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A novel synthesis and detection method for cap-associated adenosine modifications in mouse mRNA

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A method is described for the detection of certain nucleotide modifications adjacent to the 5' 7-methyl guanosine cap of mRNAs from individual genes. The method quantitatively measures the relative abundance of 2'-O-methyl and N^6,2'-O-dimethyladenosine, two of the most common modifications. In order to identify and quantitatify the amounts of N^6,2'-O-dimethyladenosine, a novel method for the synthesis of modified adenosine phosphoramidites was developed. This method is a one step synthesis and the product can directly be used for the production of N^6,2'-O-dimethyladenosine containing RNA oligonucleotides. The nature of the cap-adjacent nucleotides were shown to be characteristic for mRNAs from individual genes transcribed in liver and testis.

I n most eukaryotes, polymerase II (Pol II) dependent transcripts are modified co-transcriptionally at their 5'end by the addition of a 7-methyl guanosine (m^7G) cap to the first nucleoside of the nascent transcript. If no additional modifications are made to the cap-adjacent nucleotides, the structure is referred to as a cap0. In yeast and plants, only cap0 structures are found, however, in animals, modifications of the two nucleotides adjacent to the m^7G are possible The methylation of the first nucleotide on the ribose residue (Fig. 1a) will form a cap1 structure[1]. Cap1 messages can be converted to cap2 structures if a further 2'ribose methylation takes place on the next nucleotide following the cap1 (Fig. 1b). These methylation steps are sequential and carried out by nuclear located methylases[1–3]. In a transcript where the first nucleotide is an adenosine in a cap1 structure, a further methylation of the 2'-O-dimethyladenosine (Am) at the N^6 position of the adenine can take place to give N^6,2'-O-dimethyladenosine (m^6Am) (Fig. 1c)[4]. The methyltransferase that carries out this modification has been partially characterized and appears to be predominantly located in the cytoplasm[5]. Transcription start sites of focused promoters are usually contained within an initiator motif (Inr) of general sequence YYANWYY (Y=pyrimidine, N-any nucleotide, W=A or T) where the A is the principal start site and neighbouring nucleotides may be used to varying degrees[6]. Since, A is often the first nucleotide after the m^7G cap, its modification may have a functional role. In order to study the effect of m^6Am present in cap1 messages, the chemical synthesis of RNA oligonucleotide sequences that contain m^6Am in well-defined positions, is necessary. A number of methods exist in the literature for the preparation of N^6-methylated adenosine derivatives, with the Dimroth rearrangement being perhaps the most well known[7–9]. This transformation relies upon an initial N1-methylation of the adenine ring followed by an alkali-mediated rearrangement to give the N6-methylated adenine product. An alternative method for accessing N6-alkylated adenines has been developed, which involves the nucleophilic aromatic substitution of adenine derivitives, that are activated at the 6-position, with amine nucleophiles (i.e. $MeNH_2$)[10–13]. In order to obtain the N6-methylated phosphoramidite reagents from these adenine derivatives, a number of additional synthetic steps are required, which results in a 6 to 8 step synthesis being needed to produce each phosphoramidite reagent[10–13]. Such approaches are not realistic other than in synthetic chemistry laboratories.

