Analysis and *in vitro* localization of internal methylated adenine residues in dihydrofolate reductase mRNA

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Received April 23, 1990; Revised and Accepted July 25, 1990

ABSTRACT

A T7 RNA transcript coding for mouse dihydrofolate reductase (DHFR) was utilized as a substrate for the N^6-methyladenosine mRNA methyltransferase isolated from HeLa cell nuclei. This transcript acted as a 3 fold better substrate than either prolactin mRNA or a synthetic RNA substrate which contained multiple methylation consensus sequences. Formation of internal N^6-methyladenine (m6A) residues in the DHFR transcript was shown to increase slightly by the absence of a 7-methylguanine-2'-O-methyl cap structure. Using T7 transcripts from different regions of the DHFR gene, the majority of the m6A residues were localized to the coding region and a segment of the transcript just 3' to the coding region. This data suggests that DHFR mRNA contains multiple methylation sites with most of these sites concentrated in the coding region of the transcript.

INTRODUCTION

The formation of N^6-methyladenine (m6A) is a postsynthetic modification which occurs in the mRNA of higher eucaryotic organisms (1−6) as well as plants (7−9) and viral systems (10−12). Methylation has been shown to occur nonrandomly within the transcripts following the consensus sequence Am6AC or Gm6AC (13−15). Distribution of the methylated residues has also been shown to be localized in different regions of the mRNA molecule. Clustering of m6A residues has been shown to occur in Rous sarcoma viral genomic RNA (16) as well as in the intron regions of early and late SV40 viral transcripts (15,17). Prolactin mRNA, the only specific cellular RNA to be investigated thus far for m6A content, contains only one m6A residue in the extreme 3' end of the molecule (18,19). Interestingly, this report also demonstrated that formation of m6A residues in prolactin mRNA occurs in nonstoichiometric amounts.

While a great deal of information has been collected concerning the biochemistry of m6A formation very little data exists focusing on the enzymology or biological significance of this modification. Internal adenine methylation has however been suggested to play a significant role in the processing and/or the transport of mRNA. Inhibition of m6A formation in avian sarcoma viral RNA by treatment of infected B77 cells with cycloleucine has been shown to cause an accumulation of genome length RNA with a decrease in the levels of spliced transcripts (20). In a similar group of experiments, treatment of SV40 infected BSC-1 cells with cycloleucine also caused a decrease in the production of spliced mRNA (21). The biological significance of m6A formation in cellular mRNA has also be investigated using the methylation inhibitor S-tubercidinylhomocysteine (22). In these experiments inhibition of internal adenine methylation caused a delay in the cytoplasmic appearance of polyadenylated mRNA in cultured HeLa cells without having a significant effect on the half-life of the transcripts. While these results have suggested that m6A formation may be involved in one or more RNA processing events the fact that general methylation inhibitors were used does not rule out the possibility that methylation reactions other than m6A formation may also be involved.

Investigations involving the enzymology of m6A formation have significantly lagged behind studies dealing with the biochemistry and functional significance of this modification. Recently however, Narayan and Rottman (19) have developed an assay by which 6-methyladenine mRNA methyltransferase activity was detected in HeLa cell nuclear extracts. Using a T7 transcript coding for prolactin mRNA as the substrate, it was found that the same adenine residue methylated *in vivo* was also modified in the *in vitro* assay, indicating that the enzyme displays a high degree of substrate specificity.

In this investigation a T7 transcript coding for mouse dihydrofolate reductase (DHFR) has been investigated as an RNA substrate for the methyltransferase enzyme isolated from HeLa cell nuclei. This transcript was found to be a 3 fold better substrate than prolactin mRNA for the enzyme. In addition, subfragments of the DHFR gene were subcloned into T7 expression vectors as a means to map the regions of the transcipt where the m6A residues occur. Methylation of the transcripts resulting from these subfragments has indicated that both the coding region of the mRNA and a region of the message just 3' to the coding region serve as the best substrates for the enzyme. This data thus indicates that DHFR mRNA must contain multiple residues of m6A however the extreme 3' end of the message appears to be void of m6A (unlike prolactin mRNA).

