Unpaired terminal nucleotides and 5′ monophosphorylation govern 3′ polyadenylation by *Escherichia coli* poly(A) polymerase I

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In bacteria, most mRNAs and certain regulatory RNAs are rapidly turned over, whereas mature tRNA and ribosomal RNA are highly stable. The selective susceptibility of unstable *Escherichia coli* RNAs to 3′ polyadenylation by the *pcnB* gene product, poly(A) polymerase I (PAP I), *in vivo* is a key factor in their rapid degradation by 3′ to 5′ exonucleases. Using highly purified His-tagged recombinant PAP I, we show that differential adenylation of RNA substrates by PAP I occurs *in vitro* and that this capability resides in PAP I itself rather than in any ancillary protein(s). Surprisingly, the efficiency of 3′ polyadenylation is affected by substrate structure at both termini; single-strand segments at either the 5′ or 3′ end of RNA molecules and monophosphorylation at an unpaired 5′ terminus dramatically increase the rate and length of 3′ poly(A) tail additions by PAP I. Our results provide a mechanistic basis for the susceptibility of certain RNAs to 3′ polyadenylation. They also suggest a model of ''programmed'' RNA decay in which endonucleolytically generated RNA fragments containing single-stranded monophosphorylated 5′ termini are targeted for poly(A) addition and further degradation.

RNA decay | poly(A) tails

Escherichia coli cells mutated in the *pcnB* gene (1), which initially was discovered as a locus controlling plasmid copy number but later was found to encode an enzyme [poly(A) polymerase I (PAP I); ref. 2] that adds poly(A) tails to RNA molecules, show retarded decay of a variety of messenger and nonmessenger RNAs (for reviews, see refs. 3–5). Although the mechanism(s) by which 3′ polyadenylation accelerates RNA decay still are incompletely understood, there is evidence that poly(A) tails may act as a scaffold or ''toe-hold'' for 3′ to 5′ exonucleases (6–8).

In eukaryotes, RNA polyadenylation is restricted to mRNA and is linked directly to endonucleolytic cleavage of the primary transcript in the 3′ untranslated region; in mammalian cells, the sites of poly(A) additions are determined by the interaction of transcript sequences and/or poly(A) polymerase with ancillary proteins that include cleavage/polyadenylation specificity factor (CPSF) (9, 10), cleavage stimulation factor (CstF) (11), and poly(A) binding proteins (12). In bacteria also, 3′ polyadenylation of RNA is not a stochastic event. mRNAs (7, 13–16), bacteriophage genomic RNAs (17, 18), and RNA I, a 108-nt tRNA-like cloverleaf molecule that controls the replication of ColE1-type plasmids (Fig. 1*a*) (6, 19, 20), undergo *pcnB*-dependent (i.e., PAP I-dependent) polyadenylation *in vivo*, targeting these RNAs for rapid decay. However, mature tRNAs and ribosomal RNAs, which are highly stable within *E. coli* cells, normally lack detectable poly(A) tails (21–23).

Here, we report investigations aimed at learning the molecular basis for the differential polyadenylation of certain RNA species by PAP I. Our results indicate that, in contrast to what has been observed for eukaryotic poly(A) polymerase, which requires protein cofactors to provide polyadenylation specificity (24, 25), the ability of *E. coli* PAP I to differentially polyadenylate RNAs resides in PAP I itself. Additionally, by using substrate variants of *supF* $tRNA^{Tyr}$, which ordinarily is poorly polyadenylated, and RNA I, which has overall similarity to *supF* $tRNA^{Tyr}$ in size and secondary structure but is polyadenylated at high efficiency, we found that initiation and growth of poly(A) tails by purified PAP I *in vitro* are (*i*) dependent on the presence of unpaired nucleotides at either end of the substrate, and (*ii*) affected by the extent of phosphorylation at the 5′ terminus. Our results provide a mechanistic basis for the resistance of certain RNAs to 3′ polyadenylation and additionally suggest a model in which endonucleolytic cleavages that generate unpaired terminal sequences and monophosphorylated 5′ ends can program the cleavage products for further decay.

