Recombinant Human mRNA Cap Methyltransferase Binds Capping Enzyme/RNA Polymerase II Complexes*  

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Guanine N-7 methylation is an essential step in the formation of the m7GppPN cap structure that is characteristic of eukaryotic mRNA 5' ends. The terminal 7-methylguanosine is recognized by cap-binding proteins that facilitate key events in gene expression including mRNA processing, transport, and translation. Here we describe the cloning, primary structure, and properties of human RNA (guanine-7-)methyltransferase. Sequence alignment of the 476-amino acid human protein with the corresponding yeast ABD1 enzyme demonstrated the presence of several conserved motifs known to be required for methyltransferase activity. We also identified a Drosophila open reading frame that encodes a putative RNA (guanine-7-)methyltransferase and contains these motifs. Recombinant human methyltransferase transferred a methyl group from S-adenosylmethionine to GpppG 5' ends, which are formed on RNA polymerase II transcripts by the sequential action of RNA 5'-triphosphatase and guanylyltransferase activities in the bifunctional mammalian capping enzyme. Binding studies demonstrated that the human cap methyltransferase associated with recombinant capping enzyme. Consistent with selective capping of RNA polymerase II transcripts, methyltransferase also formed ternary complexes with capping enzyme and the elongating form of RNA polymerase II.

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† The abbreviations used are: pol II, polymerase II; pol IIO, hyperphosphorylated elongating form of human RNA polymerase II; hMet, human (guanine-7-)methyltransferase; CTD, C-terminal domain; CE, capping enzyme; HCE, human CE; EST, expressed sequence tag; Adomet, S-adenosylmethionine; ORF, open reading frame; RACE, rapid amplification of cDNA ends; bp, base pair(s); PCR, polymerase chain reaction; GST, glutathione S-transferase.

Eukaryotic mRNAs contain a 5'-terminal cap, m7GppPN (1). This important modification of RNA polymerase II (pol II)1  

transcripts occurs soon after they attain a chain length of 25–30 nucleotides (2–4). At this stage in transcription the C-terminal domain (CTD) of the pol II largest subunit is hyperphosphorylated (5, 6), and capping enzyme (CE) then binds to it (7–9). In mammals, CE is a bifunctional protein consisting of N-terminal RNA 5'-triphosphatase and C-terminal guanylyltransferase domains (7, 10). The combined effect of these activities on nascent pre-mRNAs is the conversion of 5'-terminal ppN to GpppN. Subsequent N-7 methylation of GpppN 5' ends to form m7GpppN caps is catalyzed by RNA (guanine-7-)methyltransferase (1, 11, 12). The 7-methylguanosine (m7G) moiety of the cap is a key feature in several aspects of RNA metabolism including transcript stability (13, 14), processing (15–17), transport to the cytoplasm (18, 19), and initiation of translation (1, 20).

Several functions of the cap structure are mediated by a family of cap-binding protein complexes that specifically recognize m7GpppN (19, 21). For example, in the nucleus, a cap-binding protein complex facilitates pre-mRNA splicing accuracy and efficiency and possibly nuclear export (19). In the cytoplasm, the heterotrimeric initiation factor eIF4F, which includes the cap-binding subunit eIF4E, promotes ribosomal binding and translation initiation (21, 22). Although capping stabilizes mRNAs (13, 14), cap mG is also recognized by the yeast decapping enzyme (23), and loss of the blocked 5' end leads to 5'→3' exonuclease degradation (13, 24). Consistent with the multiple effects of cap on gene expression, the RNA (guanine-7-)methyltransferase, like the RNA 5'-triphosphatase (25) and guanylyltransferase (26), is essential for viability in yeast (27).

In an effort to gain a more complete understanding of the role of the cap in mammalian RNA metabolism, we identified human ESTs homologous to the cloned Saccharomyces cerevisiae RNA (guanine-7-)methyltransferase and used them to clone a cDNA encoding the human enzyme (hMet). hMet contains several motifs that are conserved among other RNA (guanine-7-)methyltransferases. In the presence of S-adenosylmethionine (Adomet), purified recombinant hMet converted the GpppG ends of transcripts to m7GpppG. In addition, hMet formed ternary complexes with human capping enzyme (HCE) and the hyperphosphorylated elongating form of human RNA polymerase II (pol IIO).

MATERIALS AND METHODS  
Cloning of hMet cDNA—Yeast RNA (guanine-7-)methyltransferase (ABD1, accession number P32783) was used to search for homologous sequences in the EST division of GenBank© using the BLAST server at the National Center for Biotechnology Information (28). Two human ESTs were identified (accession numbers O04619 and C03306) that overlapped and displayed significant homology to ABD1. Searches with these ESTs yielded three additional, overlapping ESTs (accession numbers AA566003, AA568320, and AA642424) that were assembled to predict a partial ORF coding for 389 amino acids of hMet. BLAST searches also identified an ORF from Drosophila (accession number AC002502) that encodes a putative RNA (guanine-7-)methyltransferase with homology to other methyltransferases.

