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Regulation and function of CMTR1-dependent mRNA cap methylation

Francisco Inesta-Vaquera[1] and Victoria H Cowling[1]

[1] Centre for Gene Regulation and Expression, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK

Abstract

mRNA is modified co-transcriptionally at the 5' end by the addition of an inverted guanosine cap structure which can be methylated at several positions. The mRNA cap recruits proteins involved in gene expression and identifies the transcript as being cellular or "self" in the innate immune response. Methylation of the first transcribed nucleotide on the ribose 2'-O position is a prevalent cap modification which has roles in splicing, translation and provides protection against the innate immune response. In this review we discuss the regulation and function of CMTR1, the first transcribed nucleotide ribose 2'-O methyltransferase, and the molecular interactions which mediate methylated 2'-O ribose function.

Introduction

The mRNA cap is a critical structure in gene expression and in innate immunity. It protects pre-mRNA from exonucleases during transcription, recruits factors involved in RNA processing, nuclear export and translation, and identifies transcripts as "self" to protect against the innate immune response. Formation of the mRNA cap initiates as nascent pre-mRNA emerges from the RNA pol II complex. The rate and extent of mRNA cap formation is regulated by cellular signalling pathways resulting in changes in cell physiology and fate.

mRNA cap formation

The mRNA cap structure was first characterized in the 1970s as a 5' blocked, methylated structure in viral mRNAs and was recognised shortly after to be a feature of eukaryotic mRNA [1,2]. Nascent pre-mRNA is transcribed with a terminal triphosphate (ppp(5')N, N is the first transcribed nucleotide). The terminal phosphate is removed and GMP (guanosine monophosphate) is added to create the guanosine cap intermediate (G(5')ppp(5')N). This structure has a 5' to 5' triphosphate linkage thought to be unique to RNA pol II transcripts. Subsequently the guanosine cap is methylated on the 7'-N position and the first and second transcribed nucleotides can be methylated on the ribose 2'-O positions. More rare modifications are found in the cap structure including 6'-N methylation if the first transcribed nucleotide is adenosine [3,4]. 6'-N methylation of the 1st nucleotide adenosine is reversible and increases transcript stability by conferring resistance to decapping enzymes. Furthermore, any other RNA modification, including 1'-N adenosine methylation, may be found on the first transcribed nucleotides, thus becoming part of a cap structure[5]. Recently novel nicotinamide adenine dinucleotide (NAD^+) caps have also been isolated on mRNA, which target the transcripts for degradation [6].

The first step in mRNA cap formation is addition of the guanosine cap intermediate, (G(5')ppp(5')N). This is catalysed by the sequential actions of a triphosphatase and a guanylyltransferase. The catalytic cores of the different capping enzymes are largely conserved in eukaryotes whereas the holo-enzymes have different configurations. In this review, we focus discussion on the mammalian capping enzymes. In mammals, a single enzyme, RNGTT (RNA guanylyltransferase and 5' triphosphatase) possess both triphosphatase and guanylyltransferase activities [7,8]. Guanosine cap 7'-N methylation, creating m7G(5')ppp(5')N, is catalysed by RNMT (RNA guanine-7 methyltransferase), which is found in a complex with its activating subunit RAM (RNMT-activating miniprotein) [9-11]. Methylation of the first and second transcribed nucleotides at the ribose 2'-O position is catalysed by CMTR1 and CMTR2, respectively [12,13]. Whilst analyses are in agreement that m7G(5')ppp(5')Nm is the most abundant mRNA cap structure, the relative proportion of the

different cap methyl groups is an area of active research [6,14,15]. Previously, mass spectrometric analyses of cap structures were restricted to cell lines and large organs. With recent improvements in chromatography and mass spectrometry, analysis of mRNA caps in an increasing array of primary tissues and cells will be possible [16].

mRNA cap function

Following its discovery, the mRNA cap was found to protect mRNA from exonucleases, and to recruit protein complexes involved in RNA processing, nuclear export and translation and initiation [17,18]. In the nucleus, the binding of CBC (Cap Binding Complex) to the mRNA cap promotes pre-mRNA splicing, nuclear export and influences pre-mRNA 3' end processing and nonsense mediated decay [19]. eIF4E and the eIF4F complex also bind to the cap and promote nuclear export and translation initiation [20]. Over the last decade, formation of the mRNA cap has been recognised to be regulated in a gene-specific manner by the influence of transcription factors, signalling pathways and developmental pathways [21-24]. Regulation of mRNA cap formation results in alterations in gene expression and subsequent changes in cell function and fate. Recently CMTR1 has been recognised to be regulated in the innate immune response, and to have a critical role in the recognition of self-RNA [25]. This has reinvigorated interest in 2'-O methylation of the first nucleotide ribose.