Materials and Methods

Plasmids, Oligonucleotides, and DNA Fragments. Plasmid pHF-PAP, which carries full-length PAP I, was constructed by inserting PCR-amplified genomic *pcnB* gene *of E. coli* to plasmid p6HisF-11d (26). The primers used for PCR amplification of the PAP I fragment are as described (2). After PCR and restriction enzyme digestion, the *Nde*I–*Bam*HI fragment containing full-length PAP I was gel purified and ligated with gel-purified p6HisF-11d that had been cut with restriction enzymes *Nde*I and *Bam*HI. DNA templates for *in vitro* transcription of GGG.RNA I (27) and RNA I-t were generated by PCR amplification of pM21 (27); the plasmid used for PCR amplification of *supF* $tRNA^{Tyr}$ and tRNA-I was pJA11 (28). To amplify the DNA template encoding GGG.RNA I, the upstream primer was T7-promoter (5′-TAATACGACTCACTATAGGG-3′) and downstream primer was RNA I-3′ (5′-AACAAAAAACCACCGCTACAGCG-3′); for RNA I-t, the upstream primer was T7-promoter and the downstream primer was RNA I-C′ (5′-GGGACAGTATTTA-CAAAAAACCACCGCTACC-3′); for *supF* $tRNA^{Tyr,}$ the upstream primer was T7-promoter and the downstream primer was tRNA3′ (5′-TGGCGGTGGGGGAAGGATTCGAA-3′). Two sequential PCRs were carried out to amplify DNA template of *in vitro* transcribed tRNA-I and RNA I.20. For tRNAI, the first PCR used upstream primer I-t (5′-GGGACAGTATTTGGT-GGGGTTCCCGAG-3′) and downstream primer tRNA3′. The DNA fragment from first PCR was then used as template with upstream primer T7-I (5′-TAATACGACTCACTATAGGGA-CAGTATTTG-3′) and downstream primer tRNA3′ for the

Abbreviation: PAP I, poly(A) polymerase I.

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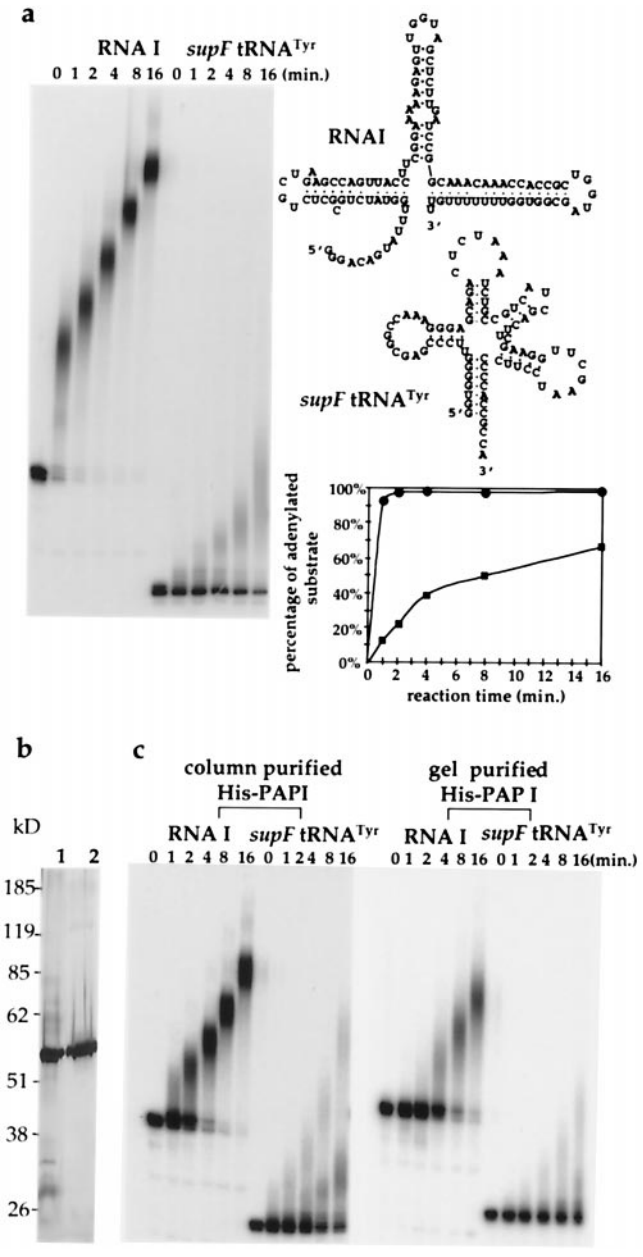

