Sequence specificity of the human mRNA N6-adenosine methylase in vitro

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ABSTRACT

N6-adenosine methylation is a frequent modification of mRNAs and their precursors, but little is known about the mechanism of the reaction or the function of the modification. To explore these questions, we developed conditions to examine N6-adenosine methylase activity in HeLa cell nuclear extracts. Transfer of the methyl group from S-[3H methyl]-adenosylmethionine to unlabeled random copolymer RNA substrates of varying ribonucleotide composition revealed a substrate specificity consistent with a previously deduced consensus sequence, Pu[G > A]AC[A/C/U]. 32P-labeled RNA substrates of defined sequence were used to examine the minimum sequence requirements for methylation. Each RNA was 20 nucleotides long, and contained either the core consensus sequence GGACU, or some variation of this sequence. RNAs containing GGACU, either in single or multiple copies, were good substrates for methylation, whereas RNAs containing single base substitutions within the GGACU sequence gave dramatically reduced methylation. These results demonstrate that the N6-adenosine methylase has a strict sequence specificity, and that there is no requirement for extended sequences or secondary structures for methylation. Recognition of this sequence does not require an RNA component, as micrococcal nuclease pretreatment of nuclear extracts actually increased methylation efficiency.

INTRODUCTION

Many messenger RNAs of higher eucaryotic cells and animal viruses contain N6-methyladenosine at internal positions (1–8). This nearly ubiquitous modification is found in hnRNA, as well as mRNA, indicating that some, if not all, methylation occurs in the nucleus before the RNA is spliced (5,9–11). Conservation of m6A in formation of adenovirus 2 mRNA has been observed (9), suggesting that methylation occurs primarily in exon sequences, however, such conservation was not observed in HeLa cell mRNA (10), and methylated adenosine residues have been localized to intron regions of SV40 and RSV RNAs (12,13,19), indicating that methylation may occur in both intron and exon sequences. Analysis of the occurrence of m6A in total polyadenylated RNA (hnRNA and mRNA) from a variety of organisms has shown that m6A occurs frequently, with an average overall content of 1–3 m6A residues per mRNA molecule (5,10,14). Recently, an in vitro assay capable of accurate methylation of bovine prolactin mRNA was reported (15). However, the function of N6-adenosine methylation remains unknown, and little is known about the mechanism and requirements of the reaction.

N6-methyladenosine is found only within the sequences Gm6AC or Am6AC, and GAC sequences appear to be methylated three to twelve-fold more frequently than AAC depending on the organism examined (11,13,16–18). In addition, the nucleotide following Gm6AC or Am6AC was never found to be a guanosine residue, indicating that the recognition sequence is GAC[A/C/U] or AAC[A/C/U]. When Kane and Beemon (19) mapped and quantitated m6A within Rous sarcoma virus RNA, they found that methylation occurred most frequently within the sequence PuGm6ACU, suggesting that the consensus may be expanded to include a purine nucleotide 5' to the core methylation sequence. One additional methylated site, which was mapped in bovine prolactin mRNA, has been found to have the sequence AGm6ACU (15), supporting this suggestion. However, the importance of a purine at this position has not been assessed in other in vivo methylated RNAs.

In general, the influence of a match to the consensus sequence on the efficiency of methylation in vivo has not been examined, presumably because of the technical difficulty involved in mapping methylated sites. Kane and Beemon (20) have shown that mutation from GAC to GAU at two known methylation sites in RSV RNA inhibited methylation of these sites in vivo, directly demonstrating the requirement for the C residue at this position. No other studies of sequence requirements have yet been reported. The deduced recognition sequence may not be sufficient for methylation, however, since simple statistical calculations indicate that PuPuAC[A/C/U] sequences occur more frequently than do m6A residues in mRNA molecules. Quantitation of m6A in several viral and cellular mRNAs (12,13,17,19,21,22) as well as precise mapping of methylated sites in RSV virion RNA (19) have confirmed that many of these sequences are unmethylated.

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These findings suggested that additional sequences or structural features may be required to define a methylation site.

In this paper, we report an assay for N6-adenosine methylation using short RNA substrates of defined sequence. These RNAs are specifically methylated in vitro and have been used to identify the precise recognition sequence required for N6-adenosine methylation, and to define some of the parameters of the reaction.