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MATERIALS AND METHODS

Materials

All tissue culture supplies including calf serum was purchased from Gibco laboratories, Grand Island, NY. S-[methyl-^{3}H] adenosyl-L-methionine (78 Ci/mmol) and α-^{32}P-GTP (3000 Ci/mmol) were obtained from Dupont/NEN, Boston, MA. Restriction enzymes, pGem vectors, T7 RNA polymerase, RNAsin and T4 DNA ligase were all obtained from Promega Biotec, Madison, WI. RNase free DNase, RNase, Nuclease P1 and mussel glycogen were from Boehringer Mannheim Biochemicals, Indianapolis, IN. ATP, GTP, CTP and UTP were purchased from Sigma Chemical Co., St. Louis, MO. Ribonuclease T2 and bacterial alkaline phosphatase were obtained from Bethesda Research Laboratories, Gaithersburg, MD. m^{7}G(5')ppp(5')Gm was obtained from Pharmacia LKB, Piscataway, NJ. pDHFR-21 was a gift from Dr. Rodney Kellems, Department of Biochemistry, Baylor College of Medicine, Houston, TX. The prolactin cDNA clone, PRL 72 FL, was kindly provided as a gift from Dr. Fritz M. Rottman, Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH.

Growth and maintanance of HeLa cells

HeLa cells were maintained in suspension in Joklik minimum essential medium containing 5% calf serum and 10 mg/lit Gentamicin. For the preparation of HeLa nuclear extract the cells were harvested at a cell density of $3.0-4.5\times10^5$ cells per ml.

Formation of T7 expression plasmids

The DHFR gene from the pDHFR-21 plasmid was recloned as a 1470 bp Pst I fragment into Pst I digested pGem-1 using T4 DNA ligase. A limited restriction map of this cDNA clone is shown in Figure 1. The resulting plasmid was then transformed into *E. Coli* DH2α cells. The positive clones resulting from the transformation were screened for T7 orientation by restriction endonuclease digestion. The resulting plasmid pGDHFR-7 was linearized with Hind III and utilized as a template in the *in vitro* transcription assay described below.

Subclones of the DHFR gene were constructed by ligating three restriction endonuclease fragments into pGem vectors. In the formation of the first clone, a 594 bp Pst I-Bgl II DNA fragment from pGDHFR-7 was recloned into Pst I-Bam H1 digested pGem-2 DNA. Ligation and transformation of all subclones were performed as described above for pGDHFR-7. The resulting plasmid, pGDHFR-PB was linearized with Eco RI for template formation. The second subclone was formed by ligating the 570 bp Bgl II-Bgl II fragment from pGDHFR-7 into Bam H1 digested pGem-2. The resulting positive clones were then screened by restriction endonuclease digestion for T7 orientation. This plasmid, pGDHFR-BB, was linearized with Eco RI for template formation. The final subclone of the DHFR gene was formed by ligation of the 310 bp Bgl II-Pst I fragment into Bam H1-Pst I digested pGem-1 DNA. The resulting plasmid, pGDHFR-BP was linearized with Hind III for template formation.

The pGMS-7 plasmid was constructed by cloning preligating double stranded DNA fragments formed by annealing complementary 35 base oligonucleotides into a pGem-1 vector. The oligonucleotides synthesized were identical in sequence to the 31 nucleotide DNA fragment of Rous sarcoma virus which was reported by Kane and Beemon (23) to contain two methylation sites. In addition to these sequences, base sequences were added to the 5' end of each oligonucleotide to produce Eco

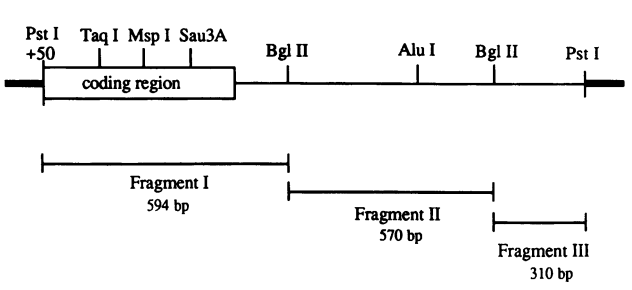

Figure 1. Restriction endonuclease map of the mouse DHFR cDNA insert from the plasmid pGDHFR-7. The boxed area represents the protein coding region. Thin lines represent the noncoding 3' end of the major mRNA species. Numbers below represent the length of the different endonuclease fragments in base pairs.

