Enzymatic characterization of mRNA cap adenosine-N6 methyltransferase PCIF1 activity on uncapped RNAs

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The phosphorylated RNA polymerase II CTD interacting factor 1 (PCIF1) is a methyltransferase that adds a methyl group to the N6-position of 2′O-methylenosine (A\textsubscript{m}), generating N6, 2′O-dimethylenosine (m\textsuperscript{6}A\textsubscript{m}) when A\textsubscript{m} is the cap-proximal nucleotide. In addition, PCIF1 has ancillary methylation activities on internal adenosines (both A and A\textsubscript{m}), although with much lower catalytic efficiency relative to that of its preferred cap substrate. The PCIF1 preference for 2′O-methylated A\textsubscript{m} over unmodified A nucleosides is due mainly to increased binding affinity for A\textsubscript{m}. Importantly, it was recently reported that PCIF1 can methylate viral RNA. Although some viral RNA can be translated in the absence of a cap, it is unclear what roles PCIF1 modifications may play in the functionality of viral RNAs. Here we show, using in vitro assays of binding and methyltransfer, that PCIF1 binds an uncapped 5′-A\textsubscript{m}\textsubscript{1} oligonucleotide with approximately the same affinity as that of a cap analog (K\textsubscript{M} = 0.4 \textit{versus} 0.3 \textmu M). In addition, PCIF1 methylates the uncapped 5′-A\textsubscript{m} with activity decreased by only fivefold to sixfold compared with its preferred capped substrate. We finally discuss the relationship between PCIF1-catalyzed RNA methylation, shown here to have broader substrate specificity than previously appreciated, and that of the RNA demethylase fat mass and obesity-associated protein (FTO), which demonstrates PCIF1-opposing activities on capped RNAs.

Modified nucleotides in mRNA are particularly enriched within the cap at the 5′ end of eukaryotic mRNAs (1) and references therein). There are at least four known cap-associated modifications: N7 methylation of the 5′ G yielding 7-methylguanine (m\textsuperscript{7}G), the two ribose 2′O-methyl modifications at the first and second templated nucleotides (N\textsubscript{m}), and the N6-methyladenine (m\textsuperscript{6}A) that is found along with 2′O-methylation when the first transcribed nucleotide after the m\textsuperscript{7}G cap is adenine, yielding N6, 2′O-dimethylenosine (m\textsuperscript{6}A\textsubscript{m}). In human cells, m\textsuperscript{7}G-capped mRNAs frequently start with 2′O-methylated A\textsubscript{m}, G\textsubscript{m}, C\textsubscript{m}, or one of the other adenosine modifications (m\textsuperscript{6}A\textsubscript{m} or m\textsuperscript{6}A), with U\textsubscript{m} being seen as well but orders of magnitude less often (2). The dimethylated adenosine m\textsuperscript{6}A\textsubscript{m} was discovered in 1975 (3), and the enzyme that generates m\textsuperscript{6}A\textsubscript{m} from A\textsubscript{m} was biochemically purified soon thereafter (4), but the gene identity (PCIF1) was only revealed recently.

PCIF1 was named as phosphorylated RNA polymerase II CTD interacting factor 1 (5), and it methylates adenosine when A is the first transcribed nucleotide after the m\textsuperscript{7}G cap (6–9). However, the reported effects of PCIF1-mediated cap-specific adenosine dimethylation vary, even among experiments using the same cell line. Specifically, PCIF1 has been reported to (a) promote the translation of capped mRNAs in human embryonic kidney 293T (HEK293T) cells (6), (b) reduce stability of a subset of m\textsuperscript{6}A\textsubscript{m}-annotated mRNAs in HEK293T cells while not substantially affecting mRNA translation (7), (c) decrease cap-dependent translation of methylated mRNAs in human malignant melanoma MEL624 cells while not altering mRNA levels or stability (8), or (d) stabilize transcripts in mouse tissues (9). These independent studies jointly indicate that a PCIF1 knockout has limited impact on the mRNA transcriptome. It is possible that PCIF1 plays additional, yet-uncharacterized roles, as it travels from 5′ to 3′ on genes, because of its eponymous direct interaction with the phosphorylated C-terminal domain of RNA polymerase II. While the function of PCIF1 in human cells is still not entirely clear, a 2021 report indicated that PCIF1 methylates viral mRNA and thereby attenuates the antiviral activity of interferon-β (10).

