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

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The *pcnB* 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 *pcnB*-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)[1] (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 pcnB* 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 *pcnB* 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*, OmpT⁻) was initially used for S-30 preparation when mRNAs encoding chloramphenicol acetyltransferase (CAT) and LacZα 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,* Δ(*lacIZYA-argF*)*U169*, *deoR* (*ϕ80dlac*Δ (*lacZ*)*M15*)) (14). pET3a-α was constructed by amplifying the DNA sequence encoding the LacZα fragment (amino acids 1–94) from chromosomal DNA of *E. coli* strain N3433 (*lacZ43, relA, spoT, thi-1*) (15) using oligonucleotides 5′-α (5′-ACAGGATCCATGACCATGATTACGGAT) and 3′-α (5′-ACAGGATCCGTGCATCTGCCAGTTTGA) and cloning into the *Bam*HI site of pET3a (Novagen). pET-3a-CAT was constructed by amplifying the CAT gene from pACYC184 (16) using oligonucleotides 5′-CAT (5′-ACAGGATCCAGGAGGCTCGAGATGGAGAAAAAAATCACTGGA) and 3′-CAT (5′-ACAGGATCCTTACGCCCCGCCCTGCCACTC) and cloning them into the *Bam*HI site of pET3a. Plasmid pLAC-RNE2 is a derivative of pPM30 (17) that directs the conditional synthesis of a full-length carboxyl-terminal-tagged form of *E. coli* RNase E under the control of the *lac*UV5 promoter. A hexahistidine-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′-GCGGCCGCAGGAGGTTACG**ATG**, the ribosome binding sequence is underlined and the start codon is in bold type). Plasmid pGL3Basic was purchased

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[1] The abbreviations used are: PNPase, polynucleotide phosphorylase; RNAP, RNA polymerase; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; HRP, horseradish peroxidase; ORF, open reading frame; GPSI, guanosine pentaphosphate synthetase I; TP, telomere binding protein; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

from Promega, Madison, WI, and pJSE371 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, T4 polynucleotide kinase, T7 RNA polymerase, and the restriction enzymes were from New England Biolabs, Beverly, MA. The oligonucleotides were from Life Technologies, Inc. [γ-^{32}P]ATP (6000 Ci/mmol), [α-^{32}P]UTP (3000 Ci/mmol), [α-^{32}P]CTP (3000 Ci/mmol), [^{3}H]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* PNPase 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 LacZα mRNA in reactions. Optimal protein production was observed at 120 nM 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 transcript was synthesized from *Hae*III-cut plasmid pTH90 using the MEGAshortscript™ T7 kit (Ambion). RNA was purified from 6% acrylamide gel containing 8 M urea. For *in vitro* synthesis of CAT and LacZα mRNAs containing no 3′-additions or containing A_{20}, A_{40}, or G_{20} *in vitro*, PCR-generated DNAs were prepared using 5′-primer (5′-TAATACGACTCACTATAGG) and 3′-primer (5′(none, C_{20}, T_{20}, or T_{40})-AAGGCTGTTAGCAGCCGGATCC) and pET3a-α or pET3a-CAT as template. PCR DNAs for coupled transcription/translation reactions were prepared as follows. First, for PCR DNAs containing CAT coding region, 5′-primer that installs the T7 promoter with different lengths of extra base pairs upstream of the T7 promoter and 3′-primer that installs 3′-tails were used to amplify CAT coding region from pET3a-CAT. The 5′-primer was 5′-TAATACGACTCACTATAGG with extra base pairs at the 5′ that are 20 random nucleotides or different lengths of upstream sequence of the T7 promoter present in pET3a-CAT plasmid (AGATCTCGATCCCGCGAAAT), and the 3′-primer was 5′ (complementary sequence of tails)-**TTA**CGCCCCGCCCTGCCA (the stop codon is in bold type). Second, for PCR DNAs containing firefly luciferase gene, the 5′-primer was 5′-GAAATTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGCCACC**ATG**GAAGACGCCA (the consensus T7 promoter is underlined, and the start codon is in bold type), 3′-primers were 5′(complementary sequence of tails)-**TTA**CACGGCGATCTTTCCGCC (the stop codon is in bold type), and the template was pGL3Basic. Third, for PCR DNAs containing G pentaphosphate synthetase I (GPSI), 5′-primer was 5′-GAAATTAATACGACTCACTATAGG (the consensus T7 promoter is underlined), 3′-primer was 5′(complementary sequence of tails)-TACGGGACG**TCA**CTGCTC (stop codon is in bold type), and template was pJSE371. Fourth, the coding region of telomere-binding protein (TP)[2] was amplified from the *Streptomyces rochei* chromosome using 5′-primer (5′-GAAATTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACAT**ATG**GTGGACTCGATCGGAGACGG (the consensus T7 promoter is underlined and the start codon is in bold type) and 3′-primers (5′(complementary sequence of tails)-**CTA**CTTGTCGTCATCGTCCTTGTAGTCCAGCTGGATCTCGATCTG, the stop codon is in bold type and the sequence for FLAG·tag is underlined). S1-depleted 30 S was prepared by the procedure published by Tal *et al.* (20). Briefly, purified 30 S was dialyzed against a low strength buffer (1 mM Tris-HCl, pH 7.5) followed by precipitation of S1-depleted 30 S. S1 protein is the largest of all 30 S ribosomal proteins, and removal of S1 protein in 30 S was confirmed by visualizing 30 S subunit ribosomal proteins in SDS-polyacrylamide gel (data not shown here).

