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Mapping of N^6-methyladenosine residues in bovine prolactin mRNA

(RNA methylation/antibodies to N^6-methyladenosine)

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ABSTRACT **N^6-Methyladenosine (m^6A) residues, which are found internally in viral and cellular mRNA populations at the sequences Apm^{6}ApC and Gpm^{6}ApC, have been proposed to play a role in mRNA processing and transport. We have developed a sensitive approach to analyze the level and location of m^6A in specific purified cellular mRNAs in an attempt to correlate m^6A location with function. Polyadenylylated mRNA is hybridized to cDNA clones representing the full size mRNA under study or fragments of it, and the protected RNA is digested and labeled with polynucleotide kinase *in vitro*. After enrichment for m^6A with anti-m^6A antibody, the [^{32}P]-pm^{6}A is separated on TLC plates, and compared with the total amount of radiolabeled nucleotides. Using this combination of *in vitro* RNA labeling and antibody selection, we were able to detect m^6A in purified stable mRNAs that cannot be readily labeled in cells with greater sensitivity than was possible by previous techniques. We applied this technique to bovine prolactin mRNA and showed that this mRNA contains m^6A. Moreover, all of the m^6A residues in this message are found within the 3′ two-thirds of the molecule and are highly concentrated (61%) within a sequence of 108 nucleotides at the 3′ noncoding region of the message. The nonrandom distribution of m^6A in a specific cellular mRNA, as demonstrated for bovine prolactin, will have to be taken into account when designing a model for m^6A function.**

The most prevalent internal methylated nucleoside in eukaryotic mRNA is N^6-methyladenosine (m^6A). This modified nucleoside is found in RNAs of higher eukaryotic organisms (1–6), plants (7–9), and viruses (3, 10–12), and occurs at two specific sequences: Gpm^{6}ApC and Apm^{6}ApC (13–17). The high degree of sequence specificity, together with the fact that m^6A is conserved during processing of the heterogeneous nuclear RNA in the nucleus (11, 16, 18, 19), argues for an important biological function. In viral RNA, internal m^6A is distributed in a nonrandom fashion. In Rous sarcoma virus, these methylated nucleosides are concentrated in a 3500-nucleotide region of the 10,000-nucleotide RNA genome (12). The highest level of internal m^6A residues in simian virus 40 (SV40) nuclear RNA is found in RNA transcripts from two specific regions of the viral genome (20). The m^6A-containing oligonucleotides of late SV40 16S and 19S mRNAs were further localized on the viral genome (15). The specificity of m^6A location in particular regions of viral RNA implies that, apart from sequence specificity, other features of the RNA may influence the location of internal methylation and is suggestive of a biological role for m^6A.

Further indication of the role of m^6A was obtained by inhibition studies. Inhibition of internal methylation of mRNA by cycloleucine in B77 avian sarcoma virus results in accumulation of genome-length RNA and decrease in the levels of spliced viral mRNA (21). Cycloleucine treatment of SV40-infected BSC-1 cells results in reduction of the amount of m^6A residues as well as inhibition of SV40 mRNA production, but almost no reduction of SV40 nuclear RNA (22). These findings argue for a role of internal m^6A in RNA processing or in modulating the processing-linked transport of viral mRNA from the nucleus to the cytoplasm. Moreover, recent findings in our laboratory suggest that internal methylation is also important for mRNA processing or transport of cellular mRNA (23). In these studies it was shown that *S*-tubercidinylhomocysteine, a potent inhibitor of *S*-adenosylmethionine-dependent methyltransferase reactions, causes a significant lag in the time of appearance of HeLa mRNA in the cytoplasm under conditions in which the level of internal m^6A is reduced by 83% (23).

