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N^6-Methyladenosine Residues in an Intron-Specific Region of Prolactin Pre-mRNA

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N^6-methyladenosine (m^6A) residues occur at internal positions in most cellular and viral RNAs; both heterogeneous nuclear RNA and mRNA are involved. This modification arises by enzymatic transfer of a methyl group from *S*-adenosylmethionine to the central adenosine residue in the canonical sequence G/AAC. Thus far, m^6A has been mapped to specific locations in eucaryotic mRNA and viral genomic RNA. We have now examined an intron-specific sequence of a modified bovine prolactin precursor RNA for the presence of this methylated nucleotide by using both transfected-cell systems and a cell-free system capable of methylating mRNA transcripts in vitro. The results indicate the final intron-specific sequence (intron D) of a prolactin RNA molecule does indeed possess m^6A residues. When mapped to specific T_1 oligonucleotides, the predominant site of methylation was found to be within the consensus sequence AGm^6ACU. The level of m^6A at this site is nonstoichiometric; approximately 24% of the molecules are modified in vivo. Methylation was detected at markedly reduced levels at other consensus sites within the intron but not in T_1 oligonucleotides which do not contain either AAC or GAC consensus sequences. In an attempt to correlate mRNA methylation with processing, stably transfected CHO cells expressing augmented levels of bovine prolactin were treated with neplanocin A, an inhibitor of methylation. Under these conditions, the relative steady-state levels of the intron-containing nuclear precursor increased four to six times that found in control cells.

mRNA from a variety of cellular and viral sources has been shown to contain methylated constituents. In addition to the modified 5′ termini of these molecules, polyadenylated [poly(A)$^+$] mRNAs possess a limited number of internal methylated nucleosides. These methylated groups occur predominantly as N^6-methyladenosine (m^6A) although small amounts of m^5C have been identified in mRNA isolated from cultured hamster cells (12). In human (38), rat (10), and mouse (23) cells, the levels of this posttranscriptional modification have been found to be 1 to 3 residues per 1,000 nucleotides, and when mapped in individual RNA species, m^6A has in the main been located in the 3′ regions of the molecules (16, 18).

In HeLa cell mRNA (39), mouse L-cell mRNA (32), and viral RNA (11, 17, 18) the m^6A residues are found in two sequences, Gm^6AC and Am^6AC. More recent observations suggest a high frequency of methylation at $PuGm^6ACU$ sequences in Rous sarcoma virus RNA (18) and bovine prolactin (bPRL) mRNA (22). These data extend the core consensus sequence observed in mouse L cells (32), where in both heterogeneous nuclear RNA and mRNA about 65% of internal m^6A occurs in the sequence $PuGm^6ACN$, with the nucleotide N rarely being a G. The method of analysis, however, could not determine the relative frequencies of the other three bases at the N position.

To date, most studies of m^6A methylation kinetics have been conducted with viral systems. The temporal relationship between RNA processing and m^6A modification in adenovirus type 2 (9) indicates that methylation occurs very soon after transcription and that these methylated nucleosides are conserved during processing. Furthermore, a role for m^6A in nuclear processing events of viral RNA has been suggested following the precise localization of m^6A in Rous sarcoma virus (18). In that molecule, the modified residues were found bracketing the *src* splice acceptor site and therefore may influence in some way the accuracy and/or efficiency of processing. Methylation of internal adenosine residues of eucaryotic cell RNA also takes place in the nucleus, as evidenced by the demonstration of m^6A in eucaryotic cell heterogeneous nuclear RNA (24). However, previous analysis indicated a conservation of m^6A residues during processing and transport to the cytoplasm, suggesting that this modification occurs mainly within exon regions (19). It was therefore of great interest to determine whether m^6A occurs in intron-specific regions of eucaryotic precursor RNA. This analysis would identify the temporal relationship between transcription, methylation, and nuclear processing and may assist in developing a concept for the role of this posttranscriptional modification in eucaryotic RNA biogenesis.

Attempts have been made to determine the influence of m^6A on processing of gene transcripts. Earlier studies with cycloleucine, an inhibitor of *S*-adenosylmethionine synthetase, suggested that m^6A affects the splicing of avian sarcoma virus genomic RNA (34) and processing of CHO cell mRNA (2). This hypothesis is supported by the similarity between the m^6A consensus sequences in both eucaryotic and viral RNA and the weakly conserved branch point sequence YNCU(G/A)AC used in intron removal (18, 27, 40). Camper et al. (5) used the mRNA methylation inhibitor *S*-tubercidinyl homocysteine to measure the effects of undermethylation on the half-life and cytoplasmic appearance of poly(A)$^+$ RNA in HeLa cells. A significant effect on processing and/or transport of undermethylated mRNA occurred with a delay in its appearance in the cytoplasm, whereas no significant alteration in the cytoplasmic half-life of undermethylated mRNA was observed. By establishing a system with elevated expression of a modified eucaryotic gene containing only one intron, studies on the influence of

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undermethylation of a single transcript are possible. Such analyses may reveal the extent to which m^{6}A is necessary in the normal maturation of cytoplasmic mRNA.