Structurally, human PCIF1 contains an N-terminal pol II CTD-binding WW domain and a large C-terminal methyltransferase (MTase) domain with a canonical Rossmann fold containing a conserved catalytic motif for SAM binding (6). Although a structure is available of human PCIF1 with SAH, lacking the WW domain and the C-terminal end, we do not yet have structural information for human PCIF1 in complex with its RNA substrates. We made use of a structure of the MTase domain of the zebrafish PCIF1 ortholog, which had been crystallized in the presence of SAH and m\textsuperscript{7}G cap (6). Surprisingly, the m\textsuperscript{7}G cap binding by zebrafish PCIF1 (Protein Data Bank: 6is0 in Fig. 1A) differs from other structurally

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addition, during viral infections, some viral genomes generate those that start with a purine mammalian mRNA fragments that begin without a cap (16). In the growth conditions used). There are also many substantial fraction of mRNAs

FTSJ3 generates internal 2J. Biol. Chem.

characterized m7G cap–binding proteins. First, the cap N7 methyl group points to the solvent when bound with zebrafish PCIF1, whereas this methyl group is buried in the protein–substrate interface in three other proteins: the euarkyotic translation initiation factor 4E (11), a viral mRNA cap–specific RNA 2’O-MTase VP39 (12), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Nsp16/10 2’O-MTase (Fig. 1, B–D). This difference may help explain results from a recent study with vesicular stomatitis virus (VSV), in which PCIF1-dependent N6-methylation of VSV mRNA was found to be independent of prior guanine-N7-methylation of the mRNA cap (10). Second, the Watson–Crick pairing edge of the cap guanine does not form the expected three hydrogen bonds when bound to zebrafish PCIF1 (Fig. 1A), whereas euarkyotic translation initiation factor 4E does provide two H-bond acceptors and one H-bond donor to assure sufficient discrimination of this base (Fig. 1B).

In this study, we first ask whether human PCIF1 is active in vitro on RNA substrates lacking a cap, since uncapped mRNA molecules do appear and can be recapped by cytoplasmic enzymes (13, 14). At least in 12 yeast species, only a fraction of RNAs were capped with m7G: ~75% of those having a purine (A or G) at the 5’ end and ~50% of those starting with a pyrimidine (15). This result implies that a substantial fraction of mRNAs—approximately 25% of even those that start with a purine—are uncapped (at least in yeasts under the growth conditions used). There are also many mammalian mRNA fragments that begin without a cap (16). In addition, during viral infections, some viral genomes generate 5’-triphasphate RNAs without a 5’-cap, either in single-stranded or double-stranded form (17–19). In summary, cells appear to contain significant amounts of uncapped mRNAs (host or viral) with adenosine in the 5’ position, and PCIF1 could thus play a role in their fates.

Second, we ask whether PCIF1 is active on internal adenosines, particularly internal 2’O-methyladenosine (A_m). This question is significant, in part, because the host 2’O-MTase FTSJ3 generates internal 2’O-methylated nucleotides (most of them adenosines) in the HIV RNA genome (20), possibly promoting HIV latency via a pol II-mediated transcription of HIV (21). Interestingly, the most strongly enriched host factor, highly selectively bound to SARS-CoV-2 RNA within infected human hepatocyte Huh7.5 cells, was the nucleary encoded and mitochondrially localized 2’O-MTase MRM2 (22). Finally, a low but significant level of internal 6mA was observed in a mutant of the RNA virus VSV defective for its own 2’O adenine MTase (VSV-LG4A) (10). PCIF1 has been reported to have ubiquitous nuclear expression (The Human Protein Atlas), but in the Discussion section, we consider its access to the cytoplasm as well.