To further understand the function of internal methylation in cellular mRNA, we were interested in analyzing the location of m^6A in purified, specific, cellular mRNAs and determining whether m^6A is randomly distributed within a given message or is specific to particular region(s) or site(s). Previous studies on the location of m^6A in cellular mRNA utilized mixed populations of mRNA and are contradictory (2, 4, 6, 17, 18). Attempts to analyze m^6A location in specific cellular mRNAs have been carried out with globin and histone mRNAs, and both messages apparently lack internal m^6A (24–27). We have developed a sensitive approach to analyze the level and location of m^6A in specific purified mRNAs. The method is designed for direct m^6A analysis of mRNAs that cannot be readily labeled in cells and is based on a combination of *in vitro* labeling of nucleotides from specific fragments of the purified RNA of interest. The [^{32}P]pm^{6}A residues are partially purified by anti-m^6A antibody binding and analyzed by thin-layer chromatography (TLC). We have applied this technique to the abundant bovine pituitary mRNAs for growth hormone and prolactin. While growth hormone mRNA contains little or no m^6A, bovine prolactin mRNA was shown to contain m^6A, and the location of m^6A residues was studied. We found that m^6A is not randomly distributed within the prolactin message. On the contrary, it is found only within the 3′ two-thirds of the RNA, and it is highly enriched in a sequence of 108 nucleotides at the extreme 3′ end of the message.

MATERIALS AND METHODS

Construction of Subclones. The bovine prolactin (bPRL) cDNA clone pBPRL72 is full length, extending from the major poly(A) site to the cap site (28). The bovine growth hormone (bGH) cDNA clone pG23 is nearly full length, extending from the poly(A) site to within 28 nucleotides of the cap site (29). Single- or double-digested restriction fragments of

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Abbreviations: m^6A, N^6-methyladenosine; SV40, simian virus 40; bPRL, bovine prolactin; bGH, bovine growth hormone.

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bPRL pBPRL72 and bGH pG23 cDNA clones were subcloned in M13mp8 or M13mp9 phage (30) to obtain full-size bPRL and bGH cDNAs or portions of them as single-stranded sequences that are complementary to mRNA. The cloning strategy and the resulting clones are presented in Fig. 1.

Preparation of RNA. Polysomal RNA was prepared from bovine anterior pituitary glands as described previously, and poly(A)-containing RNA [poly(A)$^+$ RNA] was isolated by repeated oligo(dT)-cellulose chromatography (31). When removal of the poly(A) tail of the RNA was required, a sample (11 μg) of the poly(A)$^+$ RNA was denatured (1 min, 50°C) in 45 μl of a buffer containing 50 mM Tris·HCl at pH 7.5, 100 mM KCl, 25 mM $MgCl_2$, and 20 μg of bovine serum albumin, cooled to room temperature, hybridized to 11 μg of $d(pT)_{12-18}$ (Collaborative Research) for 15 min at room temperature, and digested with 50 units of RNase H (Enzo Biochemicals, New York), for 1 hr at 30°C. The RNA was then extracted twice with phenol/choloroform, twice with chloroform, and precipitated with ethanol.

Preparation of Protected RNA Fragments. For preparation of each RNA fragment, 10 μg of bovine pituitary poly(A)$^+$ RNA or RNase H-treated RNA was heat denatured (65°C, 3 min) and hybridized to 100 μg of the respective M13 bPRL or bGH clones in 410 μl of 50% (vol/vol) formamide/0.6 M NaCl/0.01 M 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonate (Tes), pH 7.4/0.01 M EDTA/0.2 mg of yeast tRNA per ml at 42°C for 16 hr. The hybridization mixtures were then diluted 1:10 in 0.3 M NaCl and precipitated with ethanol. The pellets were dissolved in 100 μl of 0.3 M NaCl/0.02 M sodium acetate, pH 4.5, and the unhybridized RNAs were digested with 20 units of RNase T_2 (Calbiochem) at room temperature for 3 hr. RNase T_2 was removed by diluting the reaction mixture 1:10 in 0.3 M NaCl/40 mM Tris·HCl, pH 7.5, extracting twice with 2 vol of a 1:1 (vol/vol) mixture of Tris·HCl (0.1 M, pH 7.4) -saturated phenol and chloroform/isoamyl alcohol (24:1, vol/vol), once with 2 vol of chloroform/isoamyl alcohol (24:1), and precipitating with ethanol. DNA from the M13 subclones, along with the hybridized protected RNA fragments, was seprated from the oligonucleotides released by the RNase digestion by electrophoresis in a 14-cm 1% agarose gel (low gelling temperature SeaPlaque, FMC, Rockland, ME) in 40 mM Tris/5 mM sodium acetate/1 mM EDTA, pH 7.6, at 50 V for 1 hr at 4°C. The high molecular weight band, visualized by ethidium bromide