First transcribed nucleotide 2'-O methylation in gene expression

<u>First nucleotide 2'-O methylation and splicing</u>

The 7-methylguanosine cap binds to CBC which interacts with splicing factors and thus promotes splicing [26]. Whether 2'-O methylation of the first transcribed nucleotide of mRNA has a role in splicing remains to be seen. However, 2'-O methylation is present on the first nucleotide of U1, U2, U4 and U5 snRNAs and is important for their function in the mechanism of splicing [27]. snRNAs are RNA pol II transcripts and therefore receive a 7-methylguanosine cap. Following export into the cytoplasm, further methylation yields a (2,2,7) trimethylguanosine cap and the snRNA-protein complex (snRNP) is re-imported into the nucleus to function in splicing. In the nucleus, the snRNAs are further modified with pseudouridination, and first and internal nucleotide ribose 2'-O methylation. *In vitro*-transcribed U2 snRNA cannot reconstitute splicing in U2-depleted Xenopus oocytes or HeLa nuclear extract indicating that modifications of U2 snRNA are important for snRNP biogenesis and/or pre-mRNA splicing [28,29]. Only when both the (2,2,7) trimethylguanosine cap and internal modifications are acquired does synthetic U2 snRNA become fully functional [30]. Moreover, 2′-O-methyl groups at positions 1, 2, 12, and 19, but not at position 11, are individually required for the function of U2 snRNA. The function of the 2'-O methylation is not clear, but is required for the E complex formation [31]. Interestingly, snRNPs

first and second nucleotide 2'-O methylation only occurs in higher eukaryotes, indicating that CMTR1 and CMTR2 are potentially involved in these methylations [32].

First nucleotide 2'-O-methylation in mRNA translation

The 7-methyl guanosine cap is critical for eIF4E binding and translation initiation [33]. eIF4E binds to the scaffold protein eIF4G and helicase eIF4A forming the eIF4F complex, which recruits the 40S ribosomal subunit to mRNA. First nucleotide 2'-O methylation was recognised to have a role in translation shortly after its discovery; it was demonstrated to enhance ribosome binding and translation [34,35]. *In vivo*, first nucleotide 2'-O methylation was demonstrated to be important for translation during Xenopus or sea urchin development. In 1985, Caldwell and Emerson reported significant upregulation of 1st nucleotide 2'-O methylation in maternal mRNA following the fertilization of sea urchin embryos. Further studies by the Ritcher lab reported that first nucleotide 2'-O methylation is upregulated during Xenopus oocyte maturation (Kuge, 1995)[36]. Of note, c-mos mRNA translation and resultant oocyte maturation was found to be dependent upon first nucleotide O-2 methylation [36].

CMTR1: first nucleotide 2'-O-methyltransferase

First nucleotide 2'-O methyltransferase activity was initially characterised in vaccinia virus and HeLa cells [37,38]. However, the methyltransferase responsible for first nucleotide 2'-O methylation in mammalian cells, CMTR1 (Cap Methyltransferase 1), was only identified recently by Belanger *et al.* (Belanger et al, 2010) (Figure 1). *In vitro*, CMTR1 catalyses methylation of ribose 2′-OH group on the first transcribed nucleotide of guanosine-capped RNA. Although CMTR1 requires a cap guanosine structure (GpppN) in its substrates, it acts independently of N-7 methylation [12,39]. CMTR1 is also unable to methylate internal residues. Extracts of HeLa cells depleted of CMTR1 are defective for first nucleotide 2'-O methylation, indicating that there is not a completely redundant methyltransferase present in these cells [40]. Belanger *et al* also reported that knock-down of CMTR1 does not impact on global translation as measured by ^{35}S methionine incorporation, although it is a possibility that the remaining CMTR1 may be sufficient to maintain translation. As discussed above, first nucleotide 2'-O methylation has been linked to mRNA translation in several eukaryotic systems and deletion of the 1st and 2nd transcribed nucleotide 2'-O-methyltransferase in Trypanosomes results in a significant reduction in global translation [41].