Results

PCIF1 is active on uncapped RNA molecules

We synthesized two 18-nt RNA molecules with a 5’ adenosine (R1 and R2 in Fig. 2A). Both oligonucleotides contain additional internal Ade residues. Under the conditions established for the capped substrate at pH 8.0 (23), we observed PCIF1 methylation of both uncapped substrates, with higher activity on oligo R2 (Fig. 2A). Replacement of the 5’ A and 3’ A in oligo R2 (with U) resulted in only low residual activity (Fig. 2B).

Even after saturated overnight reactions, we only observed a single methylation event for oligo R1—specifically, the reaction product SAH had the same molarity as the RNA substrate used in the reaction. In contrast, oligo R2 had between one and two methylation events (Fig. 2C). Increasing the PCIF1 enzyme concentration by 10× did not alter either result (Fig. 2D). As a control, we used sinefungin (a pan methylation inhibitor) in the reaction mixture, instead of methyl donor SAM and, as expected, observed no methylation at all (Fig. 2D).

The four RNA samples shown in Figure 2D were subjected to mass spectrometry (MS) analysis. For the unmodified oligo R1 incubated in the presence of sinefungin, the observed nominal mass and nucleotide compositions matched the calculated molecular mass and expected numbers of nucleotides (Fig. 2, E and G). For the oligo R1 incubated in the presence of SAM, we observed a single major methylation.
production (~98%), with the 5’ A having been converted to 6mA (Figs. 2F and S2).

For the oligo R2, MS analysis revealed three methylated species: the fully N6-methylated 5’ A (~99%), doubly methylated products (estimated to be ~28%), with the second methylation site at the internal adenosine, three or four residues away from the 5’ end (AGCGAA) (Fig. 3, A and B) but definitely not involving the adenine from the 3’ end (Fig. S3).

To confirm the internal methylation sites, we repeated MS analysis in a separate experiment with the R2 oligo and confirmed these observations.

Knowing that the second methylation site is close to the 5’ A in R2, we reduced the distance between the first two adenosines from five residues in R1 to three residues and made a new oligo (R3) that changes the R1 5’-AGUGCGA… to R3 5’-AGCGACA (see sequences in Fig. 3C). This R3 oligo yielded nearly the same PCIF1 activity as did R2 (Fig. 3, C and D). The distance between the two methylated adenosines might reflect the two purine-binding pockets, with one in the active site (adjacent to the SAM-binding site) and the other being the cap-binding site as observed in zebrafish PCIF1 (Fig. 1A).

Consistent with R2, we observed methylation primarily at the 5’ adenosine in R3 (Figs. 3E and S4). In addition, a second methylation occurred to an internal adenosine residue, at lower intensity, at position three residues away from the 5’ adenosine (Figs. 3F and S4).

We also realized that, by chance, our design of the 5’ sequence (5’-AGCGA) is somewhat similar to the VSV viral mRNA, in which each gene contains an identical 5’ sequence (5’-AACAG) (24–26), that is subject to cap-related modifications (10, 27). We thus tested the potential activity of PICF1 on VSV RNA more specifically by synthesizing oligos R4–R6, which contain the VSV AACAG sequence. The results indicate that either one or both substitutions (5’-AGCGA to 5’-AACAG with purine-to-purine changes being underlined) reduced PCIF1 activity (Fig. 3G), suggesting that PCIF1 has some sequence preference for the 5’ end. In a previous study, PCIF1 activity was tested on a number of capped RNA
substrates, and the highest activity was on the one containing AGC as the first three residues after the m7G cap (Fig. S4B of Ref. (6)).

**PCIF1 activity on 5' adenosine is enhanced by pre-existing 2'-O methylation**

We next measured the PCIF1 kinetic parameters on oligo R2, with and without 2’O methylation, at three different pH values (5.4, 8.0, and 9.4). This pH range was used because we previously characterized PCIF1 activity on a cap analog and observed increasing catalytic efficiency from lower to higher pH that was independent of the buffering agent used (23). By varying concentrations of the RNA substrates, we determined kinetic parameters for reaction rate (kcat), RNA-binding affinity (K_M), and catalytic efficiency (kcat/K_M) (Fig. 4).