Fig. 1. Polyadenylation of GGG.RNA I and *supF* $tRNA^{Tyr}$ by PAP I *in vitro*. (*a*) Time course of reactions containing 1.2 pmol of GGG.RNA I or *supF* $tRNA^{Tyr}$ as substrates, using 1 μg of protein complex containing PAP I. Samples were taken at the times indicated. The secondary structure of GGG.RNA I and *supF* $tRNA^{Tyr}$ is as shown. The rate of initiation of polyadenylation was quantitated from gels by PhosphorImager analysis (Molecular Dynamics), and plotted for RNA I (●) and *supF* $tRNA^{Tyr}$ (■). The percentage of adenylated substrate at each time point was defined as follows: [1 − (the ratio of the quantity of nonadenylated substrate to the quantity of nonadenylated substrate at time 0)] × 100%. The adenylation initiation rate, which was defined as the time required for 50% of substrate to acquire one or more A residues, was 0.4 ± 0.1 min for RNA I and 8.0 ± 0.1 min for *supF* $tRNA^{Tyr}$. (*b*) Purification of His-tagged PAP I is shown by silver nitrate staining SDS/PAGE analysis. Lane 1, eluate of His-tagged PAP I from Ni^{2+}-immobilized metal affinity column; lane 2, same preparation further purified from SDS/PAGE. (*c*) *In vitro* polyadenylation reactions for GGG.RNA I and *supF* $tRNA^{Tyr}$ using 140 fmol of column-purified and gel-purified His-tagged PAP I.

second PCR. For RNA I.20 primers I-hpx (5′-CACTAGAAGGGGATCCCCTCGAGGGGATCCACAGTATTTGG-3′) and RNA I-3′ were used for the first PCR and the synthesized DNA fragment was the template for a second PCR in which the upstream primer T7-hpx (5′-TAATACGACTCACTATAGGATCCCTCGAG-3′) and downstream primer RNA I-3′ were used.

RNA Synthesis and Labeling. Internally [α-^{32}P]UTP-labeled GGG.RNA I, tRNA, and their variants were synthesized by using the DNA fragments mentioned above, and the Mega-Short Script kit (Ambion, Austin, TX). For synthesis of pRNA I, pppRNA I transcribed *in vitro* by using the Mega-Short Script kit was synthesized without [α-^{32}P]UTP, treated with alkaline phosphatase (New England Biolabs) to remove phosphate groups at its 5′ end, and then labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Life Technologies). Labeled RNA was electrophoresed in 8% (vol/vol) polyacrylamide gels containing 7 M urea; a gel slice containing the correct size band was soaked overnight in elution buffer [20 mM Tris·HCl (pH 8.0)/5 mM EDTA/0.2% (wt/vol) SDS/0.2 M NaCl]. The purified RNA fragment was recovered from the eluent by phenol/chloroform extraction and ethanol precipitation.

Protein Purification. PAP I that was tagged at the N terminus by 6 histidine (His) residues and a FLAG (29) octapeptide was overexpressed *in vivo* from plasmid pHF-PAP in *E. coli* BL21(DE3) cells and purified by nondenaturing immobilized metal affinity chromatography (IMAC) as described in the pET System Manual (Novagen). His-tagged PAP I was eluted from the IMAC column by using 200 mM imidazole, dialyzed three times against 1 liter of storage buffer [50 mM Tris·HCl (pH 7.9)/200 mM NaCl/1 mM EDTA/20% (vol/vol) glycerol/0.5% (vol/vol) Triton X-100/1 mM DTT], aliquotted, and stored at −70°C until used. For gel purification of PAP I, the column-purified preparation was subjected to 8% SDS/PAGE. After light staining with Coomassie blue, a gel slice containing only the PAP I band was cut from the gel. PAP I was then eluted from the gel by using Bio-Rad electro-eluter and recovered as described previously (30).

***In Vitro* Polyadenylation Assay.** Labeled RNA substrates were incubated at 37°C with PAP I in reaction buffer consisting of 250 mM NaCl/10 mM $MgCl_2$/2 mM K_2HPO_4/1 mM DTT/1 mM phosphoenolpyruvate/0.6 unit of pyruvate kinase (Sigma), 10 units of the RNase A inhibitor RNaseOut (Life Technologies), and 0.4 mM ATP. Aliquots were removed from reaction mixtures at indicated times, mixed with an equal volume of sequencing stop buffer [85% (vol/vol) formamide/10 mM EDTA/0.05% (wt/vol) bromophenol blue/0.05% (wt/vol) xylene], and denatured for 3 min at 85°C before electrophoresis on 8% polyacrylamide/7 M urea gels.