CMTR1 had previously been identified as KIA0082/ISG95, a protein implicated in the response to interferon treatment and viral infection [42-45]. Following these studies CMTR1 was characterised as a multi-domain protein with a nuclear localization signal, G-patch domain, a RrmJ/FtsJ methyltransferase domain, a non-functional cap guanylyltransferase-like domain

and a WW domain [46]. Deletion of the domains C-terminal to the methyltransferase (guanylyltransferase-like and WW domains) reduces the activity of CMTR1 *in vitro* and therefore these domains are likely to contribute to substrate recruitment and/or structural configuration [39]. The CMTR1 WW domain interacts with the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNA Pol II) [46]. Whether CMTR1 has enhanced affinity for a particular CTD phosphorylation state, remains to be determined. Since first nucleotide 2'-O-methylation does not inhibit or enhance 7'-N methylation of the cap guanosine it is not clear whether RNMT or CMTR1 methylates the cap first, or indeed whether they have an order of action.

Structural analysis of the CMTR1 catalytic domain resolved a Rossman-like fold methyltransferase domain and determined the mechanism of guanosine cap recognition [39]. In the CMTR1 active site, the methylated guanosine (m7G) is accommodated in a deep pocket and the transcribed nucleic acids adopt an L shape with nucleotide 1 located at the bend. Analysis of the positioning of the RNA in the exit of the active site suggests that substrate binding and methylation are sequence-independent. However, whether the domains N- and C-terminal to the methyltransferase domain interact with RNA and/or other proteins, potentially providing substrate specificity, will require further exploration. As discussed above, CMTR1 has other domains (G-patch, WW and guanylyl-transferase-like) which have been demonstrated to influence activity [39]. The N- and C-terminal domains of CMTR1 are intrinsically unstructured, which to date has impaired structural characterisation of the full-length enzyme.

CMTR1 and 2'-O-methylation in "self"-RNA *distinction*

In recent years, the cap1 structure (m7GpppNm) has emerged as a key factor in "self-RNA" identification during the innate immune response [47] (Figure 2). "Non-self" RNA intermediates from viruses are recognised by the cellular innate immune system as foreign, triggering cellular mechanisms which protect the cell. The role of cap1 in the innate immune response was revealed by studying the viral first nucleotide 2'-O methyltransferases in the evasion of the innate immune system [48]. First nucleotide 2'-O methylation was required for optimal infectivity; viruses lacking this methylation were unable to propagate. 2′-O methylation of viral RNA enhanced virulence through evasion of intrinsic cellular defense mechanisms involving IFN-induced proteins with tetratricopeptide repeats (IFIT proteins) [48]. This study suggested that 2'-O methylation of the 5' cap distinguishes self (cellular) and non-self (viral) RNA. In addition, this study suggested co-evolution of 2'-O methylation and viral strategies for infection.

Cap1 functions to prevent the aberrant activation of innate immune response readers, RIG-I and MDA5. RIG-I (Retinoic Acid Inducible Gene-I), an innate immune receptor, is a cytoplasmic protein which detects triphosphate RNA (pppRNA) and induces cellular signaling responses that ultimately drive to an interferon response. The impact of individual cap modifications, including cap guanosine 7'-N methylation and first nucleotide 2'-O methylation, on RIG-I activation was analysed using synthetic RNA [25,49]. First nucleotide 2'-O methylation was found to prevent RIG-I activation, in a mechanism utilizing a highly conserved histidine residue (H830). In cells, experimental interference with this "key-lock" mechanism elicits an interferon response similar to that triggered by viral infection. The RIG-I H830A mutation results in stimulation of RIG-I by endogenous mRNA. Furthermore, suppression of CMTR1 results in RIG-I stimulation in primary human fibroblasts and induction of IFN-beta mRNA in primed A549 cells. MDA-5, another RIG-like receptor, has also been linked to the induction of type I interferon by viruses deficient in 2′-*O*-methyltransferase activity. MDA-5 recognizes the internal duplex structure of long dsRNA [50]. Whether the composition of the viral and endogenous mRNA cap structure has a role in this interaction remains an open question [51]. Upon sensing of viral RNA, the cellular proteins RIG-I or MDA5 induce type I interferon (IFN) secretion, resulting in upregulation of antiviral IFN-induced proteins (IFIT) in the infected and neighboring cells [52]. These IFIT proteins include those which inhibit virus replication or protect against new infection.