In this paper, we report the presence of m^{6}A in an intron-specific region of a precursor RNA molecule for a eucaryotic cellular gene, namely, the bPRL gene. An in vitro m^{6}A methylation system was used to predict the occurrence and location of m^{6}A methylation in this bPRL intron sequence. In this segment of RNA, the predominant site of methylation is in the sequence AGm^{6}ACU, which is the same recognition motif reported for bPRL mRNA (23) and Rous sarcoma virus genomic RNA (18). When RNA from transfected cells expressing elevated levels of bPRL transcripts was analyzed, m^{6}A was detected at the same locations in the intron-specific region. As a first approach to determining the effect of undermethylation on the nuclear processing of a single eucaryotic transcript, we analyzed nuclear steady-state levels and cytoplasmic appearance of bPRL mRNA. Cells treated with neplanocin A (NPC), an inhibitor of methylation, display decreased levels of m^{6}A in nuclear and cytoplasmic RNA, and normal biogenesis of bPRL mRNA is disturbed. There was nuclear accumulation of precursor RNA and an apparent delay in the cytoplasmic appearance of the mature bPRL mRNA.

MATERIALS AND METHODS

Plasmids and plasmid construction. The bPRL minigene (bPRLMINI), containing the first four exons of the prolactin gene as cDNA, the entire fourth intron (intron D), and the final exon, together with 250 base pairs (bp) of 3′-flanking sequence was composed in the vector pBSM13(+) (Stratagene, Inc.) as a *Bam*HI-*Eco*RI fragment (pBSPRLMINI) (Fig. 1A). This minigene was also cloned in the mammalian cell expression vector pSV2dhfr (35) as a *Bam*HI-*Sal*I fragment following modification of the unique *Eco*RI site in the vector to a *Sal*I site by using synthetic linkers. Transcription of the prolactin sequences in this context was directed by a 760-bp *Sau*3A fragment of DNA containing the promoter-regulatory region of the cytomegalovirus major immediate-early gene (36). The start of transcription is 15 bases 5′ to the normal start site of the prolactin gene.

For hybrid selection of the intron D-specific region of the nuclear precursor RNA, an *Sph*I site was engineered by oligomutagenesis (41) 1 base 3′ to the splice acceptor site of exon 5 (Fig. 2) such that the entire intron from the *Bal*I site of exon 4 to the new *Sph*I site was present in an M13 vector (Fig. 1A). The reverse complement of this sequence was also obtained for use as a control. Chain termination sequencing (29) of these constructs confirmed the sequence of the intron region (R. G. Goodwin, D. F. Ayers, and S. M. Carroll, unpublished data) (Fig. 2). Plasmids were propagated in *Escherichia coli* DH5α, except when restriction at the *Bal*I site was required, and then the *dam dcm* strain GM119 (American Type Culture Collection, Rockville, Md.) was used because of the methylation sensitivity of *Bal*I. M13 plasmids were manipulated in *E. coli* JM105 and JM107. Unless otherwise indicated, all nucleic acid techniques, DNA preparation techniques, and transformation techniques were standard ones (20).