Results

Differential Polyadenylation of RNA Substrates by PAP I *in Vitro*. RNA polyadenylation in eukaryotic cells is known to be accomplished by a complex process that employs poly(A) polymerase and protein cofactors that recognize polyadenylation signals to cleave RNA and generate the 3′ terminus to which poly(A) tails are added (for reviews, see refs. 31–33). To investigate the basis for the selective RNA polyadenylation that also occurs in *E. coli*, we first determined whether a PAP I-containing multicomponent complex isolated from bacteria after gentle lysis could reproduce *in vitro* the differential polyadenylation observed *in vivo* for RNA I vs. tRNA. Prior investigations of PAP I-mediated incorporation of radioactively labeled ATP into crude preparations of tRNA (2, 34) had led to the conclusion that the action of *E. coli* PAP I, like that of mammalian poly(A) polymerase (25), is indiscriminate *in vitro*. However, we found by gel analysis that the rate of initiation of poly(A) tails by PAP I-containing complexes was more than 10-fold greater for GGG.RNA I than for

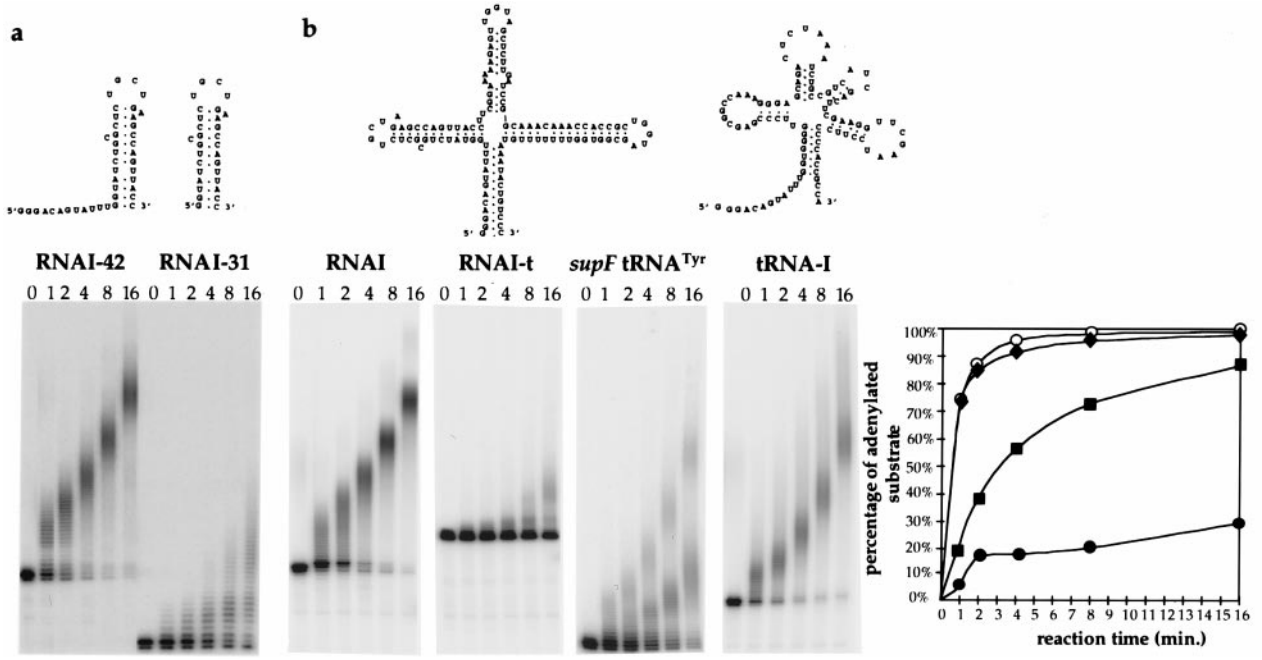

Fig. 2. *In vitro* polyadenylation of RNA I and tRNA variants by purified His-tagged PAP I. Reaction conditions were as in Fig. 1*c*. The corresponding RNA secondary structures are shown above each gel. (*a*) RNA I 42-mer and RNA I 31-mer. (*b*) GGG.RNA I, RNA I-t, *supF* $tRNA^{Tyr}$, and tRNA-I. The percentage of adenylated substrate at each time point is plotted. RNA I (◆), *supF* $tRNA^{Tyr}$ (■), RNA I-t (●), tRNA-I (○).

purified synthetic *supF* $tRNA^{Tyr}$ (Fig. 1*a*). Under the reaction conditions used, differential poly(A) addition was distributive rather than processive, in contrast to what has been observed during poly(A) addition at specific sites by mammalian poly(A) polymerase (12); initiation of tails occurred on an increasing fraction of substrate molecules whereas the length of growing tails was maintained within approximately a 20-nt range. Whereas initiation of poly(A) tails on *supF* $tRNA^{Tyr}$ by PAP I was inefficient, distributive poly(A) tail growth on this substrate appeared to be biphasic (Fig. 1 *a* and *c*). Two distinct groups of adenylated tRNA molecules were observed in gels, suggesting that the growth of poly(A) tails occurred at a more rapid rate after addition of the first few A residues.

