Polyadenylation in *E. coli*: a 20 year odyssey

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Although poly(A) polymerase was first discovered in *Escherichia coli*, polyadenylation was deemed only to occur in eukaryotes well into the 1990s. For example, in Benjamin Lewin's sixth addition of *Genes* published in 1997, polyadenylation is defined as "…the addition of a sequence of polyadenylic acid to the 3′ end of a eukaryotic RNA after its transcription." This common misconception arose in spite of considerable work throughout the 1980s on the existence of poly(A) tails in *E. coli* by the group of Nilima Sarkar. It was not until the identification of *pcnB* as the structural gene for *E. coli* poly(A) polymerase (PAP I) that it became possible to begin a careful analysis of polyadenylation in a prokaryote.

My laboratory became interested in polyadenylation in the early 1990s when we noticed that several mRNAs actually appeared to increase in size long after new transcription had been stopped by the addition of the drug rifampicin. After hearing Nilima talk about polyadenylation at a ASM sponsored mRNA decay meeting in 1992, we set out to construct a series of isogenic strains that were defective in poly(A) polymerase as well as nucleases known to be involved in mRNA decay (RNase E, polynucleotide phosphorylase [PNPase], and RNase II). Much to our surprise, the analysis of mRNA decay in these mutants showed that polyadenylation appeared to function as a targeting mechanism.

These first experiments started us on what has been a 20 year odyssey to discover the function and significance of polyadenylation in *E. coli* and other prokaryotes. At first it appeared that the analysis of polyadenylation would proceed quickly. The construction of a controlled expression plasmid allowed us to alter the intracellular levels of poly(A) polymerase and confirm a role for the enzyme in mRNA decay. However, some of the data was both unexpected and deviated considerably from what had already been observed in eukaryotes. Specifically, it appeared that 23S rRNA was a preferred target of PAP I and, more importantly, increased levels of polyadenylation were highly toxic, particularly in strains of *E. coli* that were deficient in either RNase II or PNPase.

A further surprise was that cells deficient in PAP I still contained a significant amount of poly(A) tails. The report that the *f310* gene encoded a second poly(A) polymerase proved to be incorrect. This led to the discovery that PNPase, first discovered as a potential RNA polymerase, serves as a second poly(A) polymerase in *E. coli*. This finding was originally met with considerable skepticism because of the high intracellular concentration of inorganic phosphate and the fact the PNPase had been assumed for many years to only work degradatively. For example, in the second edition of Kornberg's classic *DNA Replication*, he states that "…the function of polynucleotide phosphorylase was assigned to the salvage of nucleotides from RNA rather than their polymerization of RNA." However, the *E. coli* data were quickly confirmed in other bacteria. Specifically, in vivo PNPase synthesizes long tails that contain predominately A residues. It is interesting to note that PNPase is a highly conserved enzyme found in almost all bacteria.

Even after identifying the enzyme that was responsible for the remaining poly(A) tails in exponentially growing cultures of *E. coli* Δ*pcnB* mutants, it still was not clear how polyadenylation was affecting the stability of mRNAs. Although analysis of the *E. coli* transcriptome indicated that the majority of *E. coli* mRNAs were polyadenylated to some extent, intercellular steady-state poly(A) levels were estimated at only 1%–2%. Furthermore, it was shown that there are very few molecules of PAP I in the cell. These findings raised the question of whether there was an actual polyadenylation signal in the bacterium. Subsequent experiments demonstrated that Rho-independent transcription terminators served as polyadenylation signals.

The polyadenylation story became more complicated with the discovery that in vivo PAP I existed as part of a multiprotein complex that also contained PNPase and the RNA binding protein Hfq. Although the existence of a polyadenylating complex was not surprising, based on the fact that eukaryotic polyadenylation involves a large multiprotein complex, the presence of PNPase was rather perplexing. In the first place, PNPase can work both degradatively and biosynthetically. In the second place, the tails generated by PNPase differ in both composition and location on mRNA substrates. Currently it is not even clear whether the heteropolymeric tails synthesized by PNPase are functionally equivalent to PAP I generated poly(A) tails. The presence of Hfq has been

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rationalized based on its ability to bind to A/U regions that would be found at the 3′ ends of Rho-independent transcription terminators.

In 2008, our work on polyadenylation took an unexpected turn. In the process of examining the processing of primary tRNA transcripts, we observed that a small fraction of pre-tRNAs, which contained a few extra nucleotides downstream of their encoded CCA determinants, had short poly(A) tails 1–5 nt in length. As we examined more tRNA species, we discovered that for most tRNAs (79/86) polyadenylation competed with the 3′→5′ processing enzymes (RNase T and RNase PH) for pre-tRNA substrates. Not only was it a surprise that tRNAs were substrates for PAP I, since this has not been observed in eukaryotes, but, more importantly, PAP I in *E. coli* was actually involved in regulating the level of functional tRNAs.

The finding that pre-tRNAs were polyadenylated led us to reexamine our earlier observation that increased levels of polyadenylation were toxic to the cell. It turned out that there was a simple explanation for this phenomenon. In wild type cells, pre-tRNAs are targets for polyadenylation but mature tRNAs that can be aminoacylated are not. However, when the synthesis of poly(A) polymerase is deregulated, mature tRNAs became substrates for polyadenylation, thus interfering with aminoacylation. The block in aminoacylation leads to an inhibition of protein synthesis and subsequent cell death. We are now in the process of determining if organisms that lack PAP I activity, such as *Bacillus subtilis*, will show the same toxicity if we ectopically express PAP I in the cell.

Although I thought that we could develop a complete understanding of bacterial polyadenylation in a relatively short period of time, there are still a significant number of unanswered questions that need to be addressed. Perhaps the most important relates to the physiological role for polyadenylation in the cell. PAP I mutants show only a small defect in their growth rate in rich medium. A PAP I PNPase double mutant grows much more slowly than either single mutant but is still viable. However, we noticed some time ago that while the PAP I PNPase double mutant does not contain any poly(A) tails in exponentially growing cultures, in stationary phase long poly(A) tails reappear. These tails, which can be greater than 60 nt in length, are found at locations not seen in exponentially growing cells. Since we have yet to identify the enzyme that is responsible for their addition, it is not possible at this time to determine the phenotype of an *E. coli* cell that is totally deficient in polyadenylation.

Another issue relates to why only a limited number of bacteria contain a true PAP I activity. Is it possible that PAP I is a recent acquisition by *E. coli* or rather have other bacteria lost the enzyme because of its toxicity relating to the polyadenylation of tRNAs? tRNAs are the most abundant RNA molecule in the cell and thus could easily be targets for polyadenylation in any prokaryote that has a PAP I enzyme. *E. coli* seems to have solved the poly(A) toxicity problem by down regulating the synthesis of the protein by having a very poor Shine Delgarno sequence and a noncanonical translation start codon. For other bacteria it may have been a simpler solution to lose the enzyme entirely.

Looking back to our first experiments on polyadenylation, it is clear that what seemed like a very straightforward problem has turned out to be far more complex. However, in spite of the technical difficulties that are involved in studying polyadenylation in bacteria, the research has been both intellectually challenging and extremely rewarding.

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