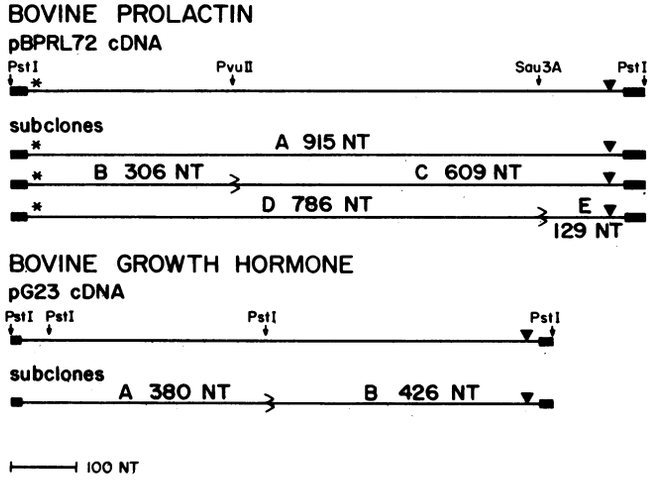

FIG. 1. Construction of bPRL and bGH subclones. Single- or double-digested restriction fragments of bPRL pBPRL72 and bGH pG23 cDNA clones were subcloned in M13mp8 or M13mp9, resulting in bPRL subclones A, B, C, D, and E, and bGH subclones A and B. The length of the protected RNA fragments is given in nucleotides (NT). *, Cap site; ▼, polyadenylylation site; ▬, oligo(G·C) tail.

staining and UV, was cut out, and the DNA and RNA·DNA hybrids were eluted from the gel by a modification of the NaI/glass powder procedure (32), as follows: The gel slices were dissolved in 3 ml of saturated NaI solution per g of gel at 37°C for 30 min and then incubated with 50 μl of glass powder at 0°C for 1 hr. The DNA–RNA–glass was washed once with cold NaI solution and twice with cold washing buffer [50% (vol/vol) ethanol/100 mM NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA]. The DNA and DNA·RNA hybrids were eluted in 10 mM Tris·HCl, pH 7.4/0.1 mM EDTA, at 37°C for 30 min, brought up to 0.3 M NaCl, precipitated with ethanol, dissolved in 40 μl of water, and stored at −80°C.

RNA Blot Hybridization. The sizes of the protected RNA fragments were analyzed by running denatured samples of the gel eluted material in agarose (1.2%)/formaldehyde (6%) gels in 0.02 M boric acid/0.2 mM Na_3EDTA, pH 8.3, and blotting onto GeneScreen (New England Nuclear) as described by the manufacturer, but omitting the NaOH hydrolysis step. The membranes were then incubated with a buffer containing 50% (vol/vol) formamide, 0.8 M NaCl, 0.02 M 1,4-piperazinediethanesulfonic acid (Pipes), 2 mM EDTA, 0.5% $NaDodSO_4$, and sonicated, denatured salmon sperm DNA at 100 μg/ml, at 42°C for 4 hr and hybridized to nick-translated bPRL (pBPRL72) or bGH (pG23) cDNA clones overnight using the same conditions. The blots were washed once at room temperature and three times at 65°C for 30 min each in 0.15 M NaCl/0.05 M Tris·HCl, pH 7.6/1 mM EDTA/0.1% sodium pyrophosphate/0.1% $NaDodSO_4$ and were then exposed to Kodak X-Omat AR (XAR-5) film at −80°C, with DuPont Cronex Lightning Plus intensifying screens.