R1 and Bam H1 restriction endonuclease sites for cloning purposes. Four, 35 base oligonucleotides were synthesized in order to ligate the two fragments in a head to tail fashion. The base sequences of the two double stranded DNA fragments are shown below.

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MS-1

     5' AATTCGAGTCCTGGACTGAAACGGACTTGTCCTTC  3'
        3' GCTCAGGACCTGACTTTGCCTGAACAGGAAGCTAG  5'

MS-2

     5' GATCCGAGTCCTGGACTGAAACGGACTTGTCCTTC  3'
        3' GCTCAGGACCTGACTTTGCCTGAACAGGAAGTTAA  5'
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(underlined bases represent consensus methylation sequences)

The purified complementary oligonucleotides were annealed by mixing in a 1:1 ratio (3 μg of each), heating the mixture at 90°C for 2 min and then allowing the DNA to slowly cool to room temperature. The synthetic double stranded DNA fragments were then phosphorylated using 10 units of polynucleotide kinase by the procedure described (24). After a one hr incubation period the reaction was stopped by heating to 65°C for 10 min. The reactions were then allowed to slowly cool to room temperature. The two DNA fragments (MS-1 and MS-2, see above) were then mixed in equal amounts (1 μg of each) and preligated at room temperature for 1.5 hr using 10 units of T4 DNA ligase. The mixture was then extracted once with phenol/chloroform (1:1) and once with chloroform. After a 1:100 dilution with 10 mM Tris (pH 8.0)-1 mM EDTA, 0.1 μg of the preligated fragments were mixed with 0.2 μg of Eco R1-Bam H1 digested pGem-1 and the DNA mixture ethanol precipitated. The dryed pellet was dissolved in 1× T4 ligase buffer, mixed with 10 units of T4 DNA ligase and incubated at 5°C for 16 hrs. The ligation mixture was then used to transform *E. Coli* DH2α cells. Positive clones were screened by hybridization selection using ^{32}P kinased MS-1 DNA as a radiolabelled probe (24).

Isolated plasmid from one of the positive clones (pGMS-7) when linearized with Hind III and transcribed in a T7 *in vitro* transcription assay produced a transcipt of 370 bases in length (data not shown). This plasmid therefore contains 10 inserted synthetic DNA fragments producing a transcript with twenty possible methylation sites.

Isolation of HeLa nuclear cell extract

HeLa cells harvested at a density described above were washed twice with five volumes of phosphate buffered saline (PBS). Cells were then lysed and nuclei salt extracted as described by Dignam et al. (25). The extract was then dialyzed and frozen in dry ice in 100 μl aliquots again as described. Protein concentration of the extract averaged 4−7 mg/ml as determined using the Pierce BCA assay.

Plasmid Isolation

Large scale plasmid preparations were performed by the method developed by Krieg and Melton as described (26). This method which employs RNase digestion and polyethylene glycol precipitation steps produces plasmid free of detectable high molecular weight DNA or RNA.

In Vitro transcription assays

Five micrograms of linearized plasmid DNA was incubated in a 50 μl *in vitro* transcription assay containing the following: 40 mM Tris-Cl (pH 7.2), 6 mM $MgCl_2$, 6 mM DTT, 10 mM spermidine, 400 μM each of ATP, CTP, and UTP, 80 μM GTP, 600 μM $m^7G(5')ppp(5')Gm$, 80 units of RNasin and 100 units of T7 RNA polymerase. After a 1 hr incubation at 37°C the reaction was stopped by adding 20 units of RNase free DNase. After an additional incubation for 10 min the reaction mixture was extracted once with an equal volume of phenol/chloroform (1:1) and once with chloroform. Thirty micrograms of glycogen was then added to the aqueous layer followed by the addition of LiCl to a concentration of 0.4 M. The RNA was ethanol precipitated and the dryed RNA pellet dissolved in 20−40 μl of diethylpyrocarbonate (DEPC) treated water.