An interaction of m^7G capped transcripts with the nuclear cap-binding complex (CBC) is important for promoting correct splicing[14] and 3'end formation[15]. After export from the nucleus, translation of most mRNAs is initiated with the recognition of the m^7G cap by eukaryotic translation initiation factor 4E (eIF4E)[16]. This cap binding step is usually rate-limiting for translation. The m^7G cap also protects the message from 5' to 3' exonuclease digestion. Compared to the well characterised role of m^7G, relatively little is known regarding the function of 2' ribose methylation on the following nucleotides. The presence of a cap1 structure may
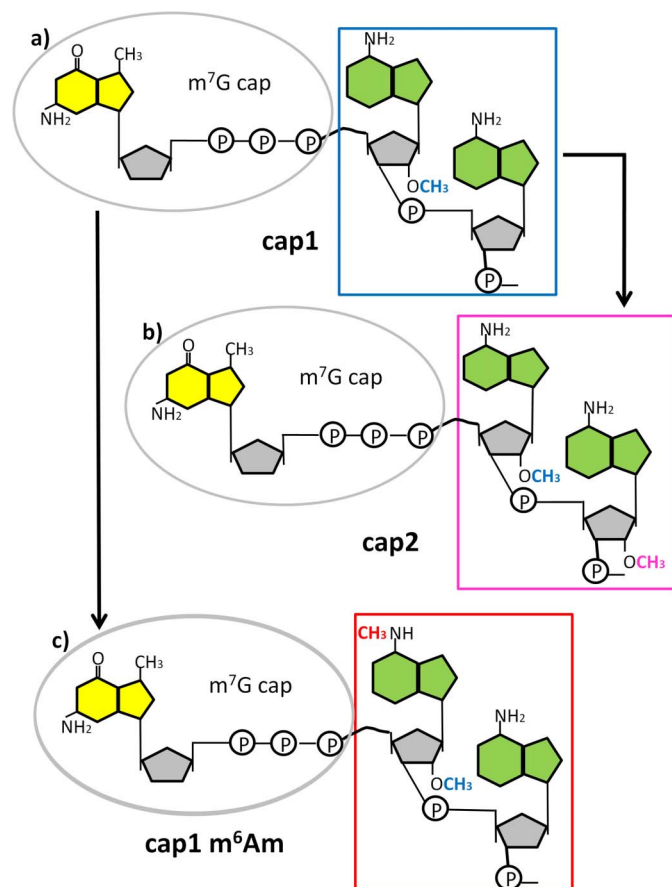
Figure 1 | Cap adjacent modification in higher Eukaryotes. Formation of the cap1 structure takes place by 2'-O –methylation on the ribose residue on the first nucleotide adjacent to the m7G cap. The example shows a structure with adenosine as the first nucleotide, adjacent to the m7G cap (a). The cap1 structure can then be converted to a cap2 by a further 2'-O-ribose methylation on the following nucleotide (b), or converted to a cap1 m6Am by placing a further methyl group on the adenosine in the N^6 position (c). The figure is a schematic representation of the mRNA cap structures. The yellow structure represents 7-methyl-guanine, the green represents adenine and the grey pentagon is ribose. On the ribose only the methylated functional groups are shown.

promote binding to ribosomes[17] and can increase translational efficiency in vitro[18, 19]. Many animal viruses encode their own cap1 methylases[20, 21] and this may also help them to limit host immune responses[22, 23]. Whilst viral cap1 methylases are common viral cap2 methylases and m6Am methylases have not been reported. However, m6Am and cap2 structures can be found in viral mRNAs in vivo, presumably as a result of the action of the host cell's enzymes[24]. The functional consequences for an mRNA of possessing m6Am in a cap1 or a cap2 structure are not known.

In order to investigate the roles of these cap-associated modifications, we have developed enzymatic techniques, in combination with thin layer chromatography (TLC) that allow the detection of m6Am and other cap nucleotides. We also describe a direct synthetic method for the preparation of the desired m6Am phosphoramidite reagent in a single step from commercially available starting material.

Through the use of this synthetic substrate we have demonstrated that a thin layer chromatography (TLC) method is quantitative for determining m6Am:Am ratios, and allows the relative proportions of Am and m6Am to be rapidly determined for mRNAs from different cell types and also for caps of messages from individual genes. We further give evidence that the relative proportion of m6Am varies in a characteristic way for mRNA from different mouse organs.

Results

Synthesis and detection of N^6, 2'-O- dimethyladenosine. We have previously used TLC assays to detect and quantify N^6-methyladenosine (m6A)[25], a modification found internally in the mRNA of many eukaryotes[26–29]. This method was modified to label and detect only the first nucleotide after the cap. In order to use this approach to compare the relative proportions of m6Am and Am in cap structures from different sources, it was first necessary to establish the mobility of m6Am in the TLC assay and to demonstrate that T4 polynucleotide kinase does not preferentially label either of these nucleotides. As neither m6Am triphosphate or m6Am phosphoramidite are commercially available, a novel method for its synthesis and incorporation into RNA oligonucleotides was developed.

In order to get quick access to the required m6Am phosphoramidite building block for solid-phase oligoribonucleotide synthesis, we performed a selective one-step methylation of the commercially available 2'-OMe-Bz-A-CE phosphoramidite. Due to its high reactivity and sensitivity towards acids and bases, Aritomo's relatively mild phase transfer catalysis (PTC) method was used[30]. Under these conditions (iodomethane, sodium hydroxide, tetrabutylammonium bromide) the N^6-methylated product was obtained as the major product in 56% yield, with the N^1-methylated isomer being formed as a byproduct in 25% yield. Due to their different Rf values, the N^6- and N^1-methylated products were easily separated via column chromatography, and the purified m6Am phosphoramidite was used directly for RNA synthesis.