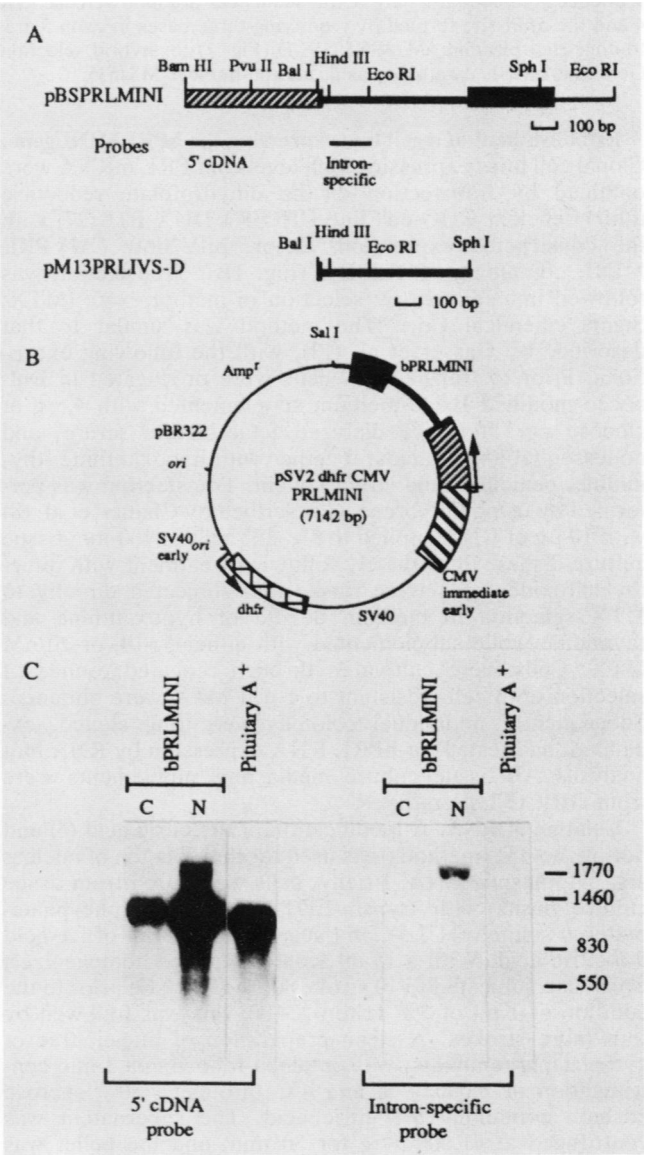

FIG. 1. Schematic outline of the modified bPRL gene pBSPRL MINI, the hybrid selection plasmid pM13PRLIVS-D, and the expression vector pSV2dhfr CMVPRLMINI, together with RNA blot analysis of the CHO 20-16 cell line expressing this gene. (A) pBSPRLMINI. Prolactin cDNA sequence (▨) from pBRPRL72 extending from the RNA cap site to the *Bal*I site at the 3′ terminus of exon 4 (30) was linked to the *Bal*I-*Eco*RI fragment from bPRL genomic clones (■) (Goodwin et al., unpublished data) such that the intron D plus the final exon and 250 bp of 3′-flanking sequence were delineated as a *Bam*HI-*Eco*RI restriction fragment of 1,766 bp in the cloning vector, pBSM13(+). pM13PRLIVS-D, the hybrid selection plasmid, constructed following generation of an *Sph*I site by oligonucleotide mutagenesis at the 3′ end of intron D (Fig. 2). (B) Structure of the expression vector pSV2dhfr CMVPRLMINI used to generate the CHO 20-16 cell line. The dhfr cDNA (⊠) is transcribed by the SV40 early promoter, and the bPRLMINI gene is transcribed by the CMV immediate early promoter (▨). The arrows indicate the start and direction of transcription of the prolactin and dhfr transcripts. (C) RNA blot analysis of 2 μg of poly(A)$^+$ cytoplasmic and nuclear RNA from CHO 20-16 cells expressing the bPRLMINI gene (lanes bPRLMINI C and N, respectively) and 500 ng of bovine pituitary cytoplasmic poly(A)$^+$ RNA (lanes Pituitary A$^+$). The left panel was probed with a 5′ cDNA probe (panel A), and the right panel was probed with an intron-specific probe (panel A). The positions of RNA size markers are indicated on the right.

