RNA capping by mitochondrial and multi-subunit RNA polymerases

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ABSTRACT
Recently, it was found that bacterial and eukaryotic transcripts are capped with cellular cofactors installed by their respective RNA polymerases (RNAPs) during transcription initiation. We now show that mitochondrial RNAP efficiently caps transcripts with ADP – containing cofactors. However, a functional role of universal RNAP – catalysed capping is not yet clear.

The discovery of non-canonical transcript capping
Capping of RNA is no longer seen as an exclusive feature of eukaryotes, thanks to the recent discovery of bacterial transcripts capped by NAD+ and 3’-dephospho-coenzyme A (DP-CoA) [1,2]. NAD+ is the only cap investigated in vivo in E. coli, and is found on a number of small RNAs (sRNAs) and messenger RNAs (mRNAs). In addition, a number of currently uncharacterised moieties were found attached to E. coli cellular RNA which could potentially also serve as 5’ RNA caps [3]. The extent of NAD+ modification (NADylation) in the cell varies greatly for different RNA species. The RNA species that are most heavily NADylated in vivo [1] are listed on Figure 1A. Even for these species, only a relatively small proportion of the transcripts are capped with NAD+ (13% in the case of most heavily NADylated species, namely RNA1 – the antisense RNA involved in the regulation of pUC19 plasmid replication [1]). More recently NAD+ capping was shown not to be unique for bacteria, as NADylated RNAs were found in vivo in Saccharomyces cerevisiae and human cells [4,5].

The search for an enzyme that can potentially NADylate RNA transcripts was relatively straightforward, as bacterial RNA polymerase (RNAP) was shown previously to use NAD+ as an initiating nucleotide (given its ADP moiety and free 3’ hydroxyl group) [6]. Studies by Bird et al., and Julius and Yuzenkova, using promoter-specific assays, demonstrated that capping can be performed by RNAP on promoters where transcription starts with A [7,8]. These studies showed that the K_m for NAD+ in transcription initiation was much lower than the in vivo concentration of NAD+ (Figure 1B). Furthermore, Bird et al. observed a strong correlation between the extent of NADylation of a chosen transcript in vivo and the efficiency of NADylation by RNAP in vitro [7]. Eukaryotic RNApol II was also shown to be able to incorporate NAD+, suggesting that the NADylated transcripts observed in vivo are also capped by RNAP [7].

Other ADP-containing cofactors were shown to be efficiently incorporated at the 5’ end of RNA by RNAP, such as FAD and 3’-dephospho-coenzyme A (but not NADP and NADPH) [7,8]. The efficiency of incorporation for these compounds and their concentration in the cell are lower than those for NAD+, suggesting that the possible abundance of these caps is also lower [9].

Cell wall precursors are potentially another class of prokaryotic capping molecules
Dinucleotides UDP-Glucose and UDP-GlcNAc, the precursors of bacterial cell wall synthesis, are even more...
abundant than NAD$^+$ in E. coli cells grown on rich media (Figure 1B). We recently found that for promoters coding for U at position +1, their RNA transcripts can be efficiently capped in vitro by E. coli RNA polymerase with UDP-GlcNAc and UDP-Glucose [8]. The relatively low $K_m$ for the incorporation of these substrates at the 5' end of the RNA transcripts by RNAP, favours the probability of in vivo capping by UDP-GlcNAc and UDP-Glucose, by analogy with NAD$^+$ (Figure 1B). Although less than 10% of E. coli promoters code for U at position +1, a link between gene expression and cell wall synthesis could be of potential significance for coordinating biomass and cell wall synthesis.

The ability of RNAP to incorporate variety of known nucleotide-containing molecules at the 5' position of transcript, as well as a number of identified but uncharacterised RNA modifying moieties [3], suggests the existence of a wide repertoire of RNA caps in the cell.