^{32}P labeled T7 transcripts were prepared as described above except the reaction mixtures were supplemented with 40 μCi of α-^{32}P-GTP. The concentrations of the unlabeled nucleotides were not altered.

6-Methyladenine mRNA methyltransferase assay

The methyltransferase assay used in these studies was identical to that described by Narayan and Rottman (19) with minor modifications. Three micrograms of an RNA substrate and 20,000 DPM'S of an identical ^{32}P labeled transcript were incubated with 10 μl of HeLa nuclear extract and 8 μCi of S-[methyl-^3H] adenosyl-L-methionine (78 Ci/mmol). The ^{32}P labeled transcripts were added in order to normalize for RNA recovery. The final volume of the assay was 50 μl. The concentration of Hepes buffer and polyvinylalcohol was as described (19), however the concentrations of KCl and $MgCl_2$ were altered to 20 μM and 2 μM respectively. The mixtures were then incubated for 30 min at 30°C. The reactions were then stopped by the addition of an equal volume of 100 mM Tris (pH 7.6) containing 20 mM EDTA, 20 mM NaCl and 0.2% SDS; extracting once with phenol/chloroform and once with chloroform. The methylated RNA was then ethanol precipitated using mussel glycogen as a carrier. Control assays were performed in which the T7 transcript was omitted from the reaction mixture.

RNA digestion and high pressure liquid chromatography analysis of methylated nucleosides

The pelleted RNA, methylated as described above, was dissolved in 38 μl of 10 mM sodium acetate (pH 5.0). Two micoliters of the RNA solution were then counted in a liquid scintillation counter in order to calculate RNA recovery values. A ratio of ^{32}P labeled RNA recovered from each assay to that added was used to determine RNA yields. The remaining RNA was then digested with 4 units of ribonuclease T2 and 4 μg of nuclease P1 for 3 hrs at 37°C as described (27). The pH of the solution was then adjusted to 9 using 200 mM ammonium hydroxide. Seventeen units of bacterial alkaline phosphatase (BAP) were added and the mixtures incubated for an additional 45 min. The reactions were then dryed in a speed vac and the nucleosides dissolved in HPLC loading buffer.

The HPLC system used was identical to that described by Backlund Jr. et al. (28) using a Altex ultrasphere ODS column. The starting buffer was 10 mM ammonium acetate containing 2.5% methanol. After 10 min, a linear gradient was started increasing to 60% methanol in 40 min. The flow rate through the entire run was maintained at 1 ml/min. One half milliliter fractions collected throughout the run were then counted for radioactivity in a liquid scintillation counter.

RESULTS

Substrate specificity for HeLa 6-methyladenine mRNA methyltransferase

Four different T7 transcripts were tested as substrates for the crude HeLa 6-methyladenine mRNA methyltransferase. These transcripts were then analyzed for methylated adenine derivatives using high pressure liquid chromatography. The chromatography system used in these studies provides excellent separation of the N-methylated adenine residues from 7-methylguanine and the 2'-O-methylated nucleosides normally found in the cap structure. Figure 2 panel A depicts a representative separation profile of methylated adenine derivatives from the parent nucleosides. T7 transcripts of two cellular genes, prolactin and DHFR, were then compared as substrates for the 6-methyladenine mRNA methyltransferase enzyme. These two transcripts were chosen as substrates because both naturally contain m6A residues (18,19,29). Panels C−F represent radioactivity incorporation into these two transcripts as well as a synthetic (MS7) RNA substrate which contained multiple consensus methylation sequences. Panel B shows the amount of m6A formed in the control reaction where no T7 transcript was added. This data demonstrates very little endogenous activity from RNA present in the HeLa nuclear extract. Panels C−F represent the pattern of radioactivity incorporated in prolactin, DHFR (capped), DHFR (uncapped) and the synthetic MS7 transcripts respectively. As seen in this figure both the capped and uncapped DHFR incorporated significantly more radioactivity than the other two transcripts.