MATERIALS AND METHODS

Methylase Assays

Hela cells were used to prepare whole cell extracts (23), or nuclear extracts and cytoplasmic S100 fractions (24). Methylase assays using random co-polymer substrate RNAs were carried out for 2 hours at 30°C in a reaction mixture containing 10mM HEPES pH 7.9, 50 mM KCl, 0.2mM EDTA, 10% glycerol, 10 mM DTT, 5μM S-[3H-methyl] adenosylmethionine, and 0.5μg/μl substrate RNA. Methylated RNA was digested with RNAsase T2 and treated with alkaline phosphatase to yield nucleosides. Nucleosides were separated by paper chromatography and newly synthesized m6A was detected by scintillation counting the region of the chromatogram containing unlabeled m6A marker.

Reaction conditions for assays using 32P labeled RNAs were identical to those described above, except that 10 μM unlabeled S-adenosylmethionine was substituted for [3H]-SAM, the final concentration of substrate RNA was 5 ng/μl, and 0.03 μg/μl poly CU was included. In general reactions were carried out for one hour at 30°C. Substrate RNAs were synthesized by T7 RNA polymerase transcription of oligonucleotides containing the T7 promoter as described by Milligan et al. (25), except that reactions contained 0.5mM ATP including α-32P-ATP at a specific activity of 6 Ci/mmole. Substrate RNAs were gel purified on 20% polyacrylamide, 7 M urea sequencing gels. Micrococcal nuclease treatment of the extracts was carried out by addition of 3 mM CaCl2 and 0.25 units/μl of micrococcal nuclease, incubation for 30 minutes at 30°C, then addition of 6mM EGTA, to inactivate the nuclease. Mock treatment was identical except that no enzyme was added.

Chromatography

Products of the methylase reactions were separated by paper chromatography on Whatman 3MM paper that was saturated with 0.4M ammonium sulfate solution and dried before use. Chromatograms were developed in 76% (v/v) ethanol (26) 4 hours (for random co-polymer assay) or 16–24 hours (for 32P labeled substrates). Appropriate mixtures of unlabeled marker nucleosides or 5′ mononucleotides were added to the sample before loading, and detected by UV absorbance. In assays using random copolymer substrates, newly synthesized m6A was detected by excising and scintillation counting 3H in the m6A marker spot. In assays using 32P labeled RNA substrates, m6AMP was detected by autoradiography of the chromatogram. Methylency efficiency was determined by comparison of the 32P counts in the AMP and m6AMP spots.

Two dimensional separations were done to confirm the identity of the m6A product. The first dimension was carried out as above. For chromatography in the second direction, the chromatogram was dried, rotated 90° and developed in isobutyric acid:0.5N ammonium hydroxide (5:3) (27). In these assays, the m6A could be distinguished from a series of methylated nucleoside markers including 2′-O-methyl adenosine, N6,N6-dimethyladenosine and N6,2′-O-dimethyladenosine.

RESULTS

Methylation of Random Co-polymer RNA Substrates

Random co-polymer RNAs, which have a defined nucleotide composition but random sequence, were used for initial experiments. These substrates were used because the random nature of their sequences gave a high probability that the RNA sequence recognized by the N6-adenosine methylase would be present in the substrate RNA, even if sequences outside of the PuAC[A/C/U] sequence were required. N6-adenosine methylase activity in Hela cell nuclear or cytoplasmic extracts was detected by transfer of a methyl group from S-[methyl-3H] adenosylmethionine ([3H]-SAM) to adenosine residues in substrate RNA as outlined in Figure 1A. The RNA was incubated with the extract and [3H]-SAM under the conditions described in Materials and Methods, then digested with RNAsase T2 and treated with alkaline phosphatase to yield nucleosides. Nucleosides were separated by paper chromatography and newly synthesized m6A was detected by scintillation counting the region of the chromatogram containing unlabeled m6A marker. Activity could be detected in whole cell extracts, as well as nuclear extracts and cytoplasmic S100 fractions, but the highest level of activity was found in nuclear extracts (data not shown), therefore all data shown is from experiments using nuclear extracts. Specificity of the methylation reaction was examined by using random co-polymers of varying ribonucleotide composition as substrate RNAs.