Preparation of 30 S Ribosomal Subunits—30 S ribosomal subunits were prepared essentially as described by Moazed *et al.* (21) except that frozen *E. coli* CA244 cells were opened by passing them through a French press at 10,000 p.s.i. twice, and a 70 S ribosome pellet was washed and resuspended twice in buffer B before being dialyzed against buffer C.

Extension Inhibition (Toeprinting) Assay—Toeprinting assay was performed as described by Ringquist *et al*. (22) using avian myeloblastosis virus reverse transcriptase at 1 unit/reaction. The primers CAT-TP (5′-GGATCCGCGACCCATTTG) and α-TP (5′-GGGTTTTCCCAGTCACGA), which are complementary to CAT and LacZα transcripts, respectively, were 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and purified from 15% acrylamide containing 8 M urea. We determined optimal conditions for binding of the 30 S ribosomal subunits to mRNA using reverse transcriptase amounts ranging from 0 to 1.6 units/reaction and mRNA to primer ratios from 0.25 to 1 (data not shown here). We then tested CAT mRNAs in this assay.

Chloramphenicol Acetyltransferase Assay—CAT activity was determined essentially as described by Nielsen *et al*. (23).

RNase E Cleavage Assay—RNase E cleavage was assayed as described by McDowall *et al*. (24).

Luciferase Assay—Luciferase assay was performed according to manufacturer's instructions, and the enzymatic activity was measured in a TD-20e luminometer (Turner).

Protein Work and Western Blotting—E. coli RNase E was purified from N3433 cells containing pLAC-RNE2 using the His·Bind purification kit (Novagen). The enzyme was eluted from columns using 80 mM imidazole, concentrated, and stored as described previously (24). CAT protein was purified from BL21 (DE3) harboring pET3a-CAT after a 1-h induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside using the T7·tag purification kit according to the manufacturer's instructions. The protein concentration was calculated using Coomassie Brilliant Blue G250 as described by Sedmak and Grossberg (25) and using bovine serum albumin as a standard. Prestained protein molecular weight standards from Life Technologies were used as size markers. Proteins were run on a 10% Tricine-SDS-polyacrylamide gel as described by Schägger and von Jagow (26), and gels were electroblotted to a nitrocellulose filter and probed as described by Hagège and Cohen (27). The dilutions used for antibodies were 1:10,000 for anti-T7·tag-HRP, 1:1000 for anti-FLAG (M2), and 1:5000 for anti-PNPase, anti-mouse IgG-HRP, and anti-rabbit IgG-HRP. When blots were used for reprobing they were stripped at 50 °C for 30 min with occasional agitation in 200 ml of stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) followed by 2× wash in Tris-buffered saline, 0.05% Tween 20 for 20 min.