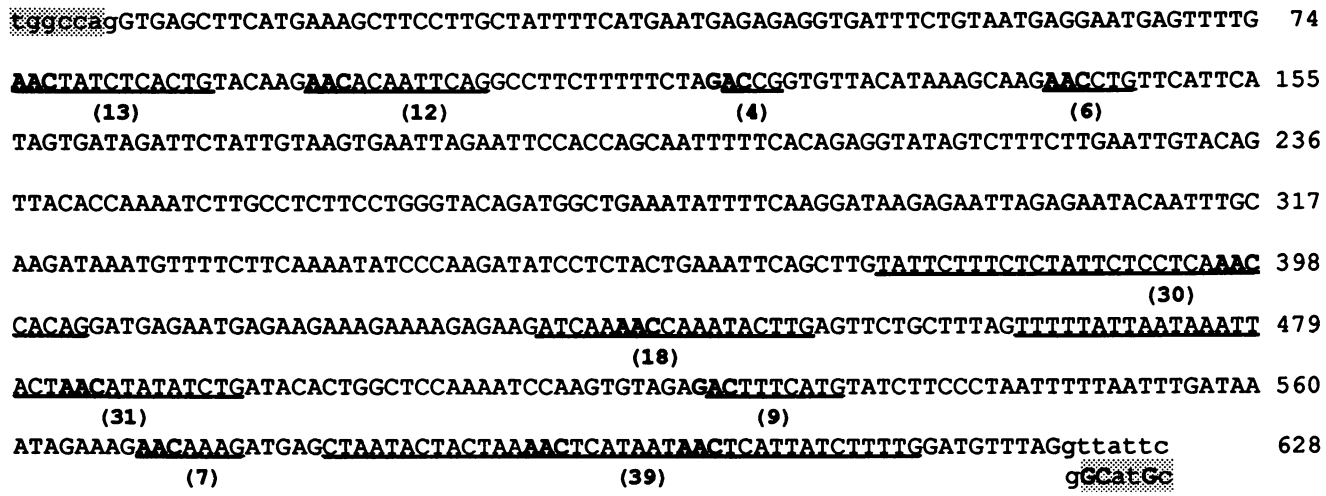

FIG. 2. Nucleotide sequence and predicted T_1 oligonucleotides of the final intron (intron D) of the bPRL gene. The nucleotide sequence of intron D (R. G. Goodwin, D. F. Ayres, and S. M. Carroll, unpublished data) shows some flanking exon sequence together with those T_1 oligonucleotides which contain putative m^6A consensus sequences (underlined with consensus sequences in boldface type). The intron sequence is in capital letters; exon sequences are in lowercase letters. Numbering refers to the intron sequence only. The *BalI* site in exon 4 and the *SphI* site formed by modifying three bases in exon 5 are boxed (modified bases are in boldface type). These two sites were used to construct plasmid pM13PRLIVS-D (Fig. 1) for hybrid selection of the intron-specific region. This sequence has been submitted to the GenBank/EMBL data bases as accession number M34535.

Establishment of a cell line expressing the bPRLMINI gene. Clonal cell lines expressing high levels of bPRL mRNA were obtained by transfection of the dihydrofolate reductase (dhfr)-deficient CHO cell line CHO K1 DUX B11 (37) with the constructed expression vector, pSV2dhfr CMVPRL MINI, containing bPRLMINI (Fig. 1B). Transfection was followed immediately by selection in methotrexate (MTX; Sigma Chemical Co.). The method was similar to that described by Gasser et al. (13), with the following exceptions. Prior to transfection, cells were propagated in Dulbecco modified Eagle medium supplemented with 4.5 g of glucose per liter, 5% dialyzed fetal bovine serum, and nonessential amino acids, together with hypoxanthine, thymidine, penicillin, and streptomycin. Transfection was performed by using Polybrene as described by Chaney et al. (8) with 10 μg of DNA applied to 5×10^5 cells in 100-mm tissue culture dishes. Immediately following treatment with dimethylsulfoxide, cells were rinsed and subjected directly to MTX selection in medium devoid of hypoxanthine and thymidine while supplemented with either 5, 10, or 20 nM MTX. Cells were cultivated through repeated rounds of selection until cells resistant to 1 μM MTX were obtained. Subsequently, individual colonies were ring cloned, expanded, and tested for bPRL RNA expression by RNA blot analysis. All tissue culture media and supplements were from GIBCO Laboratories.

Isolation of RNA. A modification of the citric acid (4) and hot-phenol (31) methods was used for the isolation of nuclear and cytoplasmic RNA. Briefly, cells were lifted from tissue culture flasks with trypsin-EDTA, rinsed in phosphate-buffered saline (pH 7.5), and suspended in 9 ml of ice-cold 5% citric acid. With a 15-ml Kontes Dounce homogenizer, cells were subjected to 10 strokes of the A pestle prior to the addition of 1 ml of 5% Triton X-100; this was followed by four more strokes. A clean preparation of nuclei, free of cytoplasmic remnants, was obtained following a 3-min centrifugation at 2,000 × *g* and 4°C through a 30% sucrose cushion containing 5% citric acid. The supernatant was centrifuged at 41,000 × *g* for 30 min, and the pellet was rinsed briefly in ice-cold 0.05 M NaCl–0.01 M sodium acetate buffer (pH 5.0) and then dissolved in 5 ml of 0.01 M Tris hydrochloride (pH 7.4)–0.1 M NaCl–0.001 M EDTA–1.5% sodium dodecyl sulfate. Following extraction with phenol-chloroform (1:1), RNA was ethanol precipitated.

Nuclear RNA was obtained from nuclei that were rinsed briefly in cold 0.05 M NaCl–0.01 M sodium acetate (pH 5.0), resuspended in 27 ml of this buffer, transferred to chilled screw-top Erlenmeyer extraction flasks, and lysed by the addition of 3 ml of 10% sodium dodecyl sulfate. Protein and DNA were extracted after 30 ml of phenol-water (90:10) at 68°C was added and the flask was shaken vigorously until the viscosity dropped. Following intermittent shaking for 3 min at 68°C, the mixture was frozen in a methanol–dry-ice bath, thawed, and centrifuged. The supernatant was subjected to two additional extractions, with the volumes of phenol being reduced to 20 and then to 15 ml. Finally, the aqueous layer was extracted with an equal volume of buffer-saturated phenol-chloroform (1:1) prior to ethanol precipitation of the RNA. Poly(A)$^+$ RNA was selected on oligo(dT)-cellulose, eluted, and precipitated by standard methods (20).

Analysis of RNA from clonal lines. Nuclear and cytoplasmic poly(A)$^+$ RNAs were separated on 1.2% agarose–6% formaldehyde gels and transferred to Zetaprobe membrane (Bio-Rad Laboratories). The RNA was hybridized with either cDNA-specific or intron-specific probes (Fig. 1A). Filters were then washed at high stringency (0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate, and 65°C) and analyzed by autoradiography.