We have used primarily five different random co-polymers: poly AC, poly ACU, poly ACG, poly AU and poly AG. Based on the apparent sequence specificity of PuAC[A/C/U] required for methylation, it was expected that the first three of these would serve as substrates for N6A methylase, while the last two would not. Figure 1B shows the results of methylation reactions carried out by a nuclear extract using these substrate RNAs. The level of methylation of endogenous RNA in the extract is shown in the no RNA sample. By comparison, poly ACG was a good substrate for N6-adenosine methylation, poly ACU and poly AC were poor substrates, while poly AU and poly AG were essentially negative as substrates, giving the same or lower incorporation as that obtained when no exogenous RNA was added. The same pattern of substrate specificity was seen in experiments using whole cell extracts or cytoplasmic S100 preparations (data not shown). The strong preference for poly ACG over poly ACU or poly AC as a substrate is consistent with the preference for GAC sites over AAC sites deduced from their use in vivo (11,13,16–19), and suggests that our assay detects the correct methylase activity. This assay was used to determine optimum conditions for pH, KCl and Mg++ concentration, which were 10 mM HEPES pH 8.0, 50 mM KCl and 0.1 mM EDTA. Methylation was significantly inhibited by Mg++ concentrations as low as 0.5 mM (data not shown).

Short RNAs of Defined Sequence Are Sufficient Substrates for Methylation.

Although the assay using random co-polymers displayed the substrate specificity expected of the N6-adenosine methylase that modifies mRNAs in vivo, the exact sequence methylated was not determined. Therefore, a second assay was developed that used short RNAs of defined sequence as substrates. Oligodeoxynucleotides containing the bacteriophage T7 promoter were used as templates for in vitro transcription of α 32P-ATP labeled RNA substrates by T7 RNA polymerase (25). Methylation of these RNAs was examined by the methods
outlined in Figure 2. RNA was methylated in reactions containing nuclear extract and unlabeled SAM, and digested with nuclease P1 to yield 5' nucleoside monophosphates, which were then separated by paper chromatography and detected by autoradiography as described in Materials and Methods.

The sequences of the RNAs used in this assay are shown in Figure 3. All of these RNAs are 20 nucleotides long, and contain the deduced consensus sequence GGACU, or a variation of this sequence with a substitution at one of the positions. The first four RNAs, shown at the top of the figure, contain four repeats of their respective five nucleotide sequences. The remaining RNAs contain a single copy of the consensus or a variation of the sequence, surrounded by sequences with no potential methylation sites. Figure 4 shows the time course of adenosine methylation using RNA 1, which contains four repeats of the sequence GGACU as substrate. A spot of m6A is barely detectable in the 5 minute sample (lane 2), but is clearly visible after 10 minutes (lane 3) and increases with time until m6A production levels off between 30 and 60 minutes of incubation (lanes 4–6). The reaction was quite inefficient, with only about 3% of the adenosine residues methylated. The time course and efficiency of methylation in these reactions are comparable to those reported for a substrate containing the 300 nucleotide fragment surrounding a natural methylation site in bovine preprolactin RNA (15), indicating that the low efficiency is not a consequence of the short substrate used in this assay. Utilization of this short RNA as a substrate for in vitro methylation demonstrates that additional sequences outside of the simple consensus are not required for N6-adenosine methylation, although surrounding

Figure 2. Schematic outline of the assay used to detect methylation of short 32P labeled substrate RNAs. RNAs labeled with α-32P-ATP were synthesized by T7 RNA polymerase, methylated, then digested with P1 nuclease to yield 5' monophosphates, among which only the adenosine nucleotides are labeled. Nucleotides were separated by paper chromatography as described in the legend to Figure 1B, and AMP and modified AMP nucleotides were detected by autoradiography.
sequences may play a role in the efficiency with which different methylation sites are utilized. Furthermore, a very short RNA sequence is sufficient for methylation, ruling out a requirement for complex secondary or tertiary structures.