RESULTS

Effects of Polynucleotide Tails on Translation of Transcripts in Vitro—In *E. coli*, translation begins on nascent mRNA during the course of its synthesis (28), so that any translational enhancement by 3′-poly(A) additions would necessarily be restricted to already completed transcripts. Accordingly, we tested the ability of full-length mRNA containing or lacking poly(A) tails to generate protein *in vitro*. Transcripts encoding CAT or the α fragment of the β-galactosidase (LacZ) protein uniformly labeled with [^{32}P]UTP were synthesized *in vitro* by bacteriophage T7 RNA polymerase using PCR-generated DNA fragments as template (see "Experimental Procedures"). The transcripts, which contained 0, 20, or 40 adenosine (A) nucleotides at the 3′-end were gel-purified and added to an *E. coli* cell 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 α and CAT, respectively), and the proteins they encode are well characterized.

Within 2 min after the addition of CAT 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 LacZα 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 LacZα 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-

[2] Bao, K. and Cohen, S. N. (2001) *Genes Dev.* **15,** in press.

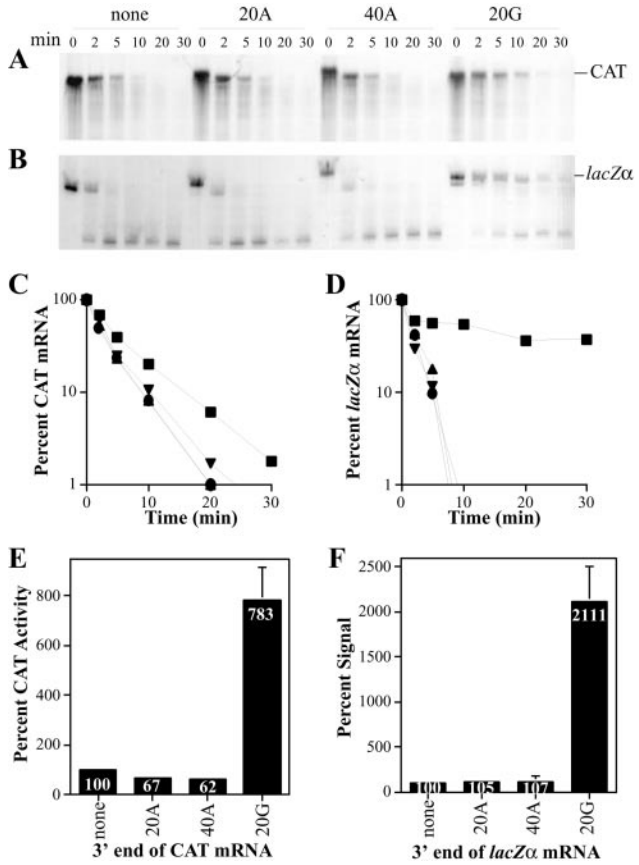

FIG. 1. **Effects of different 3′-tails of mRNA on mRNA decay and translation.** *In vitro* translation reaction was carried out at 30 °C using gel-purified, uniformly labeled CAT (*A*) or *lacZα* (*B*) mRNA with ^{32}P-UTP (120 nM) to measure mRNA decay in the reaction. Samples were removed at the times indicated, and mRNA was purified by phenol extraction and ethanol precipitation and analyzed in 6% polyacrylamide gel containing 8 M urea. *C* and *D,* the amount of full-length mRNA left in each lane was measured using a Molecular Dynamics PhosphorImager and plotted. Symbols used in graphs *C* and *D* are: ●, none; ▲, A_{20} (*20A*); ▼, A_{40} (*40A*); ■, G_{20} (*20G*) . *In vitro* translation was carried out at 30 °C for 30 min for CAT protein