In vitro methylation of RNA, HPLC, and T_1 oligonucleotide analysis. Transcripts of bPRLMINI RNA were generated by using the T7 polymerase-catalyzed transcription of the pBSPRLMINI plasmid (Fig. 1A). The plasmid was linearized with *SalI*, and capped RNA was transcribed in a reaction containing a fivefold excess of G_1 cap (m^7GpppG_m; Pharmacia, Inc.) and low levels of [α-^{32}P]UTP and [^{14}C]UTP or in the absence of radiolabeled nucleoside triphosphates. Unlabeled RNA (2 μg) was methylated by using *S*-[^{3}H]

adenosylmethionine in an in vitro methylation system consisting of a HeLa cell nuclear extract optimized for methyltransferase activity. This system has been shown previously to methylate accurately a single adenosine residue within the mature bPRL mRNA (22). Following incubation, the reaction mixture was treated with proteinase K, extracted with phenol-chloroform (1:1), and precipitated with ethanol.

Intron-specific RNA was hybrid selected on nitrocellulose filters to which M13 DNA containing the complementary strand to the intron in plasmid pM13PRLIVS-D (Fig. 1A) had been bound (21). Following hybridization, filters were treated with RNase T_1 to remove unhybridized RNA and washed and the RNA was eluted (22). A single intact RNA molecule of 632 bases was isolated, corresponding to the entire intron D segment of bPRLMINI. This was determined by the parallel reaction of RNA labeled by incorporation of [α-^{32}P]UTP (data not shown). High-pressure liquid chromatography (HPLC) analysis of the selected RNA was performed to resolve individual methylated constituents of RNA (1, 5).

T_1 oligonucleotide analysis of the [^{3}H]methyl-labeled intron D RNA to identify the exact residues possessing the modification was carried out. Methylated (^{3}H-labeled) and unmethylated (^{14}C-labeled) intron-specific RNAs were digested separately with RNase T_1. Resulting T_1 oligonucleotides were separated by polyacrylamide gel electrophoresis, transferred to Zetaprobe membrane, treated with En^{3}Hance (Du Pont Co.), and analyzed by fluorography as described previously (22). The exact sizes of the oligonucleotides generated were determined by comparison with an RNA ladder generated by alkaline hydrolysis of 3'-end-labeled RNA and analyzed in parallel (data not shown).

Methylation of bPRL intron D-specific RNA derived from CHO 20-16 cells. Methylated residues in the intron D sequence of bPRLMINI precursor RNA from transfected cells were identified by analyzing individual bases of specific T_1 oligonucleotides of intron D RNA isolated from CHO 20-16 cells, in a manner described previously (22). Briefly, nuclear precursor RNA was extracted from CHO 20-16 cells and prolactin intron D-specific RNA was hybrid selected. T_1 oligonucleotides were separated by polyacrylamide gel electrophoresis, and the positions of the unlabeled oligonucleotides generated by similar treatment of in vitro-transcribed RNA. The oligonucleotides were eluted from the gel (15), hydrolyzed to mononucleotides, and labeled at the 5' position with [γ-^{32}P]ATP and polynucleotide kinase. The 5' monophosphates resulting from P_1 nuclease treatment were then analyzed by two-dimensional thin-layer chromatography as described previously (16, 22).

Radiolabeling of CHO 20-16 cells. To determine the optimal level of NPC required for reducing the methylation of nuclear RNA, we labeled CHO 20-16 cells with L-[*methyl*-^{3}H]methionine as described previously (5, 22). Cells were grown to 75% confluence in the absence of MTX and transferred to methionine-free Dulbecco modified Eagle medium containing 5% dialyzed fetal calf serum. This medium was supplemented with 20 μM adenosine, 20 μM guanosine, and 20 mM sodium formate to prevent labeling of the purine ring (1). At 1 h, NPC was added to give a final concentration of either 10 or 100 μM. At 5 h, L-[*methyl*-^{3}H]methionine (70 to 85 Ci/mmol; ICN Radiochemicals) was added at 0.5 mCi/ml and cells were rocked gently to ensure even distribution of the medium during incubation at 37°C for an additional 4 h. The cells were rinsed thoroughly with medium and then with phosphate-buffered saline before being subjected to nuclear and cytoplasmic RNA extraction as described above. Poly(A)$^+$ RNA was prepared, and its methylation pattern was determined by HPLC analysis as described above.