We made the following two observations. First, the pH effect shows the same trend as was observed for the cap analog, with the kcat/K_M values increasing in the order of pH 5.4 < 8.0 < 9.4, irrespective of 2’O methylation. Second, the reaction rate is fairly similar between the two substrates (kcat ~10–15 h⁻¹), though the kcat at pH 8.0 is more affected by 2’O methylation than at the other pHs (9.6 h⁻¹ for A methylation versus 12.7 for h⁻¹ for Am methylation).

**PCIF1 binds an uncapped 5'-Am oligonucleotide and a cap analog with similar affinities**

Given the effects of Am versus A on PCIF1 activity, we next examined Am effects on binding affinity and made the following two observations. First, the binding affinity for the 2’O-methylated substrate is three to five times higher than for the unmodified substrate (K_M = 0.5 versus 2.3 μM at pH 8.0 and 0.4 versus 1.2 μM at pH 9.4). This is reminiscent of the observation with the cap analog of tighter PCIF1-binding affinity when Am modification was present (6).

Second, comparing the 5’-Am oligo and the Am modified cap analog, the binding affinities with and without a cap vary over an unexpectedly small range (K_M = 0.3–0.5 μM at the two higher pH values). Finally, in contrast to the relatively constant
binding affinities, the reaction rates \( k_{\text{cat}} \) are nevertheless higher for the cap analog than the 5'-0'-Am oligo, by three to five times at pH 8.0 and 9.4.

**PCIF1 activity on internal adenosine**

As shown in Figure 3, we observed that a secondary methylation event could occur to an internal adenosine in oligo R2, though at a much reduced rate. We do not know whether PCIF1 has a flanking sequence preference for an internal adenosine, and it might have none; for example, a recent publication on FTSJ3-mediated internal 2'-O-adenine methylation revealed no consensus target sequence (20). We designed short 14-mer ssRNA oligos (CAUU and CAmUU in Fig. 5) containing a single internal A and compared the PCIF1 enzymatic activities on the potential substrate (with and without 2'-O-methylation) to that of mRNA cap analog and the uncapped version under the same experimental conditions.

First, as expected, we observed increased activity on the 2'-O-methylated oligo, compared with the conventional RNA oligo (without any premodifications; Fig. 5A). Second, kinetic measurements suggest that the largest difference lies in the affinity for the premethylated Am substrate, which is greater than eightfold higher than for the unmodified substrate \( K_M = 3.1 \text{ versus } 26 \mu M \). In contrast, the reaction rate showed less than 2× variation \( (k_{\text{cat}} = 1.0 \text{ versus } 0.6 \text{ h}^{-1}) \). In other words, PCIF1 had a 16-fold gain in catalytic efficiency \( (k_{\text{cat}}/K_M \text{ values of } 0.32 \text{ h}^{-1} \mu M^{-1} \text{ for CA_mUU and } 0.02 \text{ h}^{-1} \mu M^{-1} \text{ for CAUU}) \) (Fig. 5B). Third, like the capped and uncapped 5'-A substrates, PCIF1 exhibited the highest catalytic efficiency at pH 9.4, though the difference is much smaller than for the 5'-adenosine (Fig. 5C). We note that the catalytic efficiency of PCIF1 on internal CA_mUU is about 100-fold lower than that on capped 5'-A_m \( (k_{\text{cat}}/K_M = 0.38 \text{ versus } 37 \text{ h}^{-1} \mu M^{-1}) \) and ~600 fold lower than that of the cap analog. Thus, the activity of PCIF1 on the internal adenosine is marginal, using this substrate (CAUU) and under the conditions tested, with inefficient enzymatic turnover.