production and 10 min for LacZα protein production using *in vitro* synthesized, gel-purified mRNAs (120 nM). Optimal incubation time was determined for maximum protein yield by measuring protein production at 5-min intervals. Ten-min incubation time was used for LacZα production because this peptide was degraded more rapidly in reaction mixtures. *E,* functional CAT protein was assayed as indicated under "Experimental Procedures." *F,* LacZα protein production was assayed by Western blot. Percent CAT activity and percent LacZα signal were obtained by setting values (CAT activity and Western blot signal) from reactions containing mRNAs lacking tails as 100%. The LacZα fragment of β-galactosidase was epitope-tagged with a T7·tag peptide sequence at its N terminus, and anti-T7·tag antibody conjugated with HRP was used in Western blots to detect the fusion protein. The lengths of polynucleotide tails, which were incorporated into T7-generated mRNA molecules during primary transcription, were confirmed by electrophoresis on 6% acrylamide gels by comparing the size of transcripts because of the addition of tails.

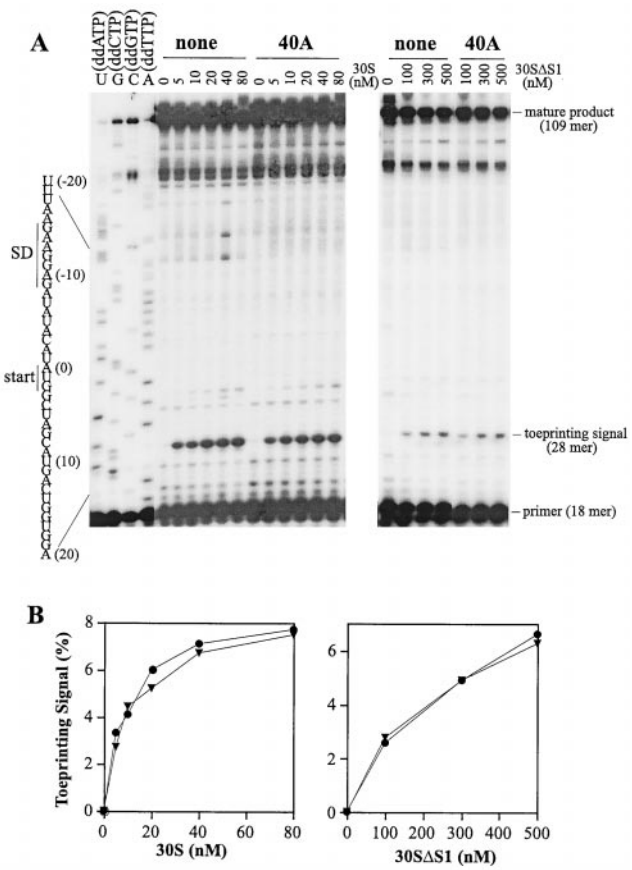

FIG. 2. **Effect of poly(A) tail on the rate of 30 S initiation complex formation.** CAT mRNA containing 40 A nucleotides or lacking any 3′-additions were used in primer extension inhibition (toeprinting) assays with varying concentrations of small ribosomal subunits with (*30S*) or without S1 (*30SΔS1*) 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 a*re 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; ▼, A_{40}.

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. 2*A*, 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 transcripts entirely lacking poly(A) tails or containing tails of 40 As showed similar binding efficiency to 30 S ribosomal subunits (Fig. 2*B*), 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 toeprint 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 LacZα 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