All oligoribonucleotides were synthesized using the standard protocol for solid-phase RNA synthesis with a 15 min coupling time per nucleotide. The last coupling step was followed by a standard DMTr-off procedure, the oligoribonucleotides were removed from the solid support, deprotected, precipitated and desalted. MALDI mass spectrometry was used to confirm the mass of all RNA oligonucleotides produced

Thin layer chromatography of modified adenosines. Radioactively 5' end labelled oligonucleotides SK-526 (m6AmGGGCUGCU) and SK-524 (AmGGGCUGCU) were digested to release pm6Am and pAm then mixed with a combination of 5' radioactive labelled pm6A and unmodified nucleotide monophosphates for which relative mobilities are well characterised. This mixture was separated by 2D TLC using the solvent systems previously described[25] to establish the position of pm6Am (Fig. 2).

To show that T4 polynucleotide kinase does not preferentially label m6Am or Am, the RNA oligonucleotides SK-524 and SK-526 were mixed in different ratios, end labelled, digested with P1 nuclease and separated by TLC. The spots corresponding to pm6Am and pAm were then quantified using phosphorimaging. The results demonstrated that both nucleotides are labelled with equal efficiency by T4 polynucleotide kinase (Fig. 3)

Labelling and analysis of the first cap adjacent nucleotide. To label the first nucleotide following the m7G, poly(A) RNA was prepared from various mouse organs then digested with tobacco acid pyrophosphatase to remove the m7G cap. The exposed 5' ends were dephosphorylated with alkaline phosphatase and after phenol/chloroform extraction and ethanol precipitation, the mRNA transcripts were radiolabelled at their 5' end using T4 polynucleotide kinase in the presence of >20 fold excess of [γ-32P] ATP. The labelled RNA was digested to monophospho-nucleotides by P1 nuclease prior to TLC separation. Using this method, new spots corresponding to the 2'-O methylated nucleotides are apparent in the labelled samples after cap removal (Fig 4A). A spot corresponding to pm6Am is readily detectable in all mRNA samples tested, and with pm6Am:pAm ratios of between 15:1 (brain) and 2:1 (liver) it appears that m6Am is more prevalent at the cap1 than is Am (Fig. 4A). Under these labelling conditions, where both ATP and polynucleotide kinase are in excess, the intensity of the spots corresponding to the unmodified
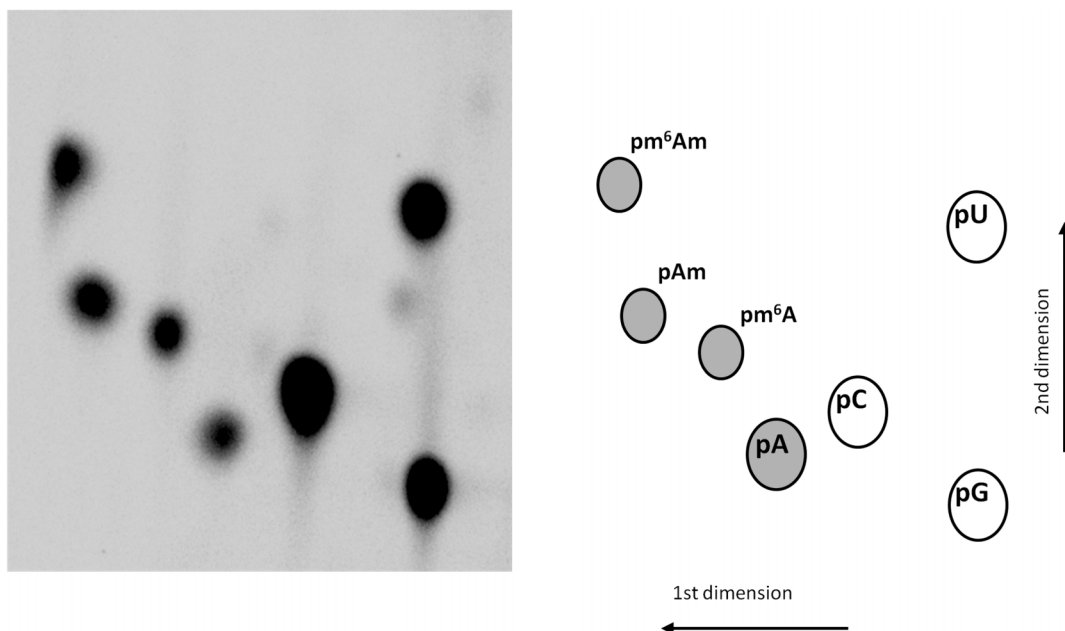
Figure 2 | Relative mobility of modified adenosines using 2 dimensional thin layer chromatography. RNA oligonucleotide fragments of known compositions were labelled at their 5' ends with[32]P, digested to 5' monophosphates and separated by 2 dimensional thin layer chromatography in order to establish the mobility of pm6Am relative to other nucleotides. Adenosine and its methylated derivatives are indicated by shading.