To learn whether the ability to differentially polyadenylate RNA I efficiently is inherent to PAP I, we overexpressed His-tagged PAP I under control of the bacteriophage T7 promoter and purified the protein to apparent homogeneity by a combination of Ni^{2+}-chelate affinity chromatography and SDS/PAGE (Fig. 1*b*). Denatured PAP I was isolated from SDS/PAGE gels and then renatured and tested for enzymatic activity. As seen in Fig. 1*c*, the recombinant PAP I protein differentially polyadenylated GGG.RNA I, whether ≈90% pure (after Ni^{2+} column chromatography) or lacking any detectable protein contaminant (after subsequent elution from SDS gels), indicating that the ability to carry out selective polyadenylation resides in PAP I itself, rather than in accessory proteins analogous to those regulating polyadenylation specificity in eukaryotes. Because column-purified and gel-purified PAP I were indistinguishable in their ability to differentially polyadenylate RNA I and tRNA, column-purified preparations were used for the investigations described below.

Efficient Polyadenylation Requires Unpaired Nucleotides at Either Terminus of an RNA Substrate. Earlier work has indicated that partially degraded fragments of RNA I (19) and mRNAs (4), as well as full-length transcripts, can undergo polyadenylation, implying that a sequence unique to RNA 3′ ends is not required for poly(A) addition. Consistent with this inference, we found, by using RNA I 42-mer (which contains the first 42 nt of RNA I but lacks the distal two-thirds of the transcript; Fig. 2*a*), that deletion of the 3′ end of RNA I had no effect on the efficiency of polyadenylation. Additionally, substitution of the 3′ terminal sequence of mature tRNA (i.e., CCA-3′) for the 3′ end of RNA I or replacement of the tRNA CCA-3′ sequence by the UU-3′ terminus of RNA I failed to alter the rate or extent of polyadenylation of either RNA species (data not shown). Remarkably however, deletion of 12 unpaired nucleotides from the 5′ end of RNA I 42-mer (yielding RNA I 31-mer) dramatically reduced both the rate of initiation of polyadenylation, as determined by the loss of non-polyadenylated substrate, and the rate of growth of poly(A) tails (Fig. 2*a*), suggesting that 5′ single-strandedness in this RNA facilitates the addition of A residues to the 3′ terminus by PAP I.

If the notion that unpaired 5′-terminal nucleotides promote efficient PAP I-mediated polyadenylation is correct, the rate of polyadenylation of GGG.RNA I should be decreased by adding 3′ nucleotides that base pair with the 5′ region. Conversely, the capacity for efficient polyadenylation should be conferred on *supF* $tRNA^{Tyr}$ by adding a single-strand segment to its 5′ end. We constructed RNA I-t, an RNA I variant with base-paired ends, and tRNA-I, a *supF* $tRNA^{Tyr}$ variant with 5′ unpaired nucleotides of GGG.RNA I (Fig. 2*b*), to test this hypothesis. Our results showed that the relative abilities of GGG.RNA I and *supF* $tRNA^{Tyr}$ to serve as substrates for PAP I were reversed by these manipulations: RNA I-t was now virtually insensitive to polyadenylation, whereas poly(A) tails were efficiently added to tRNA-I.

That the effect of 5′ unpaired nucleotides on 3′ polyadenylation is due to single-strandedness was confirmed by adding to the polyadenylation reaction mixtures a synthetic deoxyoligonucleotide that base pairs with the GGG.RNA I 5′ single-strand region. As seen in Fig. 3*a*, adding a 12-nt deoxyoligomer (oligo 12) complementary to the 5′ single-strand region of GGG.RNA I and whose base pairing with nucleotides in this region was