Characterization of the methylation reaction using these short RNA substrates gave the same results as random co-polymer substrates for pH and KCl concentration requirements (data not shown), and addition of Mg$$^{2+}$$ inhibited methylation in these reactions just as it did in reactions using random co-polymer substrates (data not shown). Addition of unlabeled random copolymer RNA to reactions containing $^{32}$P labeled RNA substrates inhibited the reaction at concentrations over 100 $\mu$g/ml. This effect was independent of whether or not the added RNA contained potential methylation sites, suggesting that it was due to non-specific binding of the methylase to RNA (data not shown). In these experiments, we observed that low concentrations of poly CU (10–30 $\mu$g/ml) stimulated the reaction, therefore it was routinely included in these reactions.

These results suggested that endogenous nucleic acids in the nuclear extracts might be responsible for the inefficiency of the methylation reaction. This was tested by pre-treatment of the extract with micrococcal nuclease (MN) to digest endogenous nucleic acids. The results shown in Figure 5 demonstrate that methylase activity is stimulated approximately 5-fold by micrococcal nuclease treatment. In this experiment, RNA 1 was methylated in reactions using untreated extract (first lane), MN treated extract (second lane) or mock MN treated extract (third lane). These results suggest that endogenous nucleic acids inhibit the methylase, providing a partial explanation for the inefficiency of the in vitro reaction. In addition, they demonstrate that the N6-adenosine methylase does not have an accessible, essential nucleic acid component.

### Sequence Specificity of the In Vitro Methylation Reaction Conforms Precisely to the Deduced Consensus Sequence.

Within the core consensus sequence, only a C to U change at the third position has been tested for its effect on methylation in vivo (20). To determine the effects of changes at positions surrounding the AC dinucleotide, a series of different RNAs (shown in Figure 3) were tested as substrates for in vitro methylation. To assure accurate comparison between different RNAs, an aliquot of each reaction was removed before nuclease P1 digestion, and analyzed by denaturing polyacrylamide gel electrophoresis. In all cases more than 50% of the RNA recovered was full length (data not shown), indicating that all of the RNAs compared were quite stable during the methylase reactions and that differences in methylation efficiency were not due to differential stability of the different RNAs. Figure 6A shows a comparison of RNAs 1, 2, 3, and 4 as substrates for methylation. In this experiment, the efficiency of methylation of RNA 1 was approximately 10%. In RNA 2, substitution of A for G at position –1 reduced methylation efficiency to 3%. This result offers an explanation for the more frequent methylation of GAC rather than AAC sequences observed in vivo. In RNA 3 U is substituted for A at position –1 and sequence specificity is somewhat altered, indicating that GAC and AAC sequences are not equivalent substrates for the methylase.

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<th>A</th>
<th>Methylation consensus sequence</th>
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| B | RNA 1: GGACU GGACU GGACU GGACU |
|   | RNA 2: GAAACU GAAACU GAAACU GAAACU |
|   | RNA 3: GAAACU GAAACU GAAACU GAAACU |
|   | RNA 4: GAAACU GAAACU GAAACU GAAACU |
|   | RNA 5: GGUCU GGUCU GGUCU GGUCU |
|   | RNA 6: GGUCU GGUCU GGUCU GGUCU |
|   | RNA 7: GGUCU GGUCU GGUCU GGUCU |
|   | RNA 8: GGUCU GGUCU GGUCU GGUCU |
|   | RNA 9: GGUCU GGUCU GGUCU GGUCU |
|   | RNA 10: GGUCU GGUCU GGUCU GGUCU |
|   | RNA 11: GGUCU GGUCU GGUCU GGUCU |

![image](https://via.placeholder.com/150)

**Figure 3.** A. Consensus sequence for N6-adenosine methylase previously derived from in vivo studies described in the Introduction. B. Sequences of the 20 nucleotide substrate RNAs used to define the sequence requirements for methylation. RNAs 1 through 4, shown above the double line, contain a single five nucleotide potential methylation sequence repeated four times, while those below the line contain a single five nucleotide potential methylation sequence surrounded by sequences devoid of adenosine residues. Underlined adenosine residues indicate potential N6-methyladenosine positions within the consensus sequence, and arrows indicate positions altered from the consensus GGACU.