Digestion and Labeling of RNA. Samples (35 μl) of the eluted material containing the selected RNA fragments were heat denatured (5 min at 100°C) and cooled on ice. The RNA was completely hydrolyzed by incubation in 0.1 M NaOH at 65°C for 1 hr, and the digestion mixtures were neutralized with HCl. The nucleotides were then 5′ end labeled by incubation in 60 μl of buffer (50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/5 mM dithiothreitol) containing 100 μCi of [γ-^{32}P]ATP (Amersham; 1 Ci = 37 GBq, 3000 Ci/mmol), and 15 units of T4 polynucleotide kinase (3′-phosphatase free, Boehringer Mannheim) at 37°C for 1 hr. The reaction was stopped by heating at 100°C for 5 min. The DNA was removed from the labeled nucleoside diphosphates by passage through a DE-52 anion-exchange cellulose (Whatman) minicolumn (200-μl bed volume) under conditions in which the DNA binds and the nucleoside diphosphates pass through (150 mM NaCl/20 mM sodium phosphate, pH 7.0). Peak fractions of the nucleoside diphosphates (600 μl) were pooled, and the 3′ phosphates were removed by digestion with 150 μg of nuclease P1 (P-L Biochemicals) in 0.04 M ammonium acetate, pH 6.0, at 37°C for 12 hr.

Antibody Binding. Anti-m^{6}A Sepharose was prepared and characterized as described (33). Samples of the 5′-labeled nucleotides (100 or 200 μl) were incubated with 25 μl (bed volume) of anti-m^{6}A Sepharose at room temperature, for 1 hr with continuous gentle inversion. After a brief wash with cold buffer (10 mM Tris·HCl/1 mM EDTA, pH 7.5), the bound nucleotides were eluted twice in 100 μl of 25% (vol/vol) ethanol at 90°C for 10 min each, pooled, and evaporated to a small volume under reduced pressure.

Analysis of Labeled Nucleotides. The total labeled nucleotides (2 μl of the original reaction) and the antibody-bound m^{6}A-enriched labeled nucleotides (representing 100 μl and 200 μl of the original reaction) were analyzed by two-dimensional TLC along with unlabeled nucleotide standards, using a modification of the method described by Nishimura (34). The chromatography was performed on 20 × 20 cm, 0.1 mm cellulose MN 300 polyethyleneimine (PEI)-impregnated

TLC plates (Sybron–Brinkmann, Westbury, NY), employing solvent systems I (isobutyric acid/0.5 M NH_4OH, 5:3, vol/vol) and II (isopropyl alcohol/HCl/H_2O, 70:15:15, vol/vol/vol). The nucleotide standards were detected by UV, and the radioactive nucleotides were located by autoradiography, using Kodak X-Omat AR (XAR-5) film at −80°C, with DuPont Cronex Lightning Plus intensifying screens. For quantitative analysis, the labeled nucleotide spots were cut out and scraped, and the radioactivity was measured in Triton X-100/toluene scintillation fluid.

RESULTS

Preparation of Protected bPRL and bGH RNA Fragments. Prolactin and growth hormone mRNAs were chosen as a model system for analysis and mapping of internal m^6A residues in steady-state, specific, cellular mRNAs. As a source of mRNA, poly(A)$^+$ RNA extracted from bovine anterior pituitary glands was used, due to the high abundance of prolactin and growth hormone messages in this tissue (31, 35). bPRL and bGH mRNAs are highly stable and thus are difficult to label *in vivo*. However, isolation of specific bPRL and bGH sequences from the pituitary mRNA population by hybridization and RNase protection provides sufficient quantities of RNA for *in vitro* labeling and for analysis of the labeled nucleotides.