While radiolabeled m6A residues were found in both cellular transcripts, DHFR incorporated 3 fold more radioactivity into the m6A peak than the prolactin transcript (Table 1). A comparison was also made between the activity of the DHFR transcript which had been capped in the T7 *in vitro* transcription assay versus that of an uncapped DHFR message. The recovery of all RNA transcripts were normalized by adding small amounts (20,000 DPM) of RNA labeled with α-^{32}P GTP to the methylation reactions. The recovery yield was then determined before RNA hydrolysis. While the amount of radioactivity incorporated into the capped and uncapped transcripts were similar (Table 1), the recovery yields (Table 1) were significantly lower for the uncapped transcript. When the methylation data was corrected for recovery, the uncapped transcript appears to serve as a slightly better methyl accepting substrate than its capped

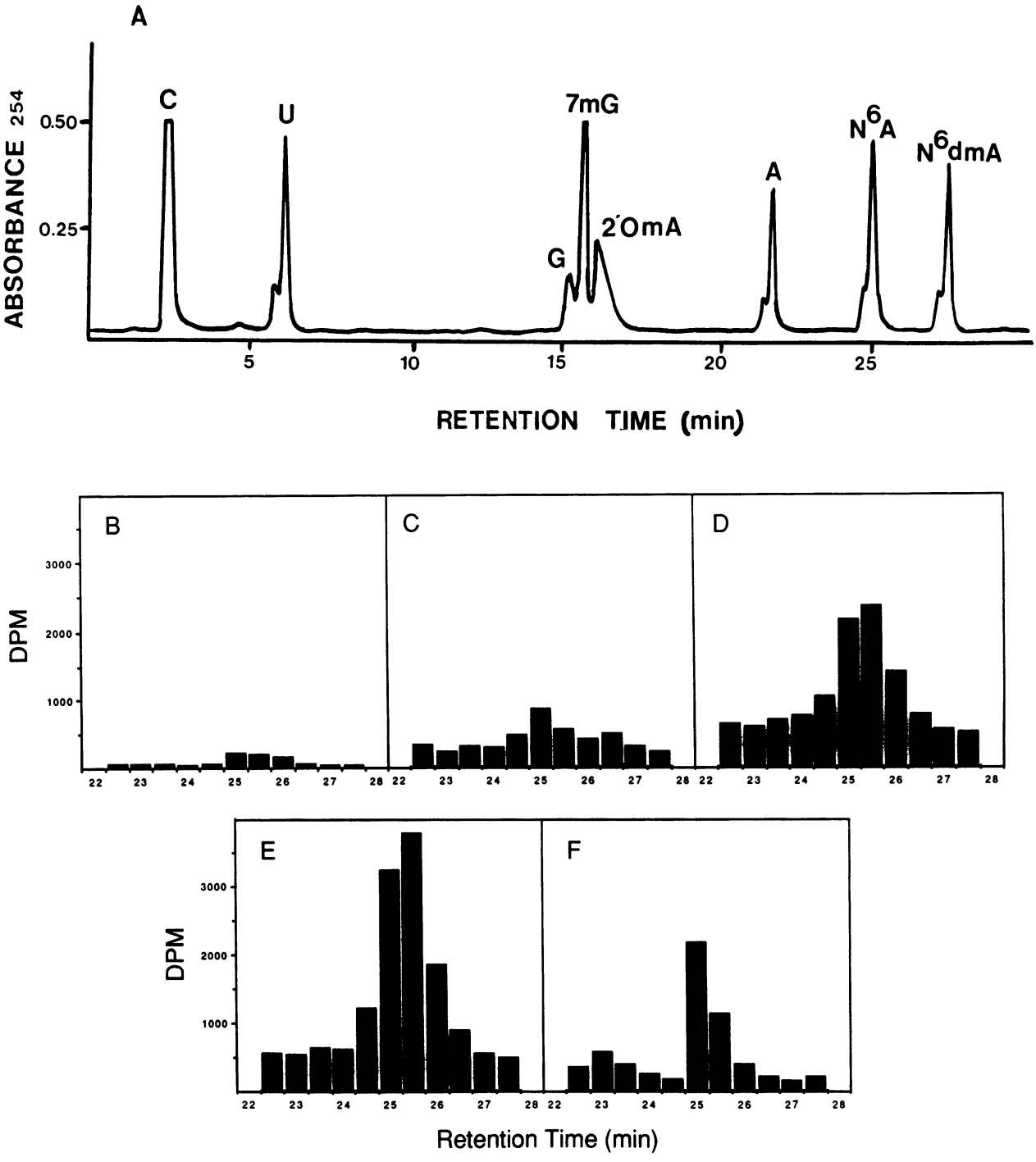

Figure 2. High Pressure Liquid Chromatograph profile of methylated ribonucleosides. Panel A, retention times (min) of standard nucleosides: 2.47, Cytosine (C); 6.18, Uridine (U); 15.41, Guanine (G); 15.86, 7-Methylguanine (7mG); 16.29, 2'-O-methyladenosine (2'OmA); 21.85, Adenosine (A); 25.04, N^6-methyladenosine (N^6A); 27.40, N^6-dimethyladenosine (N^6dmA). Panel B, radioactivity incorporation profile into the m6A peak of the control reaction (no added transcript). Panel C, radioactivity incorporated into capped prolactin transcripts. Panel D, radioactivity incorporated into capped DHFR transcripts. Panel E, radioactivity incorporated into uncapped DHFR transcripts. Panel F, radioactivity incorporated into capped MS7 transcripts.

counterpart. The differences in stability is most likely due to the presence of a cap structure. It is well known that the presence of cap structures serve as stabilizing elements for both the premRNA in the nucleus (30) as well as the mature cytoplasmic mRNA (31).