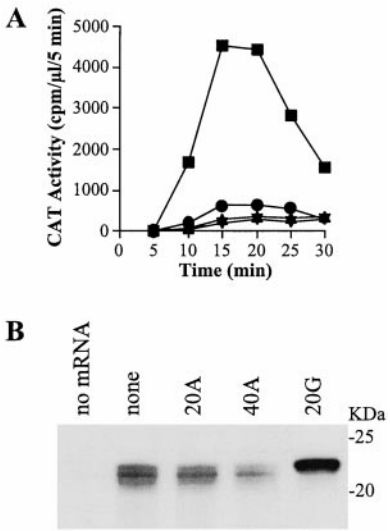

FIG. 3. **Effects of different 3′-homopolymer tails on synthesis of functional CAT protein.** *A*, functional CAT protein production *in vitro* was analyzed by measuring CAT activity in identical volumes removed from reaction mixtures at 5-min intervals and by plotting the incremental CAT activity of each sequentially removed volume. *B*, CAT protein produced in the reactions was visualized in Western blot analysis. Samples were taken after a 30-min incubation and CAT protein was detected using anti-T7 antibody conjugated with HRP. *A*, symbols used in the graph are: ●, none; ▲, A_{20} (*20A*); ▼, A_{40} (*40A*); ■, G_{20} (*20G*).

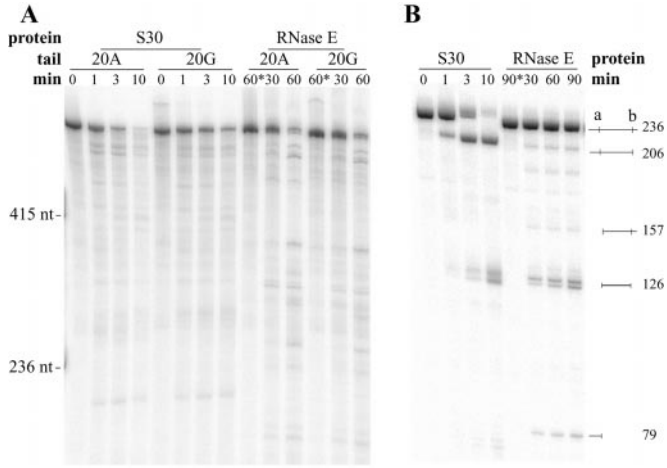

FIG. 4. **Evidence for both RNase E activity and exonucleolytic activity in 30 S fractions.** *A,* mRNA decay was measured as described in the Fig. 1 legend. Five pmol of CAT mRNA and 200 ng of affinity-purified RNase E were incubated in 1 × RNase E cleavage buffer, and samples were removed at the times indicated. *B*, gel-purified, 9 S RNA (500 nM) uniformly labeled with [^{32}P]CTP was used as substrate. The locations of RNase E cleavage sites generating bands of the sizes shown are indicated as *a* and *b*; an additional band corresponding to p5S (126 nucleotides) results from heterogeneity of the site of cleavage at *a*, as reported previously (48). All reactions were carried out at 37 °C, and reaction mixtures containing no protein are indicated as *.

step that initiates chemical and functional decay of RNA I (38, 39), a 108-nucleotide antisense repressor of replication of ColE1-type plasmids, we can find no published evidence that RNase E cleavage determines the functional half-life of mRNA in *E. coli* cells. To the contrary, earlier work by Ono and Kuwano (40) showed that mutation of the *E. coli* gene now known to encode RNase E prolongs the chemical half-life of bulk mRNA but has no effect on mRNA functional half-life.

As seen in Fig. 3, analysis of CAT protein produced by mRNA incubated with *E. coli* cell extracts for various lengths of time provided evidence that functional decay of CAT mRNA in 30 S cell fractions is not mediated by endonucleolytic cleavage. Western blot analysis of N-terminal T7-epitope-tagged CAT protein showed that CAT transcripts lacking poly(G) tails generated protein that was slightly shorter in length than the CAT protein produced by polyguanylated transcripts (Fig. 3*B*) and that the ability of poly(G) tails to reverse transcript truncation was directly related to their ability to increase the production of functional CAT protein. This result, taken together with evidence that poly(G) tails have a greater effect on CAT biochemical activity than nontailed transcripts (Fig. 3*A*), implies that inactive C-terminally truncated CAT proteins were made in these reaction mixtures by 3′ terminally truncated mRNA decay intermediates, and consequently that functional inactivation of CAT transcripts had occurred by degradation proceeding from the 3′-mRNA end.