nucleotides, as well as the other 2' methylated nucleotides, is indicative of their actual relative abundance in the de-capped mRNA sample (Supplementary Figure S1 online).

Analysis of cap structures for mRNA transcripts from individual genes. In order to assay modifications on the first transcribed nucleotide for messages from individual genes, mRNA from liver and testis was de-capped and end-labelled as described above then
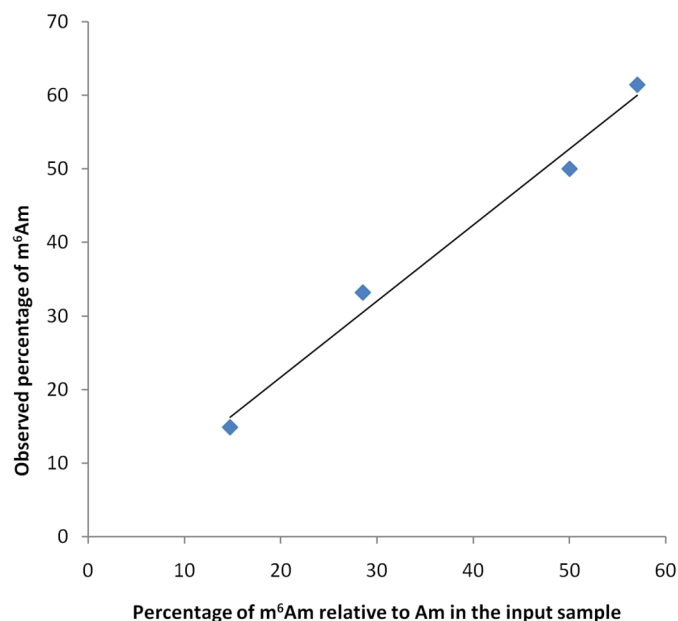

Figure 3 | T4 polynucleotide kinase does not preferentially label m6Am or Am containing ends. The oligonucleotides SK-526 (m6Am) and SK-524 (Am) were mixed in ratios of 5:1, 2:1, 1:1. 1:2 and 1:5, end labelled using T4 polynucleotide kinase, digested to 5' phosphate mononucleotides and separated by TLC. The relative intensities of the resulting spots were quantified using phosphorimaging. The calculated percentage of m6Am closely matched the actual m6Am percentage.

fragmented to ~120 nt. This was then hybridised to single stranded DNA targets corresponding to the 5' region of selected messages. These DNA targets were first cross-linked to 2 mm × 2 mm teeth cut from a Hybond N+ membrane. After hybridization and washing, the membrane was subjected to phosphorimaging (Fig. 4B). The individual teeth containing the labelled mRNA gene-specific fragments were then removed and digested to nucleotide 5' monophosphates using P1 nuclease. These samples were individually spotted onto TLC plates and developed as described (Fig. 4B). Four mRNAs were chosen for analysis, apolipoprotein A-I (*Apoa1*, BC012253), albumin (*Alb*, BC024643), protamine 2 (*Prm2*, BC049612) and poly(A) binding protein cytoplasmic 1 (*Pabpc1*, BC046233). *Alb* and *Apoa1* are predominantly liver expressed messages, whereas *Prm2* shows testis specific expression and *Pabpc1* is expressed highly in both organs. *Pabpc1* may be subjected to translational regulation, but unlike *Pabpc2*, it is present in actively translating polyribosomes of mouse testicular cells[31]. For both *Alb* and *Apoa1*, the labelled nucleotide in the cap adjacent position included A, G, U and C, as well as m6Am and Am. This is consistent with multiple alternative transcription start sites at and around the Inr[32]. In both cases, Am and m6Am appeared as the major nucleotide modifications; unlike the mixed mRNA starting material, 2'-*O*-methylcytosine was not present (compare Fig. 4A, B). The m6Am:Am ratios were 1.65:1 and 1.4:1 for *Apoa1* and *Alb* respectively, which is only slightly lower compared to that seen for the liver mRNA population as a whole. The *Prm2* mRNAs from testis tissues almost exclusively had m6Am at the cap adjacent position (Fig. 4B). With an m6Am:Am ratio of 40:1, this was four times larger than the m6Am:Am ratio for the testis mRNA population as a whole. In contrast the cap adjacent nucleotides of *Pabpc1* transcripts were predominantly unmodified, although a pronounced 2'-*O*-methyluridine was apparent in the sample from testis (Fig. 4B).