To map the m^6A-containing sequences in bPRL and bGH mRNA, specific fragments of the mRNAs were prepared by hybridization to the respective DNA subclones (Fig. 1), followed by RNase T2 digestion of the unhybridized sequences and gel purification. For measurement of the level of m^6A in full-length bPRL mRNA, the twice oligo(dT)-purified poly(A)$^+$ RNA was hybridized to the respective full-size, complement sense M13 bPRL clone (Fig. 1, bPRL A) and then digested with RNase T2. To eliminate the possibility that m^6A residues are present in the protected 21 A residues that are part of the poly(A) tail and thus contribute to the measured m^6A level, poly(A) tails were removed by RNase H digestion in one sample of the RNA (Fig. 2*A*). Hybridization of both the intact poly(A)$^+$ RNA and RNase H-treated poly(A)$^+$ RNA to the full-size bPRL clone resulted after RNase T2 digestion in the expected RNA fragments (915 and 894 nucleotides, respectively) as demonstrated by RNA blot hybridization (Fig. 2*B*, lanes 5 and 6, respectively). For mapping of m^6A within the bPRL and bGH messages, two sets of bPRL subclones, each set composed of two subclones representing the full-size bPRL sequence (Fig. 1, bPRL B and C and bPRL D and E) and one set of two bGH subclones representing the full-size growth hormone sequence (except for the first 28 nucleotides of the 5′ end) (Fig. 1, bGH A and B) were used. Poly(A)$^+$ RNA was hybridized to each subclone, and the RNase T2-protected RNA fragments were analyzed by blot hybridization. As shown in Fig. 2, the expected RNA fragments for the bPRL sequences (Fig. 2*B*, lanes 1, 2, 3, and 4) and the bGH sequences (Fig. 2*C*, lanes 1 and 2) were obtained. As a control, poly(A)$^+$ RNA was hybridized to M13 DNA containing no homologous sequences (M13mp8). No detectable bPRL and bGH sequences were found after RNase T2 digestion (Fig. 2*B*, lane 7 and Fig. 2*C*, lane 3, respectively).

Mapping of m^6A Residues in bPRL and bGH mRNA. For measurement and mapping of internal m^6A residues we utilized *in vitro* labeling of the nucleotides contained in the protected RNA fragments. Due to the fact that the m^6A levels in cellular mRNA are low, and that *in vitro* labeling will label all four nucleotides in addition to m^6A, we have designed an experimental protocol that combines *in vitro* labeling with enrichment for the labeled pm^6A nucleotides by binding to anti-m^6A antibody and purification of pm^6A by analytical TLC. Briefly, the protected RNA fragments were degraded

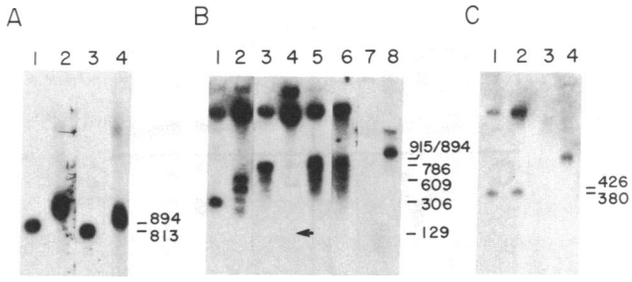

FIG. 2. Blot hybridization analysis of protected mRNA fragments. Poly(A)$^+$ RNA was hybridized to bPRL or bGH cDNA M13 clones and digested with RNase T2, and the protected RNA fragments were denatured and electrophoresed in formaldehyde/agarose gels. RNA blots were prepared and hybridized with nick-translated recombinant plasmids. (*A*) Autoradiogram of RNase H-treated (lanes 1 and 3) and intact (lanes 2 and 4) bovine pituitary poly(A)$^+$ RNA, prior to the hybridization/digestion experiment, probed with nick-translated bPRL (lanes 1 and 2) or bGH (lanes 3 and 4) cDNA. (*B*) Autoradiogram of protected RNA fragments derived by hybridization of poly(A)$^+$ RNA to bPRL subclones B, C, D, E, and A (lanes 1, 2, 3, 4, and 5, respectively), and to M13mp8 (lane 7), and by hybridization of RNase H-treated poly(A)$^+$ RNA to the bPRL subclone A (lane 6); lane 8 contains unhybridized poly(A)$^+$ RNA; all RNAs were probed with bPRL cDNA; arrow points to the position of the 129-nucleotide RNA fragment (seen better with long exposure). (*C*) Autoradiogram of protected RNA fragments derived by hybridization of poly(A)$^+$ RNA to bGH subclones A and B (lanes 1 and 2, respectively) and to M13mp8 (lane 3); lane 4 contains unhybridized poly(A)$^+$ RNA; all RNAs were probed with bGH cDNA. Numbers indicate size of the RNA, in nucleotides. For description of the subclones and expected RNA fragment sizes, see Fig. 1.