**At least two domains of bacterial RNAP determine efficiency of NAD$^+$ capping**

We showed that initiation with NAD$^+$ stabilises short transcripts and favours promoter escape by E. coli RNAP in vitro [8]. Whether this stabilisation comes via additional base pairing of cap with the -1 position of the promoter (since NAD$^+$ has a nicotine mononucleotide moiety, which may potentially interact with DNA template at -1 position) remains somewhat controversial. Bird et al. showed that the identity of the base at position -1 (-1A vs -1C) affects the efficiency of capping [7]. However, our data suggests that the base at -1 affects initiation in general, without changing the preference for NAD$^+$ [8]. Indeed, in the crystal structure of the RNAP initiation complex with a short NADylated transcript, the NMN moiety does not make contacts with DNA but rather faces the protein [7] (Figure 1C). Also, in agreement with the crystal structure, we showed that amino acid changes in the rifampicin-binding pocket of RNAP strongly affected the efficiency of NAD$^+$ incorporation, suggesting that observed stabilisation of short capped RNAs is due to interactions between the NAD$^+$ cap and the RNAP rifampicin-binding pocket [8] (Figure 1C). Therefore, different configuration of rifampicin-binding pocket may affect NADylation of RNA capping in different bacteria. In contrast to NAD$^+$, the incorporation of UDP-containing cell wall precursors was not affected.
by the amino acid substitutions in the rifampicin-binding pocket.

Cofactors bound at +1 position may potentially interact with the 3.2 region of initiation factor $\sigma^{70}$, which protrudes towards the RNAP active centre [10]. However, we found that a mutant version of $\sigma^{70}$ lacking region 3.2 ($\sigma^{70\Delta 3.2}$) had no effect on incorporation of NAD$^+$, NADH, FAD, UDP-Glucose or UDP-GlCNac. In contrast, the mature cell wall precursor UDP-MurNac-pentapeptide, was incorporated by the $\sigma^{70\Delta 3.2}$ mutant of RNAP much more efficiently, suggesting that region 3.2 of $\sigma^{70}$ may serve to prevent incorporation of advanced cell wall precursors [8] (Figure 1C). Region 3.2 is absent from many sigma factors, suggesting that alternative sigma subunits may allow capping with bulky substrates.

Decapping enzymes for non-canonical caps

The discovery of NudC (NUDIX nicotinamide pyrophosphohydrolase) as an enzyme that removes the NAD$^+$ cap from RNA made the parallel between classic eukaryotic and non-canonical capping processes even more striking. E. coli NudC was initially described as a housecleaning enzyme hydrolyzing the pyrophosphate bond of NAD$^+/NADH$ to produce nicotinamide mononucleotide (NMN$^+/NMNH$) and AMP [11]. Recently, it was shown that NudC efficiently removes the NAD$^+/NADH$ cap to produces 5′-monophosphorylated RNA [1]. In eukaryotes, the role of NudC in decapping could be played by NUDIX hydrolases NPY1 in Saccharomyces cerevisiae and Nudt19 in Oryza sativa, which both showed decapping activity in vitro [12]. The activity spectrum of the bacterial NudC is relatively wide; it can remove several ADP analogues from RNA in vitro, including DP-CoA [7], consistent with its hydrolase activity towards a broad range of dinucleotides [11].

Removal of cap by NudC was proposed to be the first stage in the degradation of capped RNA to produce a monophosphorylated species, which are a preferred substrate for endonuclease RNaseE [13]. Curiously, however, NudC was not associated with McaS (IsrA) sRNA [14], one of the most highly NADylated sRNAs in E. coli (Figure 1A), while other known components of the RNA degradation machinery, such as RNaseE, RNA helicase RhIE and PNPase, were present [14]. This may suggest that the involvement NudC in RNA maturation might be more complex. Differential decapping by NudC, and its association with target RNAs, could be influenced by the secondary structures of RNAs, as NudC is single-strand dependent [13].

NudC is orthologous to the RppH NUDIX hydrolase, which removes pyrophosphate from triphosphorylated RNA (leaving 5′ monophosphate) [15]. A number of additional poorly characterised NUDIX hydrolases in E. coli [16] suggests that there might be more potential decapping enzymes for different caps. Notably, neither NudC nor RppH are essential for E. coli under normal growth conditions, suggesting possible redundancy of the decapping activities.

Human mitochondrial RNAP efficiently caps RNA with NAD$^+$ and other ADP-containing cofactors

Recently, mitochondrial transcripts capped with NAD$^+$ were detected in human cells [5]. Mitochondria contain a major cellular pool of NAD$^+$ (up to 70%), where it is used for redox reactions and for signalling [17]. We explored the possibility that mitochondrial RNAP (mtRNAP) could cap RNA via a mechanism that is similar to multi-subunit RNAPs. We found that human mtRNAP (hmRNAP) efficiently initiates transcription with NAD$^+$, NADH, FAD and DP-CoA on the light strand promoter (LSP; one of the only two human mitochondrial promoters) in vitro (Figure 2). The efficiency of initiation with NAD$^+$ was approximately 25% compared to ATP, while the other cofactors showed of between 10 to 15%. Our results suggest that mtRNAP is likely to be responsible for adding a NAD$^+$ cap to mitochondrial transcripts. Capping in human mitochondria might have consequences for both translation and replication in these organelles. The initially transcribed sequences from both mitochondrial promoters are precursors of tRNAs. It is therefore possible that 5′ NADylation might affect their maturation process. Additionally, RNA synthesised from the LSP promoter serves as replication primer [18], and its capping might influence initiation of replication, primer removal and subsequent DNA ligation.