Could these results be due to a possible absence of active RNase E in the 30 S cell extract fractions used for transcription/translation reactions? As seen in Fig. 4A, endonuclease fragmentation of CAT mRNA was observed in these reaction mixtures, and the pattern and rate of fragmentation was unchanged by poly(G) tails. Moreover, the fragmentation pattern was similar to that produced by digestion of CAT mRNA by purified RNase E implying that endogenous RNase E is present and active in 30 S fractions. This was shown directly by using 9 S ribosomal RNA, a 236-nucleotide transcript whose site-specific cleavage by RNase E has been extensively characterized. As seen in Fig. 4*B*, 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. 4*B*) 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. 4*A* 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′-tract of G nucleotides 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 exodeoxyribonuclease-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,

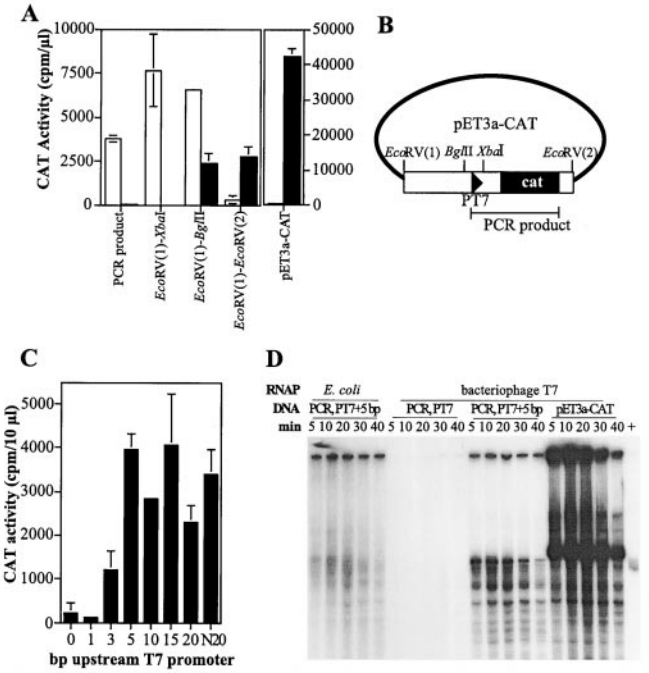

FIG. 5. **Parameters affecting efficiency of T7 promoters.** *A*, effects of base pairs upstream of the T7 promoter on T7 RNA polymerase-directed transcription. One μg of DNA was used in 20 μl of coupled *in vitro* transcription/translation reaction. The reaction was carried out either in the presence (■) or in the absence (□) of externally added T7 RNA polymerase (1 unit/μl) and rifampicin (500 ng/μl). *B*, map of plasmid pET3a-CAT showing locations of relevant restriction enzyme cleavage sites, the T7 promoter, the CAT gene, and the PCR product generated using primers indicated under "Experimental Procedures." *C*, determination of a minimum number of extra base pairs upstream of the T7 promoter required for optimal transcription by T7 RNAP. Extra base pairs were added 5′ to the T7 promoter in pET3a using PCR primers (see "Experimental Procedures"), and CAT activity was measured in coupled transcription/translation reactions. *D*, analysis of transcription and mRNA decay in coupled transcription/translation reactions. Samples were removed at the times indicated, and [^{32}P]UTP incorporation into transcripts was analyzed by 6% polyacrylamide gel in gels containing 8 M urea. Rifampicin (10 ng/μl) was added to reactions containing T7 RNAP (1 unit/μl). The DNA concentration used was 50 ng/μl. In the last *lane*, *in vitro* transcribed CAT mRNA from PCR DNA (*PT7*) was loaded as a size marker (+).