When the innate immune response is triggered, the expression of effector proteins protect the host from the external agent [52]. IFIT1 is a key effector of the innate immune response, which blocks the translation of viral mRNA by competing with the eIF4F complex for binding to the cap. Over-expression of human IFIT1 in cells or reticulocyte lysates reduces translation significantly [53]. Specific features of mRNAs were indicated to influence their sensitivity to IFIT1 (Young et al., 2016). Structural analysis of IFIT1 revealed that it forms a tight interaction with ribose 2′-hydroxyls of first and second nucleotides. Cellular mRNAs methylated at these positions are not recognised by IFIT1 thus restricting IFIT1 activity to unmethylated viral mRNAs [54]. Importantly, methylation of the first nucleotide alone is not sufficient to protect all endogenous mRNAs from IFIT1. 2'-O methylation of the second transcribed nucleotide also contributes to inhibition of IFIT1 action on cellular RNA, particularly on susceptible RNA sequences and at high IFIT1 concentrations [54]. This suggests a role for CMTR2, the second transcribed nucleotide 2'-O methyltransferase in innate immunity. Furthermore, RNA sensors may also have functional interactions with the second nucleotide, as single 2'-O methylation of the second transcribed nucleotide partially abolished RIG-I activation (Schubert et al., 2015).

Regulation of CMTR1

Since CMTR1 has important roles in translation and innate immunity, its regulation is of interest. Analysis of mouse tissues revealed differences in the extent of 1st nucleotide 2'-O methylation suggesting tissue specific regulation of CMTR1 expression or activity [15]. CMTR1 has several domains through which subcellular localisation, activity or expression may be regulated, potentially by protein:protein interactions or by post-translational modifications. The 7'-N cap methyltransferase, RNMT-RAM, has been demonstrated to be regulated at the level of expression, recruitment to chromatin and activity [21,23,24,55].

CMTR1 expression has been observed to be upregulated during the innate immune response. In four independent studies where an interferon response was elicited, the expression of CMTR1 was upregulated ~3-fold [42-45]. Despite differences in model cell lines or organisms, upregulation of CMTR1 expression is transient and efficiently cleared after the initial anti-viral response. In human fibroblasts, CMTR1 knockdown was sufficient to elicit an interferon-like innate immune response [25].

Future perspectives : ribose O-2 methylation and disease

A causative effect for CMTR1 in human diseases has yet to be elucidated. However, significant changes in CMTR1 mRNA levels in patients experiencing asthma exacerbations have been observed, suggesting a role for CMTR1 in the pathogenesis of asthma exacerbations [56]. Highly pathogenic viruses include RNA genome-based viruses which give rise to zoonotic and epidemic diseases [57]. Inactivation of flavivirus, coronavirus and poxvirus families 2'-O MTases increased sensitivity to antiviral actions of type I IFN [58]. Therapeutic targeting of 2'-O MTases may offer a new avenue to treat some of these viral infections.

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The authors declare that there are no conflicts of interest.

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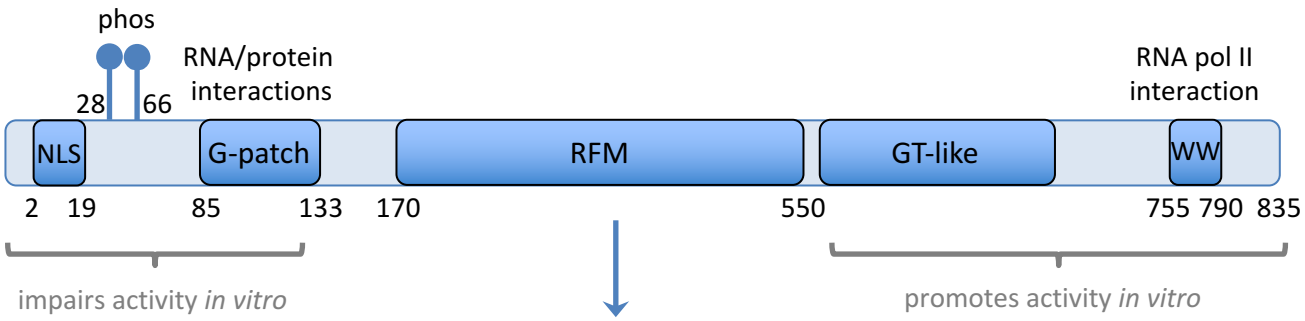

Figure 1. CMTR1 functional domains

NLS, nuclear localization signal; G-patch, glycine rich domain; RFM, Rossman-fold methyltransferase domain; GT-like, guanylyltransferase-like domain; WW, protein interaction domain; phos, amino acid 28-66 multiple phosphorylation sites (sites with more than 5 references in Phosphosite plus); (Haline-Vaz et al, 2008; Smietanski et al, 2014)