The human EST sequences facilitated design of a gene-specific primer, met-GSP1: 5' -CTGCGATGTCAAACCATTTTGCGGTCACG-3', which was used to obtain the 5'-untranslated region of hMet by rapid amplification of cDNA ends (RACE) with Marathon ready HeLa cDNA as template (CLONTECH). The resulting 1.2-kilobase 5'-RACE
product was cloned into pCR2.1 (Invitrogen) and sequenced using an Applied Biosystems-373 automated sequencer. Several different primers designed from the EST sequences failed to amplify the complete 3′ end of the hMet cDNA, possibly because of the presence of an internal A-rich stretch at codons 391–394. However, the 3′ end of hMet was obtained by identifying a motif, GTLSKSEWEA, near the C terminus that was completely conserved between Drosophila and Caenorhabditis elegans (accession number Z10383). From the codon frequency usage within the hMet gene, we designed primer met-DEG1: 5′-GCTTCCCA-TTCAGATTAGAAAAGTGTC-3′ for use in RACE to amplify the 3′ end of the cDNA.2

Northern Blot Analysis—A 734-bp fragment was obtained by PCR with HeLa cDNA as template and primers met-GSP1 and met-GSP2: 5′-GCTTCTGGACTGACATTTGAGTAA-3′ and met-A2: 5′-GGATTCTTCTCAGCATGTCAGCCTGCT-3′. The resulting PCR product was digested with BamHI and EcoRI and cloned in-frame into pGEX-2T (Amersham Pharmacia Biotech). Recombinant, GST-tagged hMet 175–302 was expressed and purified from Escherichia coli DH5a and used to raise antibodies (α-hMet) in rabbits.

Expression and Purification of Recombinant hMet and Capping Enzyme—A 390-bp DNA fragment encoding hMet 175–302 was amplified with the human EST (accession number C005306) as template and primers met-A1: 5′-GGATCCCCCTAAGAATTTAATTGGGATGAAA-3′ and met-A2: 5′-GGATTCTTCTCAGCATGTCAGCCTGCT-3′. The resulting PCR product was digested with BamHI and EcoRI and cloned in-frame into pGEX-2T (Amersham Pharmacia Biotech). Recombinant, GST-tagged hMet 175–302 was expressed and purified from Escherichia coli DH5α and used to raise antibodies (α-hMet) in rabbits.

RESULTS

Cloning and Expression of a cDNA Encoding Human RNA (Guanine-7-)methyltransferase—A human cDNA containing a single ORF of 1431 bp encoding hMet was isolated based on sequence homology to the S. cerevisiae, C. elegans, and Drosophila enzymes. The predicted protein consists of 476 amino acids with a calculated molecular mass of ~55 kDa, which is in close agreement with that reported earlier for the purified enzyme (30). hMet shows significant homology to its counterparts from yeast (27) and C. elegans (C25A1l gene product; Ref. 31). GenBank searches also revealed an ORF from Drosophila that encodes a putative RNA (guanine-7-)methyltransferase.

Fig. 1. Sequence alignment of cellular RNA (guanine-7-)methyltransferases. Deduced amino acid sequences of the human, S. cerevisiae, C. elegans, and Drosophila enzymes are compared. Dark background indicates regions of identity. Motifs I, III, and X are indicated. by Western blotting with specific antisera as indicated. Blots were developed by chemiluminescence (ECL detection system, NEN Life Science Products).

2 After we determined the sequence of full-length hMet (accession number AF067791), the same cDNA sequence from human brain was entered in the Genbank database as an unidentified gene potentially coding for a protein of unknown function.
Human RNA (Guanine-7-)methyltransferase

Activities (12) formed m7GpppGm caps (guanine-7-)methyltransferase and hMet, vaccinia virus CE complex, which contains both RNA 3′-triphosphatase, guanylyltransferase, and guanine-7-methyltransferase to form the nonmethylated cap 0 structure, m7GpppN. Although typical of yeast mRNAs (34), cap 0 structures in higher eukaryotes are usually converted in the nucleus to m7GpppN™ cap 1 structures by 2′-O-methylation (1). Additional methylation can occur in the cytoplasm to form m7GpppN™pN™ cap 2 structures (35–37). Although the possible role(s) of cap 2′-O-ribose methylation are only beginning to be elucidated (38, 39), numerous

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**Fig. 2. Northern blot analysis of hMet expression.** Poly (A)+ RNAs from human adult tissues were probed with a 734-bp PCR fragment as described under “Materials and Methods.” kb, kilobase(s).