In addition to the cellular transcripts which were analyzed as substrates for the N^6 adenine methyltransferase enzyme a synthetic RNA molecule which contained a number of potential methylation sites was also tested. As described above, this transcript was formed by cloning synthetic DNA sequences identical to regions of Rous sarcoma viral DNA into the pGem 1 vector. Transcripts produced from this DNA segment have previously been shown to contain two methylation sites (23). The rational behind constructing this plasmid was to produce a T7

Table 1. Analysis of in vitro formed 6-methyladenine residues in different T7 RNA transcripts

T7 transcript	DPM*	pmol methyl group/ pmol RNA	% Recovery
Prolactin	10,623	6.2×10^{-3}	22
DHFR (capped)	18,814	1.8×10^{-2}	41
DHFR (uncapped)	31,891	3.1×10^{-2}	34
MS7 RNA	11,573	2.8×10^{-3}	30

All values were corrected for radioactivity incorporated in the control reaction (endogenous RNA).
* DPMs and specific activity values reported were normalized for recovery yields.

transcript containing as many methylation sites as possible. The 370 base transcript produced from this template contains 20 possible methylation consensus sequences. As shown in table 1, while the transcript produced from this template served as a methylation substrate for the enzyme, the incorporation of radioactivity into the m6A peak was much less than either capped or uncapped DHFR, and 2 fold lower than prolactin mRNA.

As was reported previously for bovine prolactin RNA (19), the methylation of all four transcripts in this study occurred in substoichiometric amounts. As seen in table 1 the ratio of methyl incorporation to RNA concentration ranged from 10^{-2} to 10^{-3}. It is interesting however that the stoichiometry for capped DHFR is 6 fold that of the MS7 and 3 fold that of capped prolactin RNA. This increase in part is due to the fact that the DHFR mRNA appears to contain multiple residues of m6A (data described below) compared to only one for prolactin (19). However, such a large increase may also suggest that DHFR may be methylated at a higher stoichiometry than the other substrate transcripts.