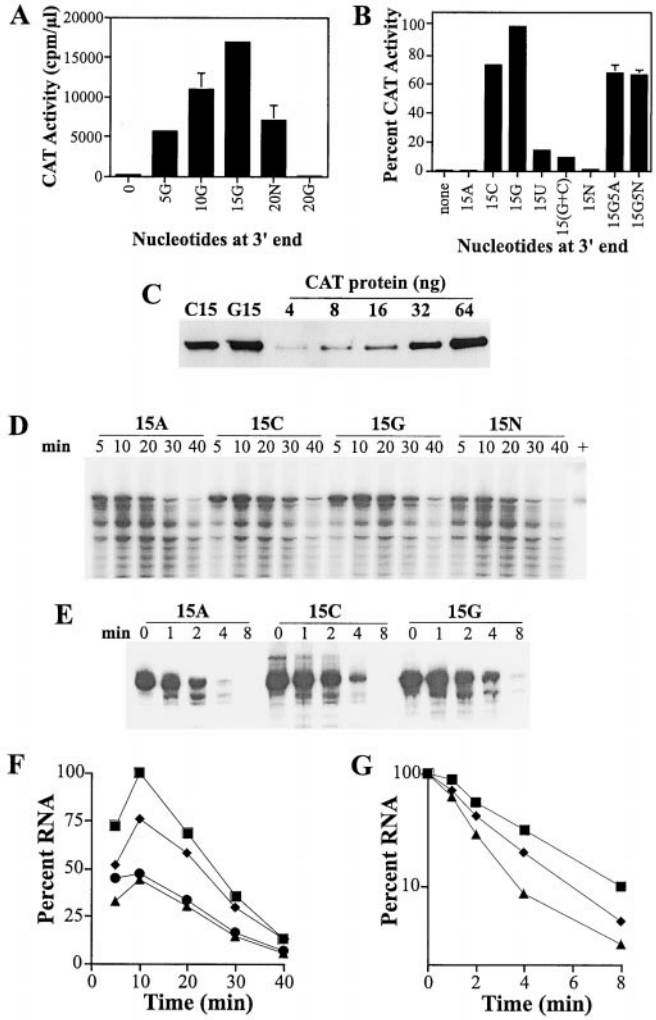

FIG. 6. **Effect of 3′-additions and inserted sequences on translation and stability of CAT mRNA.** *A*, effect of the number of G nucleotides at the 3′ terminus of mRNA on translation. PCR-generated DNAs containing 5 base pairs upstream of the T7 promoter and different tails 3′ to the CAT protein coding region were used in a coupled transcription/translation reaction, and the amount of translation product was measured using CAT assay. *B*, effects of different 3′ termini on CAT mRNA translation. 15 G nucleotides were internally incorporated at the 3′ terminus, followed by 5 A nucleotides (*15G5A*) or five random nucleotides (*15G5N*). Reactions similar to those described above were carried out except that PCR products with different tails were used in this experiment. *C*, quantitation of CAT proteins synthesized in a coupled transcription/translation reaction. Epitope-tagged CAT protein was affinity-purified from the *E. coli* cell extracts (BL21 (DE3)) harboring pET3a-CAT using anti-T7·tag antibody. The indicated amounts of purified CAT protein were loaded onto a 10% Tricine-SDS-polyacrylamide gel along with 1 μl samples taken from the *in vitro* reaction shown in Fig. 6*B* (*C15* and *G15*). CAT protein was detected in Western blot using anti-T7·tag antibody. *D*, analysis of steady-state levels of mRNA in coupled transcription/translation reaction was conducted. *F*, [^{32}P]UTP was added to reactions, samples were removed at the times indicated, and the percent of undegraded CAT transcripts was quantitated using a PhosphorImager. The percent of undegraded CAT mRNA was plotted relative to CAT mRNA containing a tail of 15 Gs present after 10 min of incubation, which was set at 100%. *E*, reaction conditions were as described in Fig. 1*A*, except a coupled transcription/translation reaction mixture was used in these experiments. *G*, the percent of RNA left in the reactions was plotted. *F* and *G*, the symbols used in the graphs are: ●, N_{15} (*15*N); ▲, A_{15} (*15A*); ♦, C_{15} (*15C*); ■, G_{15} (*15G*).