**Figure 4.** Methylation time course in nuclear extracts using $^{32}$P-labeled RNA 1, whose sequence is shown in Figure 3. Reactions were carried out as described in Materials and Methods for the indicated times, and methylated RNAs were digested with P1 nuclease and analyzed as outlined in Figure 2. The locations of AMP and m6AMP markers is indicated.
for a purine nucleotide at position −1 of the consensus. This inhibits methylation beyond the limits of detection by scintillation counting the spot, although a faint spot of m6AMP can be detected by extended autoradiography. This result substantiates the importance of a purine at this position. RNA 4 contains a G at the +3 position, which has never been observed in vivo methylated RNA. In vitro, this substitution inhibits methylation to the extent that no m6A production can be detected in this assay, even after overexposure of the autoradiograph.

Additional sequence requirements were examined within the context of a single copy of the methylation site, to allow comparison of RNAs that are identical except for a single nucleotide substitution within the methylation consensus. RNA 5, which contains a single copy of the GGACU sequence in the 20 nucleotide RNA (see Figure 3) is a good substrate for in vitro methylation (Figure 6B), demonstrating that a single copy of the GGACU sequence is sufficient to define a methylation site, although the efficiency of methylation with this substrate ranges from 1% to 3%, which is significantly lower than that of RNA 1. Figure 6B shows the results of methylation reactions with a series of substrate RNAs, each of which differ from RNA 5 at a single position. Substitution of A for G at position −2 in RNA 6, reduces methylation 5 fold compared to that of RNA 5. This indicates that G is strongly preferred at this position of the consensus, and is consistent with the observation of Kane and Beemon (20) that GGACU sequences in RSV RNA are preferentially methylated in vivo. The next two tracks in Figure 6B show that substitution at the −2 position by a C in RNA 7, or a U in RNA 8 strongly inhibit methylation, demonstrating that the methylation recognition sequence indeed extends to include a purine residue at the −2 position, as was suggested by Kane and Beemon (19).

Figure 5. Stimulation of methylase activity by micrococcal nuclease (MN) treatment of nuclear extract. Aliquots of nuclear extract were MN treated or mock MN treated as described in Materials and Methods. Methylation reactions were carried out using RNA 1 for 1 hour as described in Materials and Methods, using untreated extract (none), MN treated extract (MN) or mock MN treated extract (mock MN). The positions of AMP and m6AMP markers are indicated.

Figure 6. A. Methylation of RNAs 1, 2, 3 and 4, which contain four repeats of potential methylation sites. B. Methylation of RNAs 5−11, which each contain a single copy of a potential methylation site. Sequences of RNAs 1−11 are shown in Figure 3. Reactions were carried out for 1 hour in nuclear extracts, and products were analyzed as described in Materials and Methods. The positions of AMP and m6AMP markers are shown.
Two additional RNAs were used to examine the requirement for a C following the methylated A. RNA 9, which has a C to U substitution at this position, is methylated extremely inefficiently although a small amount of m6AMP can be detected on overexposed autoradiographs. RNA 10, which contains a C to G substitution at the +2 position, does not give detectible methylation. Our results confirm the importance of a C at this position of the consensus, and expand the results of Kane and Beemon (20), who showed that substitution of U for C at the +2 position inhibited methylation of RSV RNA in vivo.

The last track of Figure 6B shows the results of methylation of RNA 11, which has a G to C substitution at the -1 position of the consensus. This substitution strongly inhibited methylation of the RNA, which is consistent with the result obtained with RNA 3 that has a G to U substitution at this position.

**DISCUSSION**

The results presented above demonstrate that the consensus sequence deduced from methylated sequences found in cellular RNA represents an actual recognition sequence that is necessary and sufficient for the N6-adenosine methylase in HeLa cell nuclear extracts. In addition, they demonstrate that the specificity of the reaction is maintained during in vitro reactions, even when extremely short substrates are used, demonstrating that there is not a requirement for extensive secondary structure or the presence of distant sequences to form a recognition site. In fact, it is possible that such features prevent the utilization of potential target sequences, since not all A's that lie in consensus sequences are methylated in vivo.