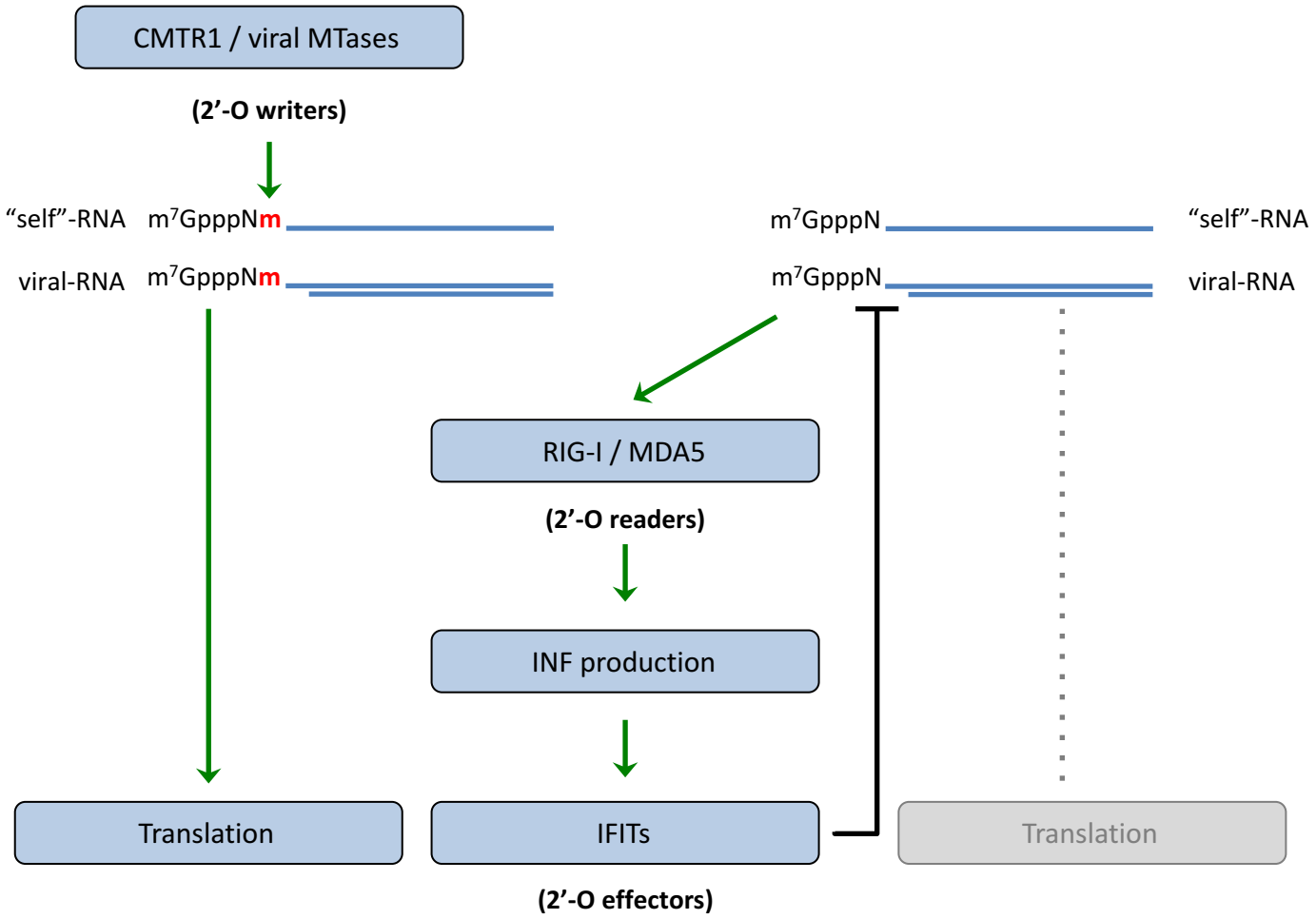

Figure 2. "Self"-RNA recognition and immune tolerance to 2'-O methylated RNA

CMTR1 and viral methyltransferases (Mtases) catalyse first nucleotide ribose 2'-O methylation, which prevents transcript recognition by RIG-I or MDA5. In the absence of ribose 2'-O methylation, RIG-I or MDA5 elicit the interferon response which includes expression of IFIT proteins. IFIT1 binds to transcripts unmethylated on ribose 2'-O to inhibit translation. m^7GpppN, 7'-N cap (N, first nucleotide); **m**, ribose 2'-O methylation; Green lines, activity or permissive effect; Black line, binding and repression; Grey dotted lines, absence of translation.