The effect of NPC treatment on the appearance of bPRL mRNA in the cytoplasm was determined by pulse-labeling experiments conducted as follows. CHO 20-16 cells were grown until nearly confluent in 100-mm culture plates. The cells were maintained for 20 h prior to pulse labeling in nonselective medium containing [^{14}C]uridine (450 to 540 mCi/mmol; Amersham Corp.) at a low concentration (180 nM) to obtain steady-state labeling of the mRNA fraction. This isotope was replenished at 8 and at 5 h prior to pulse labeling. Also at 5 h prior to pulse labeling, the medium was replaced with methionine-free medium containing 10 μM NPC. Following a 1-h incubation, the medium was made 50 μM for methionine, and incubation continued for 4 h before pulse-labeling by the addition of [^{3}H]uridine (50 mCi/ml, 50 Ci/mmol; ICN) to give a final concentration of 4 μM. At various times after this addition of uridine (0, 5, 15, and 30 min), the medium was removed and the cells were rinsed with ice-cold phosphate-buffered saline and extracted to yield cytoplasmic RNA by using Nonidet P-40 lysis buffer and vanadyl ribonucleoside complexes (20). The cytoplasmic fraction was treated with proteinase K and extracted with phenol-chloroform before ethanol precipitation. Poly(A)$^+$ RNA was selected on oligo(dT)-cellulose (20). BPRL mRNA was hybridized to filters to which single-stranded M13 DNA containing the complementary sequence to the full-length bPRL mRNA had been bound (21). Hybridization was performed for at least 20 h, filters were washed at high stringency (0.1× SSC, 0.5% sodium dodecyl sulfate, and 65°C) and air dried, and radioactivity was counted for 5 min in the presence of Ecoscint A (Du Pont, NEN Research Products) scintillation cocktail.

S1 analysis of bPRL precursor RNA. Total nuclear RNA was isolated from cells either untreated or treated with 10 μM NPC for 8 h. The RNA was hybridized to a 5'-end-labeled cDNA fragment corresponding to the *Eco*RI-*Sph*I sequence in Fig. 5. Preparation of the probe required subcloning this fragment into polylinker sites of pBSM13(+), restricting the plasmid with *Sph*I, and treating it with T4 DNA polymerase in the presence of only dCTP, thereby producing a 9-base 5' overhang. This 5' end was then dephosphorylated with calf intestinal alkaline phosphatase, and the fragment was labeled with [γ-^{32}P]ATP and polynucleotide kinase. Restriction at the *Pvu*II site in the vector released a fragment of 870 bp, which was gel purified prior to use as a probe in hybridization. RNA was hybridized for 16 h at 39.5°C in a 10-μl volume as described elsewhere (6). After digestion with S1 nuclease (100 Vogt units for 1 h), protected DNA fragments were separated on a 6% polyacrylamide denaturing gel and analyzed by autoradiography.

RESULTS

In vitro methylation at specific locations of intron D-specific RNA. We have previously described an in vitro system in which accurate methylation of internal adenosine residues in bPRL mRNA was observed (22). In the present study we have analyzed an intron-specific region of bPRL sequence for N^6-methyladenosine modifications. A capped in vitro transcript of 1,766 bases corresponding to exons 1 to 4 of the bPRL gene, intron D, and the final exon and 250 bases of 3'-flanking sequence was used as a substrate in this in vitro system (Fig. 1A). Following in vitro methylation in a HeLa cell nuclear extract, the presence of methylated nucleosides

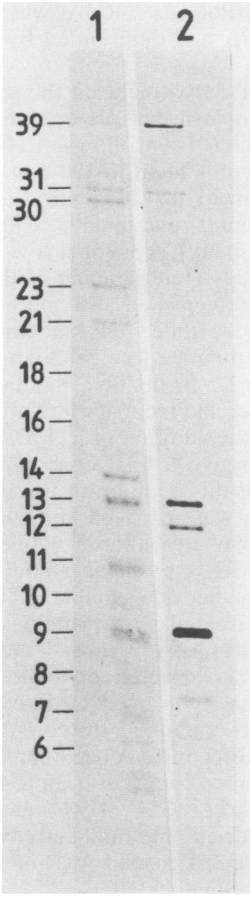

FIG. 3. Prolactin RNAs transcribed in vitro by using the T7 promoter of the construct pBSPRLMINI (Fig. 1) were either unmethylated (^{14}C labeled) or methylated (^{3}H labeled) by a cell-free methylation reaction. The intron D-specific RNAs were hybrid selected and digested separately with RNase T_1. The resultant oligonucleotides were separated by electrophoresis on a 15% polyacrylamide gel under denaturing conditions, transferred to Zetaprobe, treated with En^{3}Hance, and analyzed by fluorography. Lanes: 1, unmethylated RNA; 2, methylated RNA. Size markers were determined by comparison with an RNA ladder generated by alkaline hydrolysis of 3'-end-labeled RNA (not shown).

in the hybrid-selected intron region was determined by HPLC analysis. The elution profile indicated that between 85 and 90% of the radioactive methyl groups were incorporated into internal adenosine residues as N^6-methyladenosine. No other base modifications were observed for this segment of RNA (data not shown).