Discussion

Core promoters of Pol II transcribed genes may be focused, directing transcription initiation at a single site or cluster of adjacent sites, or they may be dispersed and have multiple start sites over a region of 50–100 nucleotides[6]. In case of focused promoters the transcription
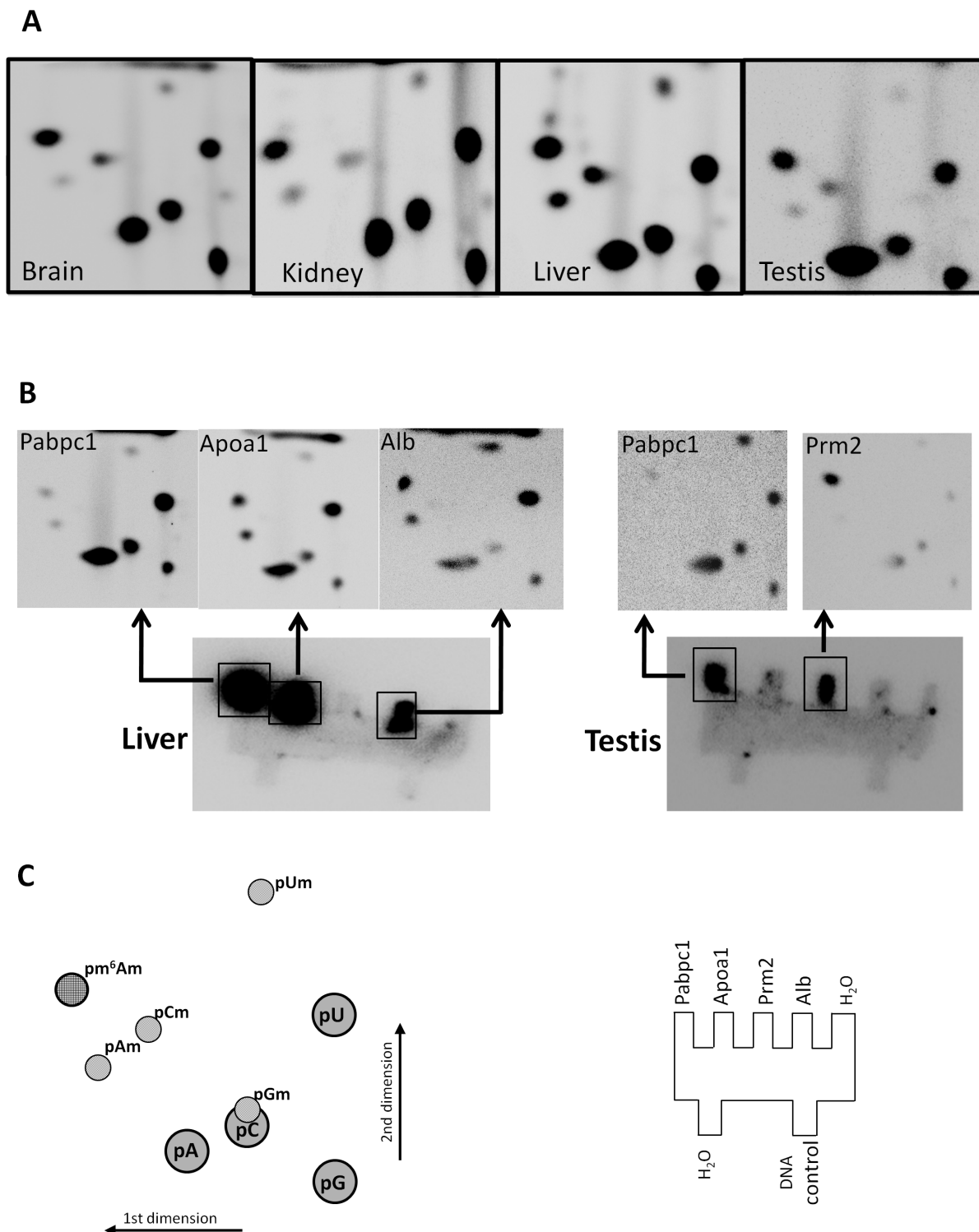
Figure 4 | Relative abundance of modified adenosines in mRNA from different mouse organs and in individual mRNAs. (A) 2'-*O*-methylated nucleotides are present in the mRNA populations from various mouse organs. Arrow indicates m⁶Am. Different organs have characteristic m⁶Am:Am ratios; brain (15:1), kidney (2.4:1), liver (2:1), testis (10:1). (B) Transcripts from individual genes have characteristic nucleotides or nucleotide modifications at the first nucleotide position; *Pabpc1* (poly(A) binding protein cytoplasamic 1), *Apoa1* (apolipoprotein A-I), *Prm2*, (protamine 2), *Alb* (albumin). (C) Schematic indicating the relative position of the nucleotide spots (left) and the location of the cold single stranded target sequences on the hybridisation template (right). Single stranded DNA from the yeast gene IME2 was used as a negative control DNA in the hybridisations.