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**Fig. 3. RNA (guanine-7-)methyltransferase activity of recombinant hMet.** A, T7 polymerase run-off transcripts containing G32pppG 5′ ends were incubated as described under “Materials and Methods” with 2.5 μg of bacterially expressed, purified GST-hMet bound to glutathione beads (lane 1) and the same amount of GST-hMet in solution (lane 2). Purified GST (2.5 μg, lane 3) and vaccinia virus CE (3.4 units, lane 4) were included as controls. P1 nuclease-digested samples and the indicated standards (Amersham Pharmacia Biotech) were analyzed by TLC and autoradiography. B, His6-tagged full-length hMet (1 μg, lane 1) and hMet 1–389 (1 μg, lane 2) were immobilized on protein A beads using monoclonal antibody to His6 (CLONTECH) and assayed for methyltransferase activity as in A. Vaccinia virus CE (3.4 units) was assayed as a control (lane 3).

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**Fig. 4. Ternary complex formation of hMet with HCE and the elongating form of RNA pol II in vitro.** Recombinant HCE bound to antibody-protein G beads was mixed with HeLa RNA pol II (lane 2), recombinant hMet and pol II (lane 3), or hMet alone (lane 4). Immunoprecipitates were washed extensively and analyzed by 4–20% gradient SDS-polyacrylamide gel electrophoresis together with the input pol II containing approximately equal amounts of hyperphosphorylated pol IIo and unphosphorylated pol IIa (lane 1). Western blot analysis was done with antibodies against HCE (lower panel), hMet (middle panel), or the CTD of the largest subunit of pol II (upper panel).

DISCUSSION

The cap structure that is characteristic of RNA pol II transcripts consists of m7G linked via a 5′-5′-triphosphate bridge to the first residue in pre-mRNAs. Capping of nascent transcripts proceeds by the sequential action of RNA 5′-triphosphatase, guanylyltransferase, and guanine-7-methyltransferase to form the nonmethylated cap 0 structure, m7GpppN. Although typical of yeast mRNAs (34), cap 0 structures in higher eukaryotes are usually converted in the nucleus to m7GpppN™ cap 1 structures by 2′-O-methylation (1). Additional methylation can occur in the cytoplasm to form m7GpppN™pN™ cap 2 structures (35–37). Although the possible role(s) of cap 2′-O-ribose methylation are only beginning to be elucidated (38, 39), numerous
important biological consequences have been described for m$^7$G.

Many if not all aspects of mRNA synthesis and function depend on cap-binding protein complexes that specifically recognize m$^7$GpppN. Ribosome binding during initiation of translation is facilitated by interactions of eIF4F with cap (21, 22). Nuclear events such as in pre-mRNA splicing, 3’ end formation, and nucleocytoplasmic transport are mediated by a different cap-binding protein complex (19). In addition, guanine N-7 methylation of 5’ ends can influence transcript stability both positively and negatively. Guanine N-7 methylation blocks pyrophosphorylosis of GpppN structures that protect transcripts against 5’-exonucleolytic degradation (13). On the other hand, the 7-methyl group contributes to the substrate specificity of the yeast mRNA decapping enzyme, Dep1p (23), which increases transcript susceptibility to exonuclease.

Cap methylation studies have focused on virion-associated enzymes (11, 12) and on the yeast methyltransferase (27). Although cap methyltransferases have been purified from mammalian sources (30, 40), the corresponding gene was not previously cloned and characterized. Here we report the identification and primary structure of a human cDNA that encodes a protein with activity and sequence motifs characteristic of RNA methyltransferases (32, 33). A C-terminal truncation mutant of hMet (1–389) that retained previously identified methyltransferase motifs but not motif X was without enzymatic activity. However, motif X is not present in ABD1, and thus its possible role in RNA methylation needs to be further investigated. In this context, it is interesting to note that C-terminal deletion of as few as 55 amino acids from the 436-amino acid ABD1 protein was shown to be lethal (27). Comparison of the human and yeast sequences in this region shows several conserved residues (Fig. 1), but which if any are specifically required for methyl group transfer from Adomet to caps remains to be determined.

Selective capping of RNAs transcribed by pol II, but not pol I and III, was recently explained by the finding of specific association between capping enzymes and the hyperphosphorylated CTD of RNA polymerase II (7–9). Yeast RNA (guanine-7-)methyltransferase has also been shown by affinity chromatography to bind specifically to hyperphosphorylated GST-CTD (8). Although we did not detect binding of full-length hMet to pol II in immunoprecipitation experiments, preliminary studies of hMet 1–389 indicate that the truncation mutant can bind pol IIo.3 Thus association of hMet with pol II may be hindered by C-terminal residues (including motif X) that are not present in ABD1. Binding of hMet to HCE and to HCE/poIIo complexes suggests that it is recruited to the transcription machinery by association with HCE.

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