes in the panel are sequencing ladders; the same CAT mRNA (no tail) and primer were used in toeprinting assays and sequencing. The location of the Shine-Dalgarno sequence and translation start codon are shown in the CAT sequence depiction. B, symbols used in the graph are: ●, none; ▲, A40; ▼, A60; ●, G40.

transcripts entirely lacking poly(A) tails or containing tails of 40 As showed similar binding efficiency to 30 S ribosomal subunits (Fig. 2B), indicating that 3'-polyadenylation has no detectable effect on the initiation of translation. Additionally, whereas ribosomes depleted of S1, which binds to poly(A) tails and has been speculated to play a role in translation by recruiting the 30 S subunit to poly(A)-tailed mRNA (33), produced an expected decrease in toeprinting signal (34), the magnitude of the decrease was unaffected by the presence or absence of poly(A) tails (Fig. 2).

Effects of Poly(G) Tails on Degradation of CAT mRNA—As seen in Fig. 1, enhancement of CAT protein synthesis in vitro by 3'-polyguanylation is associated with retardation of CAT mRNA chemical decay. However, the effect of poly(G) tails on CAT mRNA functional half-life as reflected by the production of active CAT and LacZa proteins was 4–6 times greater than their effect on the chemical decay of mRNA (cf. Fig. 1, C and D versus E and F). This finding suggested that poly(G) tails might prove useful in investigating the mechanisms involved in mRNA functional decay. The initial step in the chemical decay of a variety of E. coli mRNAs is endonucleolytic cleavage by RNase E, the principal endonuclease of E. coli (for reviews, see Refs. 35 and 36), and poly(G) previously has been found not to affect such cleavage (37). Whereas RNase E cleavage is also the

says, which measure the rate of formation of translation initiation complexes between mRNA and 30 S ribosomal subunits, have proved useful in evaluating factors that affect translation initiation (22). We found that neither poly(A) tails nor primary transcripts were detectably degraded over a 30-min period in vitro in toeprinting assay mixtures that used highly purified ribosomes and reverse transcriptase, allowing the use of toeprinting to test for the possible enhancement of translation efficiency by poly(A) tails. As seen in Fig. 2A, a toeprint signal produced by the binding of CAT mRNA to 30 S ribosomal subunits was detected at a characteristic position (32), 15 nucleotides from the 5'-most nucleotide of the start codon. CAT
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As seen in Fig. 4A, incubation of the 236-nucleotide 9 S transcript in transcription/translation reaction generated the 206-nucleotide and 126-nucleotide fragments characteristically produced by RNase E cleavage. The additional presence of exonucleolytic activity in these extracts was shown by evidence that fragments generated by RNase E cleavage of 9 S RNA were themselves degraded (Fig. 4B) and that the combined radioactivity detected in full-length 9 S RNA and identifiable RNase E-generated fragments of this substrate diminished with time (69% remained after 10 min). Further experiments presented in Fig. 4A specifically confirmed earlier evidence (37) that the rate and pattern of cleavage of CAT mRNA by purified RNase E is not affected by the presence of a 3’-triphosphorylated nucleotide on the substrate (cf. 20G versus 20A).

Installation of Poly(G) Tails on Transcripts Encoded by PCR Products during Coupled Transcription/Translation in Vitro—
The ability of poly(G) tails to significantly protect mRNA from functional decay during in vitro protein synthesis suggested that the installation of poly(G) tails onto mRNA molecules might be useful as a strategy for increasing the yield of active protein in coupled transcription/translation reactions in vitro. If so, we hypothesized, poly(G)-tailed transcripts made by in vitro transcription of PCR-generated templates containing genomic open reading frames (ORFs) potentially could facilitate the synthesis of functional proteins encoded by genes on unfractionated DNA templates. The approach we devised to test this idea involved the synthesis of run-off CAT gene transcripts by the highly efficient bacteriophage T7 RNA polymerase (41). The 5’-primer used to generate the template by PCR installs the bacteriophage T7 promoter (42) near the 5’-end of the template, and the 3’-primer installs a 20-nucleotide stretch of poly(C) at the template’s 3’-end. This was expected to lead to synthesis of homopolymeric G tails on T7-generated transcripts. The E. coli cell extracts employed for these experiments were prepared from an endodeoxyribonuclease-deficient (recD) strain (SL119) to minimize degradation of linear DNA templates.