start sites are contained within and around the Inr (YYANWYY), where the A is the principal start site and surrounding nucleotides are utilised to varying degrees[6]. Recent high-throughput sequencing from full length mRNAs, suggests that purine instead of A may be a better representation of the Inr consensus. However, most core promoters have several closely arrayed transcription start sites with different initiation rates rather than initiating at a single nucleotide[32]. Thus, most genes will give rise to a population of mRNAs that differ in their starting nucleotides. For example the *Prm2* message initiates predominantly at the central A of the Inr as well as a second A 3

nucleotides upstream, whereas, *Apoa1* strongly initiates at the A of the Inr but also uses surrounding U, G, C and sites at a significant level (Supplementary Figure S2 online) and (Fig. 4B).

Conversion of an adenosine following the m7G cap to Am (cap1) can be followed by further conversion to m6Am (Fig. 1a, and 1c). It is not known if this Am to m6Am conversion preferentially targets certain messages, or if the Am:m6Am ratio for messages from any one gene is merely a reflection of the general m6Am methylase activity of a particular cell type. The enzyme responsible for the formation of m6Am has been partially characterised[5]. It appears to be a cytosolic enzyme and is distinct from METTL3/MT-A70, the nuclear enzyme responsible for the formation of m6A at internal positions within mRNA[33].

Whilst there is evidence that cap1 methylation may increase translation efficiency[18, 19], the consequences of converting a cap1 Am to m6Am are largely unknown. Some crystallographic observations suggest that the first nucleotide after the m7G may influence interaction with eIF4E or other cap binding proteins. Crystallographic analysis of eIF4E in a complex with the dinucleotide m7GpppG, show the G in a disordered arrangement[34]. However, with m7GpppA as substrate, the A interacts with a C-terminal loop of the protein[35]. A key interaction of this C-loop is a hydrogen bond between Thr[205] and the amino group in the N^6 position of the adenosine. It is this amino group that is targeted for methylation by the m6Am methylase. Thus, N^6 methylation of a cap1 adenosine might influence the flexibility and orientation of the eIF4E C-terminal loop region. The ability to synthesize RNA oligonucleotides that contain m6Am in well-defined positions should facilitate future structure-function analysis. Therefore a direct synthetic method for the preparation of the desired m6Am phosphoramidite reagent, in a single step from commercially available starting material via direct N^6-methylation, dramatically improves access to these materials for biological evaluation.