Emerging physiological roles of non-canonical capping

The first experimentally confirmed role for non-canonical NAD$^+$ cap in bacteria is an increased
resistance to degradation, shown for RNAI in the absence of NudC processing [7]. However, this remains controversial, since in other studies [1] deletion of NudC did not affect the overall stability of the RNAI and GcvB populations, the two RNAs most heavily NADylated in vivo. Moreover, overall stability of NADylated sRNAs varies widely in wild type E. coli, and there is no direct correlation between NADylation and stability (Figure 1A). Notably, in contrast to E. coli, NADylation in eukaryotes promotes mRNA decay [5] via decapping by the DXO enzyme, which might additionally supply its 5’-3’ degradation activity.

The existence of subpopulations of capped RNA may play a potential role in bistability, the creation of phenotypic variability among clonal population that bacteria use in processes such as dormancy, persistence and sporulation [19]. Capping with dinucleotide analogues might play role in number of regulatory processes involving unstable regulatory RNAs. One example of such process is the type I toxin-antitoxin systems in bacteria, based on translational repression of toxin mRNA by an antisense RNA. This idea is supported by high in vivo NADylation of QUAD (sib) RNA (Figure 1A) – antitoxin sRNA preventing the production of the small protein that depolarises the cellular membrane [20].

The extent of capping could be responsive to the changes in cellular metabolism. For example, in E.coli, the proportion of NAD⁺ capped RNAI found in stationary phase compared to exponential phase was two-fold higher [1]. Similarly, in yeast, more capped RNA was found in cells grown on synthetic media compared to those grown on the rich media [4]. Since the NAD⁺/NADH balance plays key role in cellular redox homeostasis, capping could connect transcription directly to the cell’s redox state. Given the affinity for NAD⁺ is roughly the same as for NADH (Figure 1B), changes in their cellular concentrations will be directly mirrored by the capping of RNA with NAD⁺ or NADH. The functioning of such signalling of course would depend on a mechanism recognising NADylated from NADHylated RNAs. UDP-Glucose and UDP-GlcNAc are the initial substrates for the cascade of reactions leading to the synthesis of cell wall components. It would be tempting to speculate that expression of some cell wall synthesising enzymes could be controlled directly by the pool of
UDP-GlcNAc via capping of +1U transcripts. In general, being rare, RNA modification with cell wall precursors might provide a better regulatory potential, compared to ubiquitous capping with ADP analogs.

Capping might potentially influence translation initiation on a leaderless mRNA.

Another potential cellular role of capping could be the targeting of a specific RNA species, via its cofactor cap, to a protein with affinity for the cognate cofactor, or to a specific subcellular location, e.g. to the vicinity of the membrane in the case of UDP-GlcNAc capped RNA.

Intriguingly, we showed that a number of rifampicin resistant RNAPs, including the most widespread clinical isolates, are deficient in capping [8]. This deficiency may contribute to the overall fitness reduction, characteristic for a rifampicin resistant strains [21].

To conclude, despite recent progress, the understanding of non-canonical RNA capping by RNAPs is still patchy. More information is needed to put this type of RNA 5’ modification into the category of functional capping, rather than a side reaction of RNAPs. Currently it is hard to envisage a “classic” regulation by the stochastic process of capping. Nevertheless, this “unavoidable” side reaction has to be either used to some advantage or, alternatively, fought against. Both scenarios would have wide ranging cellular consequences with multiple regulatory mechanisms involved. It seems that RNAP has a limited ability to control capping process, apart from alternative sigma factors exchange. It is more feasible to regulate amounts of capped RNA post-transcriptionally, by the linked processes of decapping, alternative folding and RNA chaperons binding. Being a stochastic process, capping might generate variability in a clonal population, which can be exploited at a population level to benefit the organism in adaptation to rapid change in growth conditions. At present, an exact roles of various non-canonical caps in bacteria, eukaryotes and mitochondria are still to be established, as well as full repertoire of enzymes that process non-canonically capped RNAs are to be characterised.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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