Following the discovery of m^6A in this intron-specific region, it was necessary to determine whether this modification was restricted to specific locations within the intron sequence. By digesting the intron-specific RNA with RNase T_1, a diagnostic pattern of oligonucleotides was obtained. The distribution of T_1 oligonucleotides expected from this analysis was verified by analysis of unmethylated RNA labeled with [^{14}C]UTP (Fig. 3, lane 1). The differential intensity of the bands reflects both the number of oligonucleotides of that specific size and the number of labeled bases in those individual oligonucleotides.

Within the 628 nucleotides of the intron-specific region, there exist 11 methylation consensus sequences of either AAC or GAC contained in 10 unique T_1 oligonucleotides (locations and sizes are shown in Fig. 2). When the T_1 oligonucleotides obtained from in vitro methylated RNA (^{3}H labeled) were analyzed as described above, a predominant band corresponding to the 9-base oligonucleotides was observed (Fig. 3, lane 2). The intron sequence contains two 9-base T_1 oligonucleotides, only one of which possesses an m^6A methylation consensus sequence, and this is located approximately 100 bases from the 3' splice site (Fig. 2). The 12-, 13- and 39-base oligonucleotides gave much weaker signals, and upon longer exposure of the autoradiograph, extremely weak signals were observed for the 6-, 7-, 18-, 30-, and 31-base oligonucleotides. However, no signal was observed for the 4-base oligonucleotide. Thus, 9 of the 10 oligonucleotides which contain methylation consensus sites were methylated to some degree in this reaction. The variation in methylation efficiency may be due in part to different primary sequence surrounding the consensus sites.

Determination of m^6A in intron D-specific RNA derived from CHO 20-16 cells. To determine whether the methylation observed in the in vitro system is an accurate reflection of the in vivo situation, an attempt was made to demonstrate methylation of the intron D sequence of bPRL precursor in vivo. However, the transcription rate of the bPRL gene in endogenous tissue is very low (6), and it is not possible to obtain adequate amounts of a specific pre-mRNA to analyze for m^6A. Therefore, stably transfected cell lines were established by using the mammalian cell expression vector pSV2dhfr CMVPRLMINI (Fig. 1B). Transfectants were selected directly in MTX, and gene amplification was achieved by application of increasing concentrations of MTX. Cell line CHO 20-16 was chosen on the basis of (i) high levels of expression of bPRL precursor RNA and mRNA as determined by RNA blots with intron-specific and cDNA-specific probes, respectively (Fig. 1); (ii) production of the prolactin hormone as determined by an enzyme-linked immunosorbent assay (data not shown); and (iii) normal CHO cell growth characteristics. Cells were maintained in 1 μM MTX until 48 h prior to RNA extraction, at which time MTX was omitted. For precursor RNA analysis, 2×10^8 CHO 20-16 cells were harvested, nuclear RNA was prepared, and intron D-specific RNA was hybrid selected. This RNA was digested with RNase T_1 and separated by polyacrylamide gel electrophoresis in parallel with ^{32}P-labeled synthetic RNA corresponding to intron D. Oligonucleotides of 9, 13, 21, 23, and 39 bases were extracted from the gel, and component nucleotides were analyzed by thin-layer chromatography. Oligonucleotides of 9 bases did indeed contain m^6A residues (Fig. 4, top), whereas the 21-base oligonucleotide, lacking a consensus site, revealed the absence of this modification (Fig. 4, bottom). In addition, the 13- and 39-base oligonucleotides possessed m^6A residues; however, the level was substantially lower than that of the 9-base oligonucleotide. The 23-base oligonucleotide, also lacking a consensus site, did not contain detectable levels of m^6A. To estimate the level of m^6A in the 9-base oligonucleotide, areas corresponding to m^6A and A were removed and the radioactivity was counted. By this method it was calculated that 24% of the consensus site A residues in the 9-base oligonucleotide were modified.

To analyze the methylation pattern of mature-form mRNA from the bPRLMINI gene, we examined the m^6A content and location in the 3' terminus of prolactin mRNA (data not shown). The only consensus sequence found to be methylated was the same T_1 hexanucleotide reported to be methylated in this segment of pituitary-derived bPRL mRNA (22). Therefore, the presence of an adjacent intron sequence