Methods

Preparation of 2'-OMe-Bz-m6A-CE phosphoramidite. 5'-(4,4'-Dimethoxytrityl)-6-*N*-benzoyl-6-*N*-methyl-adenosine,2'-*O*-methyl-3'-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (2'-OMe-Bz-m6A-CE phosphoramidite) was prepared as follows: tetrabutylammonium bromide (260 mg, 807 μmol) and aq. NaOH (1M, 7 ml) were added to a stirring solution of 2'-OMe-Bz-A-CE phosphoramidite (Link Technologies, 708 mg, 797 μmol) and iodomethane (200 μl, 3.21 mmol) in dichloromethane (7 ml). After vigorous stirring for 30 min, ether and water were added and the resulting layers were separated. The aqueous layer was extracted with ether, the combined organic extracts were dried over Na$_2$SO$_4$ and the volatiles were removed in vacuo. 2'-OMe-Bz-m6A-CE phosphoramidite was readily purified by column chromatography (1:1 petrol/ether→9:1 ether/methanol) affording 2'-OMe-Bz-m6A-CE phosphoramidite (403 mg, 56%, white foam, 1:1 mixture of diastereoisomers) and 2'-OMe-Bz-m1A-CE phosphoramidite (182 mg, 25%, white foam, 1:1 mixture of diastereoisomers). Data for 2'-OMe-Bz-m6A-CE phosphoramidite and for 2'-OMe-Bz-m1A-CE phosphoramidite are presented in Supplementary Table S1. online.

Synthesis of oligoribonucleotides SK-524 and SK-526. Oligoribonucleotides SK-524(AmGGGCUGCU) and SK-526(m6AmGGGCUGCU) were synthesized on an ABI 394 DNA/RNA synthesizer and mass spectra were recorded using a Bruker Ultraflex III mass spectrometer *via* MALDI-TOF. Columns (SynBase™ CPG 1000Å, RNA: 0.2 μmol), apart from the 2'-OMe-Bz-m6A-CE phosphoramidite, standard RNA-phosphoramidites and reagents for the synthesizer were purchased from Link Technologies Ltd., MeNH$_2$ solution (33 wt.% in ethanol) was obtained from Fluka, NEt$_3$•3HF, *N*-methylpyrrolidinone (NMP), 3-hydroxypicolinic acid (HPA) and Dowex™ 50WX8-200 were purchased from Aldrich, illustra Nap™-10 columns were obtained from GE Healthcare Europe GmbH. Dichloromethane and acetonitrile were freshly distilled from CaH$_2$ before use on the synthesizer.

The RNA oligonucleotides were synthesized using a standard 0.2 μM scale protocol, but with a 15 min coupling time for each nucleotide addition step. The polymer-bound oligoribonucleotide was transferred from the synthesis column to a 1.5 ml microfuge tube and suspended in MeNH$_2$ solution (1 ml). The mixture was heated to 65 °C for 10 min, cooled to room temperature (water/icebath) and centrifuged for 1 min (10,000 g). The supernatant was separated from the CPG beads, the beads were washed with RNase free water (2 × 0.25 ml), all supernatants were combined and dried (2 h under nitrogen stream, then freeze dried). The oligoribonucleotide was resuspended in anhydrous NEt$_3$•3HF/NEt$_3$/NMP solution (250 μl of a solution of 1.5 ml NMP, 750 μlNEt$_3$ and 1.0 ml NEt$_3$•3HF), heated to 65 °C for 1.5 h, cooled to room temperature and quenched with 3M NaOAc solution (25 μl). *n*-BuOH (1 ml) was added to the mixture, which was then thoroughly mixed, cooled

to −70 °C for 1 – 2 h to encourage further precipitation and centrifuged for 30 min (4 °C, 13 000 g). The supernatant was removed, the pellet washed with 70% EtOH (2 × 500 μl) and then dried *in vacuo* (30 min). The dry precipitate was dissolved in RNase free water (1 ml) and desalted using a Nap™-10 column following the standard protocol. The resulting solution was freeze dried over night leaving the oligoribonucleotide as a white foam/powder. Samples for MALDI-mass spectrometry were prepared as follows[36]: Dowex™ ion-exchange beads were rigorously cleaned with dilute HCl, washed with water, then treated with dilute NH$_3$ and finally washed with water again to generate Dowex-NH$_4$$^+$. Diammonium citrate (DAC) (100 mg) was dissolved in water (1 ml) and HPA (34.8 mg) was dissolved in 1:1 acetonitrile/water (1 ml). The HPA solution was filtered through Dowex-NH$_4$$^+$ and the DAC solution (100 μl) was added to prepare the matrix stock. Prior to MALDI-MS acquisition, matrix stock (20 μl) was mixed with Dowex-NH$_4$$^+$ (5 μl) and each oligoribonucleotide sample (1 μl) was mixed with Dowex-NH$_4$$^+$ (19 μl). After 30 min the matrix, and after drying, the sample solution (0.5μl) were spotted onto the sample well and allowed to dry prior to confirmatory analysis by MALDI-MS.