Using the coupled transcription/translation system described above, no chemical or functional stabilization of CAT mRNA by poly(G) tails was observed. This raised the possibility that synthesis of poly(G)-tailed transcripts by T7 RNA polymerase (RNAP) was not occurring in these E. coli cell extracts.
consistent with earlier evidence (11) that transcription of PCR-generated DNA fragments containing the T7 promoter is mediated by the E. coli RNAP rather than by T7 RNAP in coupled transcription/translation reaction mixtures. In such a case, the inability of the E. coli enzyme to efficiently transcribe homopolymer sequences (43) could lead to the absence of poly(G) tails on transcripts. This interpretation was tested and confirmed by the finding that the addition of rifampicin, an inhibitor of E. coli RNAP but not of T7 RNAP (44), to reaction mixtures sharply decreased protein production in vitro (Fig. 5A). However, in contrast to our results using PCR-generated templates, Nevin and Pratt (45) observed that linearized plasmid DNA containing the T7 promoter was efficiently transcribed in E. coli cell extracts in the presence of rifampicin. We compared the sequence of Nevin and Pratt’s template with ours and found that theirs contained additional base pairs 5’ to the T7 promoter. Thus, these nucleotides are crucial to the ability of the T7 RNAP to initiate transcription on linear DNA templates as shown in Fig. 5. Whereas rifampicin-independent transcription occurred on a template containing 17 base pairs 5’ to the T7 promoter (i.e. the restriction endonuclease-generated BglII-EcoRV DNA fragment), a PCR-generated DNA fragment that included the same promoter but lacked additional upstream base pairs failed to function as a template for T7 RNAP.

Further experiments showed that CAT protein synthesis encoded by transcripts generated by the E. coli RNAP (i.e. those made in the absence of rifampicin) decreased as the length of the template increased (Fig. 5A). Also, as few as 5
Our investigations of a possible effect of poly(A) tails of mRNA on bacterial mRNA translation revealed no evidence that 3'-polyadenylation alters the ability of transcripts to produce proteins in vitro. However, we observed that 3'-poly(G) additions to transcripts can increase the chemical and, even more dramatically, the functional half-life of mRNA in E. coli cell extracts, yielding up to an 80-fold increase in coupled transcription/translation reaction during 1-h incubations (Fig. 6B).

Guanine-rich nucleic acid segments are known to form a structure termed a “G quartet” (46), which commonly is found within telomeres. It was shown previously that G tails inhibit the binding and action of PNPase (5), one of two major 3'-to-5'-exonucleases of E. coli. However, poly(G) tails do not affect cleavage by RNase E (37), the principal endoribonuclease of E. coli, and in our experiments did not alter the rate or pattern of fragmentation of CAT mRNA by RNase E present in the reaction mixtures used for in vitro protein synthesis. Instead, they protected against the C-terminal truncation of protein encoded by the mRNA. Whereas there is substantial evidence that RNase E initiates the chemical decay in vivo of a variety of mRNAs (for reviews, see Refs. 35 and 36) and both the chemical and functional decay of RNA I (38, 39), published evidence for an effect of RNase E cleavage on the functional half-life of mRNA in E. coli cells is lacking. Our conclusion that functional inactivation of CAT mRNA in E. coli cell extracts occurs by a mechanism other than endonucleolytic cleavage is consistent with the finding that mutation of the E. coli rne (formerly known as ans) gene affects bulk mRNA half-life in vivo but not functional decay (40). Nevertheless, interference by poly(G) tails with 3'-to-5'-exonucleolytic decay may not entirely explain their effect on the functional inactivation of mRNAs, as the synthesis of active CAT protein paradoxically was observed to decrease when the length of the tail extended past 15 nucleotides. Additionally, the effect of poly(G) tails on protein synthesis decreased as the length of the primary transcript increased, suggesting that functional decay of CAT mRNA in vitro may not be entirely independent of endonucleolytic cleavage.

We found during our investigations that at least five nonspecific base pairs 5' to the bacteriophage T7 promoter is required for efficient transcription by T7 RNAP. This effect and also the effect of poly(G) tails on mRNA functional half-life were observed for a commercially available transcription/translation reaction mixture (PROTEINscript-PRO™, Ambion) as well as for the E. coli cell extracts we prepared. Using as template a DNA that contained the CAT ORF, a 5'-primer that installed the T7 promoter and additional base pairs at the 5'-end of the PCR-generated CAT ORF-containing template, and a 3'-primer that installed a poly(G) tail on run-off transcripts synthesized by T7 RNA polymerase, our reaction mixtures yielded a level of protein that was comparable with that reported for in vitro protein synthesis systems employing genes cloned on circular plasmid DNA (47).

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