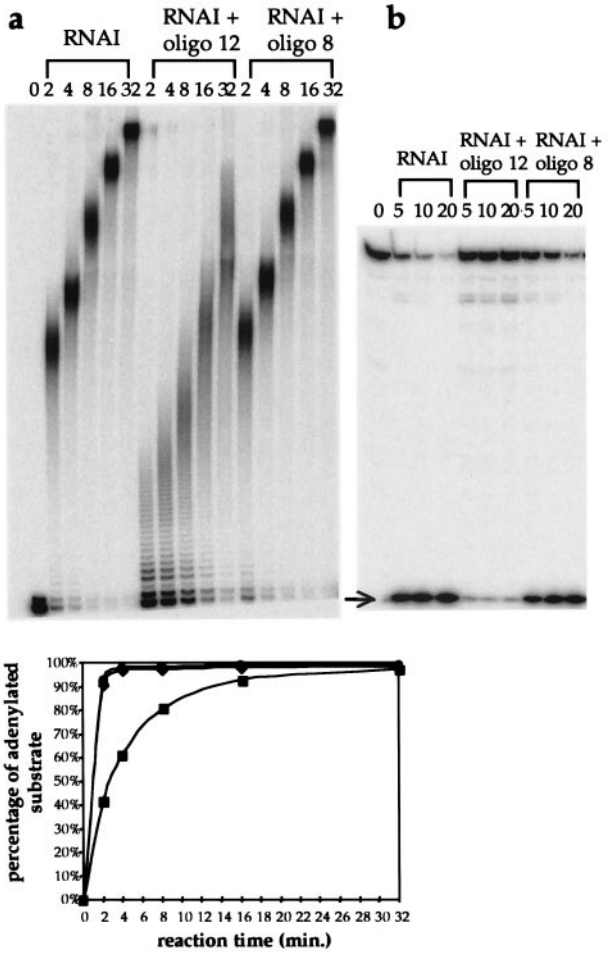

Fig. 3. Effect of oligonucleotides complementary to the 5′ single-strand region of RNA I on PAP I activity. (*a*) *Upper*, polyadenylation reactions for RNA I, RNA I + oligo 12 (5′-AAATACTGTCCC-3′), and RNA I + oligo 8 (5′-AAATACTG-3′). *Lower,* plot of adenylation rates for the three reactions [RNA I (○), RNA I + oligo 12 (■), and RNA I + oligo 8 (◆)]. (*b*) RNase E cleavage assays of the substrates shown in *a*. →, the 8-nt RNase E cleavage product. One picomole of GGG.RNA I 5′-labeled with [γ-^{32}P]ATP was used in adenylation or RNase E cleavage reactions. RNA I was incubated with either water or 100 pmol of oligonucleotides for 10 min before addition to the reaction mixture. PAP I (280 fmol) was added to each polyadenylation reaction at time 0. RNase E cleavage assay was carried out with 1 μg of recombinant RNase E (53) at 30°C in polyadenylation reaction buffer except that ATP, phosphoenolpyruvate, and pyruvate kinase were omitted.