In addition to the CAUU sequence, we tested RNA molecules known to be substrates for other RNA adenosine MTases: a 14-nt oligo containing an mRNA consensus sequence GAGCU for MettL3-14 (Fig. 5D), a short linear 14-nt RNA oligo corresponding to the sequence (AACAA) surrounding A1832 of 18S rRNA for MettL5-Trm112 (Fig. 5E), and a 29-nt hairpin oligo corresponding to the hairpin of small nuclear RNA (snRNA) for MettL16 (Fig. 5F). As with CAUU, we observed minimal activity with an apparent \( K_{\text{app}} \) of 1 to 2 h^{-1}. Among these substrates, the hairpin RNA shows the highest activity with a \( K_{\text{app}} \) value of 2.2 h^{-1}, suggesting that more than one adenosine is being methylated in the substrate (ACAGAGAA).
Discussion

PCIF1 has broad substrate specificity

Here, we report that PCIF1 is enzymatically active on an array of RNA substrates, from cap-proximal adenosine to internal adenosine, in 2’O-methylated A or unmodified A, with sequences derived from mRNA, rRNA, and snRNA. This is a substantially broader substrate range than had been appreciated. Given the apparent significance of RNA 6mA methylation on a variety of human diseases (e.g., (28–31)), accurate understanding of the activities of RNA MTases is a high priority.

Potential significance of methylation on viral RNA

The potential relevance of PCIF1 methylation of RNA adenosines in viruses, which was first observed in the 1970s (reviewed in Ref. (32) and references therein), is very recently illustrated in VSV. In contrast to the methylation order on cellular mRNA, VSV cap-proximal adenosine methylation proceeds in the opposite order: it requires ribose-2’O-methylation but not guanine-N7 methylation (10). In fact, ribose 2’O methylation of the VSV mRNA cap, catalyzed by the viral MTase (protein L), precedes and facilitates subsequent guanine-N7 methylation (33). The PCIF1-dependent modification of VSV mRNA cap structures may help viral mRNA appear more host like (i.e., for the differentiation between “self” and “non-self” RNA during viral infection), and we show here that PCIF1 binds an uncapped 5’-Am oligonucleotide with approximately the same affinity as that of a cap analog (Fig. 4C).

Like VSV, SARS-CoV, Middle East respiratory syndrome coronavirus, and SARS-CoV-2 have an adenosine as the cap-proximal nucleotide of the nascent mRNA (m7GpppA). It will be interesting to investigate whether SARS-CoV-2 has dimethyladenosine (m6Am) in its m7Gpppm6AmUUAAA cap, and whether PCIF1 is involved in the generation and biological functions of the cap modifications in regulation of SARS-CoV-2 infection (or of other clinically relevant viruses).
PCIF1 is mainly localized to nucleoplasm (The Human Protein Atlas) (9), is associated with active chromatin (at least in HeLa cells) (34), and its protein network comprises mostly factors involved in nascent RNA synthesis by RNA polymerase II (35), where cellular mRNA-capping modifications proceed in an ordered fashion from m^7G to A_m to m^6A_m.

The existence of a cytoplasmic pool of PCIF1 has not been reported. In order for PCIF1 to act on RNA from RNA viruses, PCIF1 has to be able to localize to cytoplasm. This might occur via changes in nucleocytoplasmic transport during infection (36), though the nuclear localization signal of PCIF1 has not yet been characterized and does not seem obvious (37). Alternatively, nuclear and cytoplasmic contents mix during mitosis, when nuclear envelope disassembly occurs (38). We also note that the processes of nuclear envelope assembly/disassembly are modulated by some viruses (39).

**Fat mass and obesity-associated protein appears to oppose documented activities of PCIF1**

Intriguingly, we note that the methylation activities of PCIF1 *in vitro* seem to fully oppose to the fat mass and obesity-associated protein (FTO) demethylase activities on N6-methyladenosinelines (Fig. 6).