Reaction conditions required for methylation are relatively simple, with the one unusual observation being inhibition of the reaction by even low concentrations of MgCl2. These results vary somewhat from those previously reported by Narayan and Rottman (15), who included 4mM MgCl2 in their in vitro methylation reactions using a fragment of the bovine prolactin mRNA. Under these conditions, these authors demonstrated in vitro methylation of the authentic site utilized in vivo, indicating that the Mg2+ does not affect specificity of the reaction. The differences in optimal Mg2+ concentration for methylation of this bovine prolactin mRNA fragment and those observed for the two types of substrates used in our experiments probably reflects differences in potential secondary structure or other features of the individual RNAs. It is clear from our results, however, that Mg+ is not essential for the in vitro methylation reaction. A possible precedent for this involves pre-mRNA 3' end cleavage, which does not require Mg2+ in vitro, although one very long pre-mRNA was shown to display a Mg+ requirement (28).

Accurate definition of the sequences that constitute the recognition site for N6-adenosine methylase is the first step toward understanding the mechanism and functional significance of this relatively common modification of mRNA and its precursor. The results presented in this paper demonstrate that the consensus sequence previously derived by mapping in vivo methylation sites represents a true recognition site for the N6-adenosine methylase. Deviation from the recognition site at any position tested resulted in substantial inhibition of methylation. This degree of sequence specificity in a factor that interacts with a pre-mRNA substrate is unusual. Perhaps most analogous is the factor that recognizes the AAUAAA hexanucleotide required for pre-mRNA 3' cleavage and polyadenylation (29-32). Many single base changes within the AAUAAA sequence have been shown to inhibit the cleavage/polyadenylation reaction (reviewed in 33). In contrast, splicing factors that recognize 5' splice sites or branch point sequences in mammalian pre-mRNAs can utilize sequences that deviate significantly from the consensus sequences (reviewed in 34). In these cases, the interactions are mediated at least in part by base-pairing between the RNA sites and RNA components of snRNP particles (reviewed in 35). The micrococcal nuclease insensitivity of the N6-adenosine methylase indicates that the enzyme does not contain an accessible RNA component, and suggests that recognition occurs by a mechanism that does not involve RNA-RNA base-pairing.

Substrate recognition by N6-adenosine methylase appears to be distinct from those of the methylases that modify nucleotides within the 5' cap of mRNAs. These enzymes appear to recognize the cap as a structural feature, but no consensus sequence adjacent to the cap has been identified (reviewed in 36). The sequence specificity for N6-adenosine methylation indicates that mutation of authentic methylation sites in hnRNAs will inhibit methylation of the RNA, with little chance of activating cryptic sites as is seen with splice site mutations. Such mutational analyses may aid in assessing the possible functions of N6-adenosine methylation in vivo.

Methylation does not appear to be absolutely required for mRNA maturation and function since there are some mRNAs, such as globin and histone mRNAs, that lack m6A (37,38). The effect on RNA metabolism of inhibiting SAM-dependent methylation has been examined in several cell types. Treatment of avian cells with cycloleucine, which inhibits the synthesis of SAM, was shown to reduce N6-adenosine methylation of total cellular RNA by over 90%, although newly synthesized RNA was still transported to the cytoplasm and associated with polyribosomes (39). Subsequent experiments using avian sarcoma virus infected cells demonstrated that splicing of the viral RNA was significantly inhibited by cycloleucine treatment (40). A similar reduction in the appearance of SV40 late region 19S RNA was observed in cycloleucine treated cells (41). Similar studies on the effect of methylation inhibition in HeLa cells showed that transport of undermethylated RNA to the cytoplasm was delayed, but it was eventually transported and appeared to be fully functional (42). While it is not possible to attribute these effects directly to reduction of m6A in the mRNA, since all SAM-dependent methylation is affected by these inhibitors, the results of these experiments indicate that m6A is not absolutely required for the production of functional mRNAs, but that it does appear to affect processing of certain RNAs.

The ability to achieve site specific methylation in vitro means that the possible functions of methylation can be tested directly for specific RNAs. For example, it will be possible to determine whether or not methylation of alternatively spliced pre-mRNAs will affect the splicing pattern. Most importantly, the in vitro assay can be used to purify the N6-adenosine methylase.

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