to nucleoside 2′- and 3′-monophosphates (Np's) by alkali treatment. The 5′-OH groups were then phosphorylated by polynucleotide kinase in the presence of [γ-^{32}P]ATP, resulting in nucleoside 3′,5′-diphosphates labeled at their 5′-phosphate ([^{32}P]pNps). To obtain radioactive nucleoside 5′-monophosphates ([^{32}P]pNs), the 3′-phosphates were removed by nuclease P1 treatment. To prevent any interference by the M13 DNA, the high molecular weight bacteriophage DNA was removed from the labeled [^{32}P]pNps by anion-exchange chromatography (DE-52 cellulose) prior to the nuclease P1 digestion. The radioactivity incorporated into the nucleotides of each RNA fragment was estimated by TLC of a 2-μl sample of the [^{32}P]pNs followed by autoradiography, cutting and scraping the nucleotide spots, and measuring their radioactivities in a scintillation counter. A representative TLC autoradiogram of total labeled nucleotides is presented in Fig. 3*A*. The pm^6A level is below the detection limit of the analytical TLC when total labeled nucleotides are analyzed prior to m^6A antibody enrichment. To control for background levels of radioactivity, poly(A)$^+$ RNA was hybridized to M13mp8 DNA, treated with RNase T2, and processed as described above. The level of radioactivity in the nucleotide spots as visualized by TLC was negligible (autoradiogram not shown). The average cpm in total nucleotides for each of the protected RNA fragments as well as the control is presented in Table 1.

To enrich for [^{32}P]pm^6A, 100- and 200-μl samples of the labeled nucleotides were bound to anti-m^6A antibody, under conditions of antibody excess. Since we did not want to risk any loss of specific pm^6A, we chose to wash the antibody-bound material only once. Consequently some nonspecifically bound labeled nucleotides remained with the eluted [^{32}P]pm^6A. These were resolved from the pm^6A by analytical TLC. The TLC plates were autoradiographed, the pm^6A spots were visualized by UV absorption of the marker nucleotide, cut out, and scraped, and the level of radioactivity was measured by a scintillation counter. Background [^{32}P]pm^6A