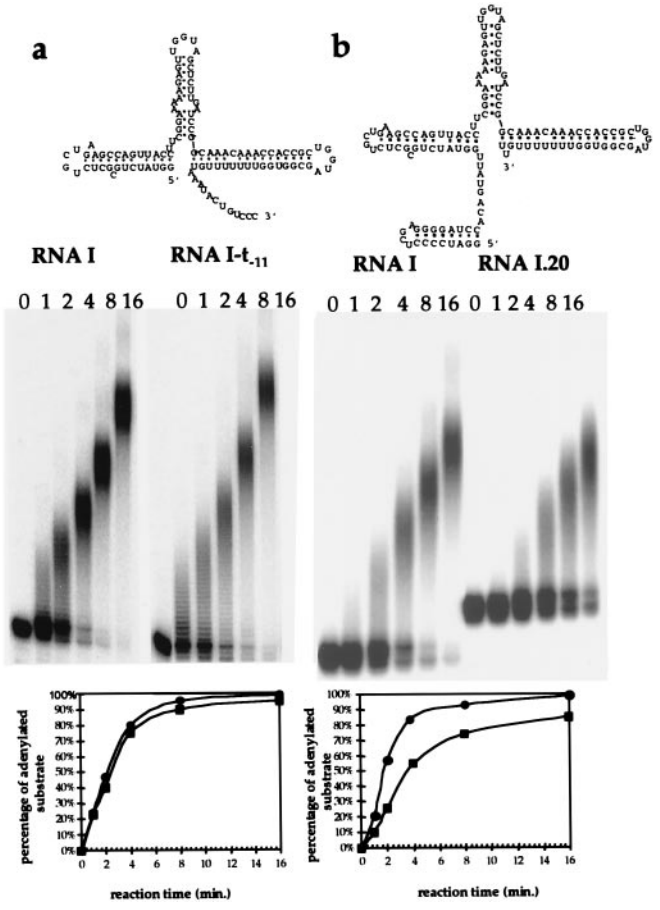

Fig. 4. The effect of 3′ end or internalized single-strand region in polyadenylation efficiency by PAP I. The reaction conditions were as in Fig. 1*c*, and 70 fmol of PAP I was used for each reaction. The corresponding secondary structure of RNA is as shown above each gel. (*a*) Polyadenylation of RNA I and RNA I-t$_{-11}$. The initiation rate of adenylation is as plotted [RNA I (●) and RNA I-t$_{-11}$ (■)]. (*b*) Polyadenylation of RNA I and RNA I.20. The initiation rate of adenylation is as plotted [RNA I (●) and RNA I.20 (■)].

demonstrated by the loss of sensitivity of GGG.RNA I to cleavage by the single-strand endonuclease RNase E (Fig. 3*b*), reduced the initiation of poly(A) tails on GGG.RNA I. Addition of an identical amount of an oligonucleotide that is complementary to only 8 nt (oligo 8) of the 5′ single-strand region of GGG.RNA I and that failed to protect GGG.RNA I from RNase E cleavage had no effect on polyadenylation, indicating that the observed action of oligo 12 resulted from base pairing rather than from oligonucleotide addition.

The above results demonstrate that 5′ single-strandedness is sufficient to promote 3′ polyadenylation. 3′ single-strandedness also promotes polyadenylation; the removal of 12 nt from the 5′ end of RNA I-t, which has paired ends and was poorly adenylated (Fig. 2*b*), yielded a substrate (RNA I-t$_{-11}$) that was polyadenylated as efficiently as GGG.RNA I (Fig. 4*a*). Taken together with the findings described above, this result indicates that unpaired nucleotides at *either* end of RNA can facilitate PAP I-mediated addition of poly(A) tails and also that polyadenylation is independent of the particular sequence at either terminus. Further supporting these conclusions was evidence that addition of a 5′ stem-loop structure that is predicted to internalize the unpaired segment at the 5′ end of RNA I (construct RNA I.20; ref 35) reduced both the initiation rate of poly(A) tail additions and the rate of growth of tails (Fig. 4*b*).

5′ Monophosphorylation Enhances PAP I Efficiency. Earlier work has shown that the extent of 5′ phosphorylation of RNA molecules can affect RNA I decay *in vivo* (36) as well as the *in vitro* enzymatic activities of polynucleotide phosphorylase and RNase E (6, 37). Both of these enzymes have been shown to attack poly(A) tails on RNA molecules (6, 38). During the course of our experiments, we observed that 3′ poly(A) additions by PAP I to RNA I derivatives having unpaired 5′ ends are also strongly affected by 5′ phosphorylation. Like the above mentioned nucleases, the RNA polymerase activity of PAP I prefers RNA I molecules that contain 5′-monophosphate termini (Fig. 5). As seen, the rates of initiation and growth of poly(A) tails were increased 5- and 3-fold, respectively, on a substrate containing a 5′-monophosphorylated vs. 5′-triphosphorylated terminus.