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. 5*A*). 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. That these nucleotides are crucial to the ability of the T7 RNAP to initiate transcription on linear DNA templates is 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 *Bgl*II-*Eco*RV 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. 5*A*). Also, as few as 5

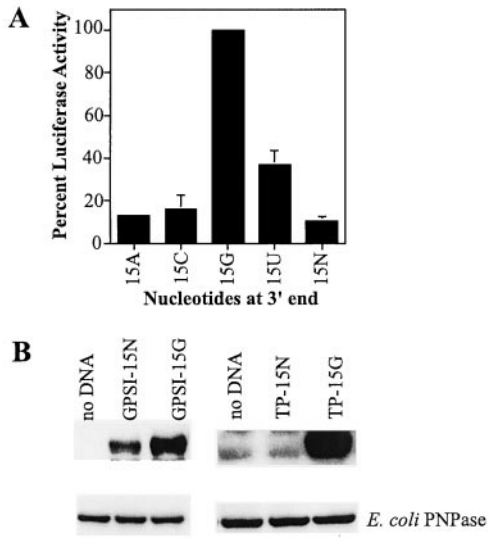

FIG. 7. **Effects of poly(G) tails on translation of luciferase, GPSI, and TP mRNA.** *A*, effects of different 3′ termini on translation of luciferase mRNA. Reaction mixtures were similar to those described in Fig. 6*B* except that PCR products contain luciferase protein sequence. The amounts of luciferase protein produced in reactions was measured and compared by setting the amount of luciferase activity encoded by transcripts containing a G_{15} tail as 100%. *B*, effects of poly(G) tails on translation of GPSI and TP. GPSI- and TP-coding regions were amplified either from plasmid (GPSI) or directly from *S. rochei* chromosome (TP), and the translation product was detected in Western blots using anti-T7·tag (GPSI) or anti-FLAG·tag antibody (TP). The same membrane was reprobed with antibody to *E. coli* PNPase antibody to produce a control for possible variations in loading.

base pairs upstream of the T7 promoter on the template DNA fragment were sufficient to promote rifampicin-independent synthesis of CAT to a level that was much higher than that achieved by *E. coli* RNAP (Fig. 5, *C* and *D*). This effect was independent of the nucleotide sequence of the 5′ base pairs (the 20-base-pair natural sequence *versus* randomly inserted base pairs) (Fig. 5*C*, *N20*) and was due specifically to transcription by T7 RNAP (Fig. 5*D*).

Stabilization of mRNA Function during Coupled Transcription/Translation in Vitro—PCR-generated CAT gene templates containing the T7 promoter and 5 upstream base pairs plus 3′-sequences that generate different types of tails on the transcripts encoded by these templates (Fig. 6, *A* and *B*) were used for the synthesis of active CAT protein *in vitro* during coupled transcription/translation. As shown, the number of G nucleotides required for the maximum yield of translation product peaked at 15. Installation of 15 G nucleotides internal to the transcript and 3′ to the ORF yielded about 70% of the protein production observed with 15 G nucleotides at the 3′ terminus. Whereas the same length of homopolymeric C nucleotides also increased the production of functional CAT protein to 30–60 μg/ml of the reaction (Fig. 6*C*), this effect was not observed for other mRNAs we tested. The observed steady state level and rate of decay for CAT mRNA containing poly(G) and poly(C) tails (Fig. 6, *D–G*) correlated well with the effects of homopolymeric additions on CAT protein produced by transcription/translation of templates synthesized by PCR.

Poly(G) tails also increased the yield in coupled transcription/translation reactions of proteins specified by other PCR-generated templates. These included firefly luciferase and *Streptomyces* chromosomal ORFs encoding GPSI or a 21-kDa *S. rochei* telomere binding protein[2] (Fig. 7). The effect of 3′-polyguanylation on the synthesis of biologically active 80-kDa GPSI by poly(G) tails was 2-fold, whereas production of the 21-kDa TP was increased 22-fold by the poly(G) tail. For these proteins, as well as for luciferase and CAT, the extent of functional stabilization of transcripts by poly(G) was inversely related to transcript length.

DISCUSSION

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. 6*B*).

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

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