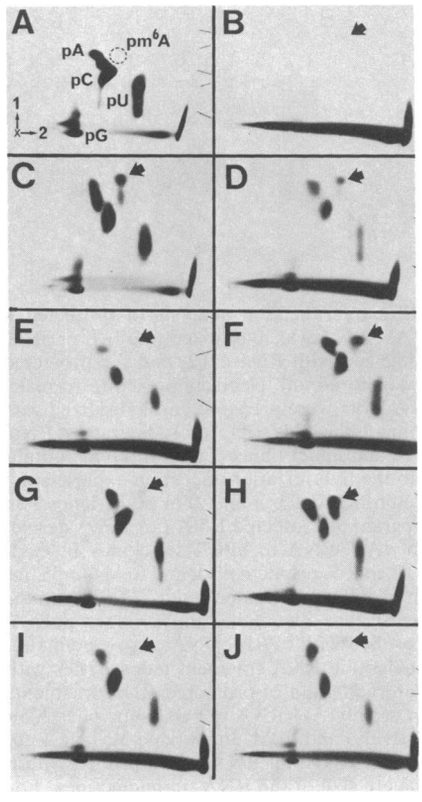

FIG. 3. Two-dimensional TLC of antibody-bound labeled nucleotides. Protected bPRL and bGH mRNA fragments were digested and treated with polynucleotide kinase *in vitro*. The resulting nucleotides (100 μl) were purified with anti-m^{6}A antibody and separated on TLC plates in two dimensions. ×, origin; 1, first dimension; 2, second dimension; pA, pC, pU, pG, and pm^{6}A indicate the nucleotide spots; arrows indicate positions of the pm^{6}A spots. (*A*) Nucleotide distribution before antibody purification (2-μl sample), as demonstrated for the bPRL full-size protected mRNA. (*B*) Antibody-bound M13mp8 control (no protected RNA); (*C–J*) antibody-bound labeled nucleotides of the respective protected RNA fragments: (*C*) bPRL full-size (915 nucleotides); (*D*) RNase H-treated full-size (894 nucleotides); (*E*) 5′ 306 nucleotides; (*F*) 3′ 609 nucleotides; (*G*) 5′ 786 nucleotides; and (*H*) 3′ 129 nucleotides; and (*I*) bGH 5′ 380 nucleotides; and (*J*) 3′ 426 nucleotides.

levels were low, as shown for the M13mp8 hybridization reaction in Fig. 3*B* and Table 1. The levels of [^{32}P]pm^{6}A in each of the bPRL and bGH protected fragments are qualitatively demonstrated in Fig. 3 *C–J*, representing RNA fragments covering bPRL full length (915 nucleotides), full length missing the 21 nucleotides of the poly(A) tail (894 nucleotides), 5′ 306 nucleotides, 3′ 609 nucleotides, 5′ 786 nucleotides, and 3′ 129 nucleotides, and bGH 5′ 380 nucleotides and 3′ 426 nucleotides, respectively. The amounts of radioactivity in the pm^{6}A spots as well as the normalized relative amounts of pm^{6}A in each fragment are presented in Table 1. When the full-size bPRL protected mRNA (915 nucleotides) is taken as 100%, it is clear that removal of the otherwise protected tail of 21 A residues by RNase H treatment did not reduce the level of detected m^{6}A (106%), hence, all the internal m^{6}As in bPRL mRNA are within the transcribed sequence. Analysis of the first set of bPRL RNA fragments (bPRL 5′ 306 and 3′ 609) shows that the m^{6}A residues are present only in the 3′ two-thirds of the molecule. Interestingly, the majority of these (61%) reside within a short fragment (129 nucleotides) representing the extreme 3′ end of the bPRL mRNA (see fragments bPRL 5′ 786 and 3′ 129). The bGH mRNA, on the other hand, as shown with bGH 5′ 380 and bGH 3′ 426 RNA fragments, is very poor in m^{6}A, with pm^{6}A levels approaching those of background control (Table 1).

Table 1. Amount of pm^{6}A in bPRL and bGH RNA fragments

Subclone used for hybridization	Orientation and RNA size, nucleotides	Mean cpm in total nucleotides at 1× (2 μl)	cpm in pm^{6}A at 50× (100 μl)	cpm in pm^{6}A at 100× (200 μl)	Relative amount of pm^{6}A,* %
		bPRL			
A	915	63,300	775	1385	100
A	894†	15,500	200	340	106
B	5′ 306	16,700	55	112	2
C	3′ 609	28,000	495	833	91
D	5′ 786	39,100	209	289	27
E	3′ 129	22,600	1128	2049	61
		bGH			
A	5′ 380	27,500	90	105	NA
B	3′ 426	20,800	115	198	NA
		Control M13mp8			
	—	700	53	74	NA

NA, not applicable.

*The amount of pm^{6}A per fragment of RNA was calculated by dividing the net (cpm above M13mp8 control) mean cpm in pm^{6}A by the net mean cpm of total nucleotides (both corrected to 1× starting material) and multiplying this fraction by the number of nucleotides per fragment. For normalization, the amount of pm^{6}A in full-size bPRL mRNA was taken as 100%. For example, the relative amount of pm^{6}A in the bPRL 3′ 129-nucleotide RNA fragment is

$$\frac{\frac{[2(1128-53)+(2049-74)]\div 2}{100(22{,}600-700)}\times 129}{\frac{[2(775-53)+(1385-74)]\div 2}{100(63{,}300-700)}\times 915}\times 100 = 61\%.$$

†The poly(A)$^+$ RNA used in this experiment was pretreated with RNase H to remove the poly(A) tails.