RNA purification. Total RNA was prepared from mouse (C57BL/6) tissues using Trizol reagent (Invitrogen). The poly(A) RNA was purified twice using oligo(dT) cellulose (Fluka) followed by oligo(dT) magnetic beads (Invitrogen). All samples were quantified and assayed for poly(A) purity using an Agilent Bioanalyser.

Analysis of gene-specific mRNA cap structures. Regions corresponding to the first 300 to 500 nucleotides of the target messages were PCR amplified from cDNAs using the following oligonucleotides:
*Apoa1*forward GCTCCGGGGAGGTCACCCACACCT and
*Apoa1*reverse CAATGGGCCCAGCCGTTCCTGCAGC;
*Alb*forward CCCCACTAGCCTCTGGCAAAATGAAGTG and
*Alb*reverse GGCTGGGGTTGTCATCTTTGTGTTGCAG;
*Prm2*forward GCTGGGTGTGCGCGAGTCAGGGGCTC and
*Prm2*reverse CTTGTGGATCCTATGTAGCCTCTTACG;
*Pabpc1*forward CGGCGGTTAGTGCTGAGAGTGCGGAG and *Pabpc1*reverse GAAGTTCACGTACGCGTAGCCCAAGG. Prior to amplification, the forward oligonucleotides were 5' phosphorylated, the amplification products were subsequently digested with lambda nuclease (New England BioLabs) to leave the single stranded antisense DNA strand. 100 ng of this ssDNA (2 μl) was spotted onto 2 mm × 2 mm teeth cut from a Hybond N$^+$ membrane (Amersham) (Fig. 2B,C), and UV cross-linked (Stratalinker). Membranes were prehybridised at 42 °C in 40 % formamide with 5 × Denhardt's, 3 % SDS, 0.3 M NaCl, 50 mM sodium phosphate buffer (pH 7.0) and 0.1 mg ml^{-1} sonicated salmon sperm DNA.

600 ng of poly(A) RNA was digested with 20 units of Tobacco Acid Pyrophosphatase (Epicentre) for 30 minutes at 37 °C. The 5' phosphate of the exposed cap adjacent nucleotide was removed by the addition of 10 units of Alkaline Phosphatase (Fermentas) and incubation for a further 15 minutes at 37 °C. After phenol-chloroform extraction and ethanol precipitation, RNA samples were resuspended in 20 μl of sterile distilled water and 5' ends were labelled using 30 units T4 polynucleotide kinase (PNK, Fermentas) and 7.4 MBq [γ-^{32}P] ATP at 37 °C for 30 minutes. The PNK was heat inactivated (70 °C for 15 min) and the reaction made up to 60 μl with sterile distilled water then passed through a P-30 spin column (Bio-Rad) to remove unincorporated isotope. A 1 μl aliquot was taken, added to 9 μl of nuclease P1 buffer and digested with P1 (Sigma) for one hour at 37 °C. 1.5 μl of the released 5' monophosphates from this digest was then analysed by 2D TLC as described previously[25]. The remaining end labelled RNA was fragmented to lengths of approximately 120 nt by the addition of Na$_2$CO$_3$ to a final concentration of 60 mM and NaHCO$_3$ to a final concentration of 40 mM followed by incubation for one hour at 60 °C.12 μl 3M sodium acetate pH 5.2 was then added and the RNA precipitated with ethanol. The pellet was resuspended in 200 μl of pre-hybridisation buffer then added to the membranes (final volume 2 ml) and hybridised overnight at 42 °C. Membrane washings were carried out with 2 ×, 1 × and 0.2 × SSC, 0.1% SDS. Two final washes were carried out at 60 °C, with 0.2 × SSC but with the SDS omitted. Hybridised membranes were exposed to storage phosphor screens (K-screen; KODAK) and imaged using Bio-Rad Molecular Imager FX in combination with Quantity One 4.6.3 software (Bio-Rad). Individual teeth containing the target mRNAs end labelled at the cap adjacent position were cut off and digested with P1 nuclease (Sigma) in a final volume of 3 μl. All of this digestion mix was applied to a cellulose coated TLC plate (20 × 20 cm, MERCK) and developed as described previously[25].

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Author contributions

SK and CH developed and carried out the novel chemical synthesis of phosphoramidites. SZ, JB and RF developed the cap labeling protocols. ZB and RF wrote the manuscript. MA provided materials and useful comments. All authors reviewed the manuscript.

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