Localization of the m6A residues within the DHFR transcript

It has been well documented that not all methylation consensus sequences are modified both *in vivo* or *in vitro*. In the case of prolactin mRNA one adenine residue was shown to be modified in the 3′ end of the molecule out of a total of 3 possible consensus sequences (19). Because the main objective of this research is to utilize DHFR mRNA as a model system to determine the biological function of m6A residues; it was important to localize the modified residues to specific regions of the transcript. These experiments were done by subcloning three restriction endonuclease DNA fragments of the DHFR gene into pGem vectors. The resulting plasmids were linearized and used as templates in the synthesis of capped transcripts. These transcripts were then utilized as substrates in the methyltransferase assay. In addition to these experiments, the DNA fragments were also linearized with restriction endonucleases which digest at different sites within the DHFR gene. Transcripts resulting from these templates varyed in length and were used to map the location of m6A residues with in the DHFR transcript. HPLC analysis of the methylated transcripts showed all to act as substrates for m6A formation to varying degrees. As shown in table 2, the 594 and the 570 base transcripts derived from the Pst I-Bgl II and Bgl II-Bgl II fragments respectively incorporated the greatest amount of radioactivity as m6A. The small 310 base Bgl II-Pst I fragment also acted as a substrate in m6A formation however the counts in this fragment were only slightly above the control reaction. From this data it can be concluded that DHFR mRNA contains at least two residues of m6A with at least one localized in the 3′ noncoding region.

An attempt was then made to map the location of the m6A

Table 2. Localization of 6-methyladenine residues in DHFR mRN

T7 transcript	*pmol methyl group/ pmol RNA
Pst I-Pst I (1470 base transcript)	1.8×10^{-2}
Pst I-Bgl II (594 base transcript)	1.0×10^{-2}
Taq I	1.6×10^{-3}
Msp I	2.3×10^{-3}
Sau3A	5.6×10^{-3}
Bgl II-Bgl II (570 base transcript)	4.0×10^{-3}
Alu I	5.3×10^{-3}
Bgl II-Pst I (310 base transcript)	3.2×10^{-4}

*Values were corrected for endogenous RNA activity and normalized for recovery yields.

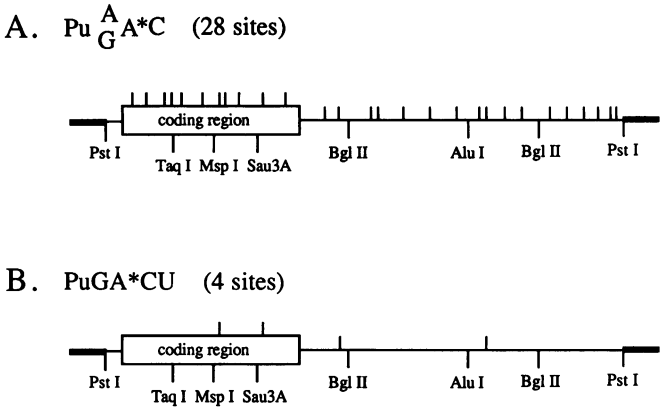

Figure 3. Map of methylation consensus sequences in DHFR mRNA. In map A, vertical lines represent all AAC and GAC consensus methylation sequences for a total of 28 sites. In map B the preferred PuGACU sequences are represented by vertical lines (4 total sites). * represents the methylated adenine residue in these sequences.

residues in the Pst I-Bgl II and the Bgl II-Bgl II transcripts since these were found to incorporate the most m6A. The pGDHFR-PB plasmid containing the 594 base pair Pst I-Bgl II insert was digested in three different reactions using Taq I, Msp I and Sau3A (cleavage sites of all three enzymes are shown in figure 1). Templates resulting from these digestions produced run off capped transcripts of 137, 250 and 359 bases respectively. When these transcripts were analyzed for methylation activity it was found that all three acted as substrates for the enzyme (table 2). The fact that the specific activity of the methylation decreased with the length of the transcripts, suggests that the transcript derived from the Pst I-Bgl II fragment contains multiple methylation sites located throughout the molecule. A similar experiment was performed with the pGDHFR-BB plasmid containing the Bgl II-Bgl II fragment. This plasmid was digested with Alu I and utilized as a template in the formation of a 352 base run off transcript representing the 5′ half of this RNA fragment. This transcript also served as a methylation substrate (table 2) displaying a specific activity slightly larger than that of the entire 570 base parent transcript. This data therefore suggests that the Bgl II-Bgl II transcript is void of m6A residues 3′ to the Alu I digestion site.