DISCUSSION

We have developed a sensitive approach to analyze the level and location of m^{6}A in specific purified mRNAs in an attempt to correlate location and function of internal methylation in cellular mRNA. The method is designed for direct m^{6}A analysis of mRNAs that cannot be readily labeled in cells. It is based on a combination of *in vitro* labeling of nucleotides from specific fragments of the purified RNA of interest, and analyzing the [^{32}P]pm^{6}A by anti-m^{6}A antibody binding and TLC. We have applied this technique to the well-characterized bovine pituitary mRNAs growth hormone and prolactin. We were interested in determining whether m^{6}A in these cellular mRNAs is randomly distributed or is specific to particular region(s) or site(s).

The measured amount of pm^{6}A in the bGH message was very low and close to background levels. This is similar to findings reported for two other eukaryotic cellular mRNAs (globin and histone) that were analyzed for m^{6}A content and were found to lack or have very low amounts of this internal methylated residue (24–27). The possibility that bGH contains m^{6}A in the 28 nucleotides at the extreme 5′ end that were not protected by our bGH cDNA subclones is still an open one. Nevertheless, this short sequence contains only two possible methylation sites out of the total 26 ApApC and GpApC sequences that are present in bGH mRNA.

bPRL mRNA, on the other hand, had significant levels of m^{6}A, and the location of these residues was studied. As was found for viral RNAs (12, 15, 20), the m^{6}A in bPRL mRNA is not randomly distributed. On the contrary, all the m^{6}A residues in this message are found within the 3′ two-thirds of the

molecule. More detailed mapping indicated that the majority of the m^6A residues (61%) are localized within a very short sequence of the 3′ portion of the molecule. This sequence corresponds to 129 nucleotides of the extreme 3′ end of the protected RNA. Since this region includes 21 A residues as part of the poly(A) tail of the message, it was important to demonstrate that the enrichment for m^6A in this region is not due to a contribution from the poly(A) tail. The fact that RNase H-treated poly(A)$^+$ RNA, when analyzed for m^6A content in bPRL, has the same level of m^6A as untreated RNA, eliminates this possibility. Thus, most of the m^6A residues in bPRL mRNA are specific to a 108-nucleotide transcribed sequence at the extreme 3′ noncoding region of the message. This region contains two ApApC sequences and one GpApC sequence out of a total of 27 possible methylation sites that are present in the bPRL message. The specificity of m^6A location in a particular region of the bPRL RNA implies that, apart from sequence constraint (i.e., ApApC and GpApC) other features of the RNA influence the location of internal methylation. We are presently interested in pinpointing the m^6A within the 108-nucleotide sequence in an attempt to find out whether the internal modification in this short sequence is site specific or specific for the 3′ end region as a whole. Once the methylation sites are identified, it will be interesting to apply site-directed mutagenesis in studies designed for further understanding the role of m^6A.

The levels of m^6A within the bPRL RNA are apparently nonstoichiometric, namely less than one per molecule. This could be due to technical limitations. As a control for the accuracy of the measurements, we applied the *in vitro* labeling/antibody-binding technique to different RNAs of known m^6A levels [e.g., HeLa cell poly(A)$^+$ RNA], and in all cases our measurements of m^6A content were in agreement with the reported values (unpublished results). Therefore, the relatively low levels of internal m^6A in the bPRL mRNA may represent the situation *in vivo*. Nonstoichiometric levels of m^6A have also been reported for cellular and viral RNAs (14, 36, 37). A reduction of m^6A levels in cytoplasmic RNA with time was also noted (38). The possibility of a cytoplasmic demethylase that would demethylate m^6A residues, as was suggested by Sommer *et al.* (38), is an attractive one and could explain the nonstoichiometric levels of m^6A in this stable message.

We have presented here a general method for mapping m^6A residues in specific mRNAs and have used it to demonstrate the location of m^6A in a specific region of bPRL mRNA. Using this approach, it will be interesting to study several other well-characterized mRNA species and look for common patterns or a consensus nucleotide sequence. Results of such a study may indicate features of the mRNA that determine methylation sites and may further our understanding of the biological function of internal methylation. The nonrandom distribution of m^6A in a specific mRNA, as demonstrated for bPRL, will have to be taken into account when designing a model for m^6A function.

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