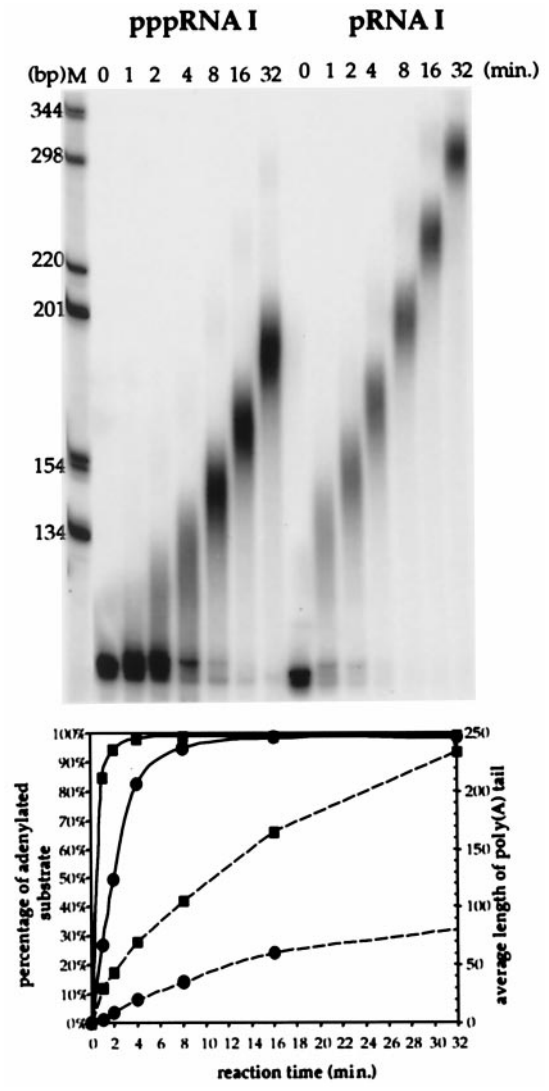

Fig. 5. Differential polyadenylation of pppRNA I and pRNA I. Universally ^{32}P-labeled pppRNA I (1.2 pmol) and 5′ end kinase-labeled pRNA (1.2 pmol) were used as substrates. M, 1-kb DNA marker (Life Technologies), which was labeled with [α-^{32}P]dATP by using Klenow fragment of *E. coli* DNA polymerase I. The rate of initiation of poly(A) tails (solid lines) and the average length of poly(A) tails (dashed lines) are shown for pppRNA I (●) and pRNA I (■). Reaction conditions were as described in Fig. 1, except that 70 fmol of PAP I was used and an extra time point (32 min) was taken. The times required for adenylation of 50% of the substrate were 2.2 ± 0.1 min for pppRNA I and 0.4 ± 0.1 min for pRNA I. The average rate of poly(A) tail growth, which was defined as the average length of the poly(A) tail at each time point divided by the number of minutes, was 3.75 nt/min for pppRNA I and 11.68 nt/min for pRNA I.

Discussion

RNA decay in bacteria can occur by a vectorial process that degrades substrates in the 3′ to 5′ direction (39). Such decay is aided by the addition of poly(A) tails to primary transcripts or to transcript fragments generated either by endonucleolytic cleavages or digestion by 3′ to 5′ exonucleases (6, 7, 13, 14, 19, 40). Whereas stem-loop structures at or near the 3′ ends of RNA molecules impede the progress of 3′ to 5′ exonucleases (41–43), stem-loops at 5′ termini can also reduce RNA decay (44–46), in part by affecting occupancy by ribosomes (47). Our results, which demonstrate that base pairing of 5′ terminal sequences interferes with efficient polyadenylation, now suggest an additional possible mechanism for the effects of 5′ stem-loop structures on RNA decay.

PAP I recently has been found to interact with RNase E (ref. 48 and our unpublished data), which is believed to endonucleolytically initiate decay of most mRNAs (5, 49–51). By decreasing the opportunity for intramolecular base pairing within RNA molecules and generating 5′-monophosphate termini on fragments 3′ to cleavage sites, endonucleolytic digestion by RNase E may program the cleavage products for efficient polyadenylation and consequent 3′ to 5′ exonucleolytic degradation. Thus, polyadenylation of *E. coli* RNAs *in vivo* may occur largely on RNA decay intermediates, rather than on full-length primary transcripts. Evidence that 30 of 32 clones of polyadenylated RNA I molecules lacked the native 3′ terminus (19) is consistent with this notion. Rapid turnover of these polyadenylated RNA decay intermediates may explain the low steady-state level of polyadenylated RNA (52) isolated from wild-type *E. coli* cells.

Our *in vitro* findings imply that reduced poly(A) tail initiation by PAP I on small stable RNAs may account at least in part for their insensitivity to polyadenylation *in vivo* (22) and suggest a molecular basis for the differential polyadenylation observed for some cellular RNAs. Whereas 5S rRNA and certain other stable small RNAs have monophosphorylated 5′ termini produced by endonucleolytic processing or exonucleolytic digestion, the length of unpaired terminal nucleotides on these molecules may be too short for PAP I to act efficiently. Similarly, a very short sequence of unpaired nucleotides (UU) at the 3′ end of RNA I derivatives may account for a rate of polyadenylation that is slightly greater than observed for substrates that totally lack unpaired nucleotides at their termini. Our finding that polyadenylation of tRNA is biphasic suggests that once the length of the 3′ unpaired terminus of tRNA is increased by the addition of the first few A residues by PAP I, tail elongation can occur more rapidly. Thus, the relative resistance of small stable RNAs to polyadenylation by PAP I appears to depend largely on a reduced rate of poly(A) tail initiation. Overproduction of poly(A) polymerase *in vivo* can result in the addition of poly(A) tails to ribosomal RNA species that ordinarily do not contain such tails in wild-type bacteria (16).

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