FTO has Fe(II) and α-ketoglutarate-dependent dioxygenase activity (40). FTO was initially characterized as a repair enzyme active on 3-methylthymidine (3mT) in ssDNA (40) or on 3-methyluracil in ssRNA (41). Later, five additional FTO activities were identified: (a) a demethylase that removes the methyl group from internal m^6A residues in mRNA (42), (b) a cap-dependent demethylase that preferentially removes the N6 methyl group from m^6A_m (43), (c) a demethylase active on internal m^6A and m^6A_m in snRNA, and a demethylase of (d) 1-methyladenosine in tRNA (44), and of (e) N6-methyldeoxyadenosine in ssDNA (45).

Besides the important effects on the stability of those mRNAs having m^6A_m at the 5’ end (at least in HEK293T cells (43)), FTO modulates mRNA splicing, is required for adipogenesis (46), and mediates stem-like properties in colorectal cancer cells (47). Structures have been determined for human FTO, bound to the mononucleotide 3mT (48) or to ssDNA containing an internal N6-methyldeoxyadenosine (45). However, these structures do not explain the preference of FTO for either mRNA caps or for 2’O-methylation. (We have not tested PCIF1 activity on 3mT, 3-methyluracil, or 1-methyladenosine on either RNA or DNA substrates.)

The cytoplasmic pool of FTO impedes cancer stem cell growth (47); specifically, low FTO levels in patient-derived cell lines are associated with high levels of m^6A_m in mRNA and elevated tumorigenicity and chemoresistance. The hypothesized apparently full complementarity between PCIF1 and FTO activities (Fig. 6) suggests that their expression ratio may be a key regulatory parameter. The present study suggests that another key regulatory parameter is their respective activity levels on their diverse substrates.

**Experimental procedures**

**Recombinant enzymes**

The highly purified recombinant enzymes used in this study were recently characterized in our laboratory: human PCIF1 (pXC2055) (23), human MettL3-14 (49, 50), human MettL5-Trm112 (pXC2062-pXC2076), and human MettL16 (pXC2210) (23). The example of purified PCIF1 and its activity is shown in Fig. S1, A–C.

The oligonucleotides were synthesized by Integrated DNA Technologies. The cap analog was purchased from TriLink BioTechnologies (catalog number: N-7113). The sequence of RNA oligo R1 was taken from a study of human RNase H1 complexed with an RNA–DNA hybrid (51). The sequence of

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**Figure 6. Reactions tested here in which PCIF1 methylates in opposition to the activities of the demethylase FTO (demonstrated by others, see Discussion section).** FTO, fat mass and obesity-associated protein; PCIF1, phosphorylated RNA polymerase II CTD interacting factor 1.
PCIF1 activity on uncapped RNA substrates

RNA oligo R2 was taken from a study of Wilms tumor protein WT1 (52).

Methylation assays

Methylation reactions for LC–MS analyses were carried out in a 60 μl mixture containing 5 μM PCIF1, 10 μM RNA oligo R1 or R2 (Fig. 2A), 60 μM SAM, 60 U recombinant RNasin (an RNase inhibitor; Promega), in a reaction buffer that included 20 mM Tris–HCl, pH 8.0, 50 mM NaCl, and 1 mM DTT. For control samples, 60 μM sinefungin was added instead of SAM. Reactions were performed at room temperature (~22 °C) with overnight incubation. A 5 μl aliquot of the reaction mixture was serially diluted three times to ensure that the byproduct SAH was in the linear range (Fig. S1, D and E) for the Promega bioluminescence assay (MTase-Glo) (53). The RNA samples after reaction were purified by Monarch RNA Cleanup Kit (New England BioLabs; catalog number: 2040S) using a modified protocol for RNA with length >15 nt.

Kinetic assays were performed in a 20 μl mixture at three pH values (5.4, 8.0, or 9.4), in 20 mM citric acid and 20 mM Bis–Tris propane, 50 mM NaCl, 1 mM DTT, 10 U RNasin, 20 μM SAM, 0.2 μM PCIF1 (for RNA with 5′-adenosine) or 1 μM PCIF1 (for detecting RNA methylation at internal adenosines), with varied RNA concentrations. The reactions proceeded at room temperature for 15 min. The dependence of product SAH formation per enzyme molecule on RNA substrate concentration was analyzed according to Michaelis–Menten kinetics using GraphPad Prism 8 (GraphPad Software, Inc).