It was surprising that DHFR mRNA appears to contain multiple methylation sites considering that prolactin, the only other cellular mRNA investigated thus far, contains only one. Whether the location of the methylated residues formed *in vitro* match those

which are formed *in vivo* (29) is not known at this time. It must be stressed that the location of methylated residues formed *in vitro* by these studies may be effected by the possible secondary and/or teritiary structure of the transcript. The location of the methylated residues may therefore be different than those found in future *in vivo* studies. Also the stoichiometry of methylation may be significantly altered with the different transcripts used in these studies. This data therefore suggests the location of the modified residues. However, it must be noted that the accuracy of the *in vitro* methylation reaction has not been characterized well enough to determine the location of the methylated residues without *in vivo* information.

DISCUSSION

With the exception of prolactin (18,19) and Rous sarcoma viral RNA (16,23), very few specific RNA molecules have been analyzed for m6A content. Recent techniques which enable precise mapping of m6A residues (18,19) and the development of an *in vitro* methylation assay (19) have opened the door for studies investigating m6A levels in specific mRNA molecules as well as the biological significance of this modification. In this study T7 transcripts coding for mouse DHFR have been investigated as substrates for the HeLa nuclear 6-methyladenine mRNA methyltransferase enzyme. The results of these investigations have showed that DHFR mRNA is a highly methylated message possibly containing several residues of m6A located in different regions of the transcript. In addition to these findings it was observed that the 7-methylguanine cap structure had no effect on the ability of the transcript to act as a substrate for the methyltransferase enzyme.

While the GAC and AAC consensus methylation sequences occur with a high degree of frequency in mRNA only a small population are modified (13- 15,19,32). DHFR mRNA contains 28 consensus sequences which appear throughout the transcript at random (figure 3). One such sequence PuGACU has been previously suggested to be a preferred methylation sequence (16,33). This sequence appears 4 times in the entire message (figure 3). It is therefore likely that in addition to a primary sequence, the 6-methyladenine mRNA methyltransferase enzyme must require a certain secondary and/or tertiary structure in order for methylation to occur. This theory is supported by the fact that MS-7 RNA which contains 20 proven methylation sites per molecule of RNA is a relatively poor substrate for the enzyme. Alteration of secondary structure may also expose a methylation sequence (not normally methylated by the methylase enzyme) for modification. Data such as this obtained *in vitro* must therefore be used as a guide to advance *in vivo* investigations of this problem.

It is interesting that in the transcripts from the Bgl II-Bgl II fragment, all of the m6A residues are present between the Bgl II- Alu I sequences. Since no major PuGACU sites are present in this transcript, the methylation must be occuring in the AAC sequences. Similarly, the AAC sequences also appear to be modified in the Pst I- Msp I derived transcripts (table 2). These sites however appear to be methylated to a lower extent than the PuGACU sites located downstream. It will again be interesting if future *in vivo* experiments using DHFR mRNA confirm these AAC methylation sites.

The low stoichiometry of m6A formation as described in this report for DHFR mRNA as well as for prolactin mRNA (19) is especially puzzling when attempting to assign a biological function to this modification. One possible explanation could be the formation of m6A residues may be a means by which the cell labels certain populations of transcripts. This labeling procedure may therefore greatly effect both the function or the metabolism of the transcripts by altering cytoplasmic transport, stability, translation efficiency or possibly other processing events.

The development of an *in vitro* system to investigate m6A formation has provided the means to make an extensive effort into investigating the biological function of this modification without utilization of general methylation inhibitors. The fact that DHFR mRNA is a highly methylated substrate and may contain multiple methylation sites as demonstrated in this report makes it an interesting model for these investigations. It is hoped that in the future a more detailed map of the location of m6A residues can be obtained both *in vitro* and *in vivo* for DHFR and that this information can be used in such studies to understand the biological significance of m6A formation in DHFR mRNA.

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