Methylation assays of PCIF1 on different RNA oligonucleotides were conducted in the reaction buffer containing 20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM DTT, 20 μM or 100 μM SAM, with various PCIF1 and substrate concentrations. Methylation assays of MettL3-MettL14, MettL5-Trm112, and MettL16 were conducted under the optimal conditions for each enzyme as previously characterized in our laboratory (23, 49, 50).

To calibrate SAH concentration and luminescence, the SAH standard solution within the MTase-Glo Methylation Assay Kit (Promega) was subjected to serial twofold dilutions, starting from 4 μM. Luminescence signals from a standard concentration curve of SAH were generated according to the manufacturer’s protocol. An aliquot of 5 μl of the sample was transferred into a low-volume 384-well plate, and the luminescence signal was detected using a Synergy 4 Multi-Mode Microplate Reader (BioTek). A linear regression of the SAH standard was plotted against luminescence (Fig. S1, D and E).

MS

Base composition analysis was initiated by digestion of RNA oligos by treatment at 37 °C for 1 h with the Nucleoside Digestion Mix (NEB; catalog number: M0649S). The resulting nucleoside mixtures, without further purification, were directly analyzed by reversed-phase LC–MS. Nucleoside analysis was performed in duplicate on an Agilent 1290 Infinity II UHPLC equipped with G7117A diode array detector and a 6135XT single quadrupole detector operating in positive electrospray ionization (+ESI) and negative ESI (−ESI) modes. UHPLC was performed using on a Waters XSELECT HSS T3 X0 column (2.1 × 100 mm, 2.5 μm) with the gradient mobile phase consisting of aqueous ammonium formate (10 mM at pH 4.5) and methanol. MS data acquisition was recorded in total ion chromatogram mode. The relative abundance of each nucleoside was determined by dividing the UV absorbance by the corresponding extinction coefficient.

Intact mass analysis and modification mapping were performed by LC–MS/MS on a Vanquish Horizon UHPLC System equipped with a diode array detector and a Thermo Q-Exactive Plus orbitrap mass spectrometer operating under – ESI mode. UHPLC was performed using a Thermo DNA PAC RP Column (2.1 × 50 mm, 4 μm) at 70 °C and 0.3 ml/min flow rate with a gradient mobile phase consisting of hexafluoroisopropanol, N,N-diisopropylethylamine aqueous buffer, and methanol. UV detection was performed at 260 nm. Intact mass analysis was performed under full scan mode at a resolution of 70,000 (full width at half maximum) at m/z 200. ESI–MS raw data were deconvoluted using Promass HR (Novatia, Inc). Modification mapping was performed in parallel reaction monitoring mode with a normalized collision energy of 15% at a resolution of 70,000 (full width at half maximum) at m/z 200. The top three ions from each peak were chosen for parallel reaction monitoring analysis. ESI–MS raw data were processed with the Nucleic Acid Search Engine in Open-MS (version 2.6.0) (54).

Data availability

The experimental data that support the findings of this study are contained within the article.

Supporting information—This article contains supporting information.

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Conflict of interest—N. D., E. J. W. and I. R. C. are employees of New England Biolabs, Inc, which is a manufacturer and vendor of molecular biology reagents, including several enzymes and buffers used in this study. This affiliation does not affect the authors’ impartiality,
adherence to journal standards and policies, or availability of data. The other authors (D. Y., J. Z., T. W., R. M. B., X. Z., and X. C.) declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: 3mT, 3-methylthymidine; A_m, 2′O-methyladenosine; ESI, electrospray ionization; HEK293T, human embryonic kidney 293T cell line; m6A, N6-methyladenine; m6A_m, N6, 2′O-dimethyladenosine; m7G, 7-methylguanine; MS, mass spectrometry; MTase, methyltransferase; PCIF1, phosphorylated RNA polymerase II CTD interacting factor 1; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; snRNA, small nuclear RNA; VSV, vesicular stomatitis virus.

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