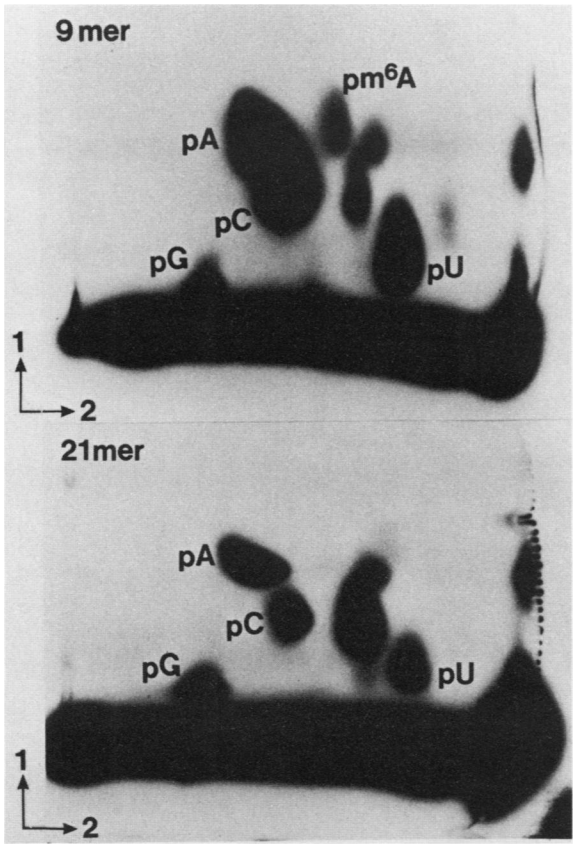

FIG. 4. Analysis of T_1 oligonucleotides from intron D-specific RNA isolated from CHO 20-16 cells. Oligonucleotides produced by RNase T_1 treatment of hybrid-selected intron D RNA isolated from nuclear precursor RNA produced in stably transfected CHO 20-16 cells were separated by electrophoresis on a 15% polyacrylamide–7 M urea gel. Following elution from the gel, hydrolysis to mononucleotides, 5′ labeling with [γ-^{32}P]ATP, and removal of 3′ phosphates, the 5′ monophosphates were analyzed by two-dimensional thin-layer chromatography. Analyses of the 9-base oligonucleotide (9mer; upper panel) and 21-base oligonucleotide (21mer; lower panel) are shown. Abbreviations: pA, AMP; pG, GMP; pU, UMP; pC, CMP; pm⁶A, N^6-methyladenosine-5′-monophosphate.

in the minigene construct had no apparent effect on the methylation pattern of the exon sequence.

Influence of NPC on CHO 20-16 nuclear RNA methylation. The methylation inhibitor NPC is a structural analog of adenosine. It has been demonstrated to affect RNA methylation (14) and has been shown previously to markedly lower m⁶A and 2′-*O*-methylnucleoside levels in cellular cytoplasmic mRNA from CHO cells (26). Prior to examining perturbations of nuclear processing due to undermethylation, we determined the optimal level of NPC for reducing the methylation of nuclear RNA. Cells were treated with either 0, 10, or 100 μM of NPC for 8 h, during which time RNA transcripts were labeled with L-[*methyl*-^{3}H]methionine. Nuclear and cytoplasmic poly(A)$^+$ RNA was prepared and digested to nucleosides, and the methylation pattern was determined by the HPLC analysis. The level of m⁶A in RNA from each cellular compartment is expressed as a ratio of m⁶A residues per cap structure (Table 1). When cells were treated for 8 h with 10 μM NPC, the amount of internal m⁶A methylation in both the cytoplasmic and nuclear RNA fractions was greatly reduced (83 and 78%, respectively). Furthermore, there was a concurrent decrease in the level of Cap I structures, although it was more marked in the cytoplasmic fraction than in the nuclear fraction. NPC at 100 μM did not further reduce the m⁶A/cap ratio in the cytoplasmic fraction, while significantly increasing the level of Cap 0 structure. Therefore, 10 μM NPC, which resulted in approximately 80% inhibition of internal m⁶A methylation, was chosen to examine the influence of undermethylation on the nuclear processing and transport of bPRL RNA in these cells.

TABLE 1. Effect of NPC on the methylation status of poly(A)$^+$ RNA isolated from CHO 20-16 cells[a]

RNA type and NPC concn (μM)	m⁶A/cap cpm ratio[b]	Cap I/total cap cpm ratio (%)[c]
Cytoplasmic		
0	3.5	99
10	0.6	53
100	1.3	25
Nuclear		
0	2.3	74
10	0.5	68

[a] Cells were cultured in the absence or presence of NPC for 8 h and with L-[*methyl*-^{3}H]methionine for 4 h prior to RNA isolation. HPLC analysis of digestion products resolves labeled internal methylated constituents, Cap I [m^7GpppN_{1m}] and Cap 0 [m^7GpppN_1] structures.

[b] Cap counts per minute were normalized by dividing counts in each cap peak by the number of methyl groups in the residue. The m⁶A/cap ratio was determined by dividing the counts per minute in the m⁶A peak by the sum of the normalized counts per minute in all the caps.

[c] Cap I counts per minute are the sum of normalized Cap I values, including moieties from Cap II structures which resolve as Cap I + $N_{2m}p$.

Altered cytoplasmic appearance of bPRL mRNA in NPC-treated cells. Camper et al. (5) had shown that undermethylation of HeLa cell RNA delayed the appearance of poly(A)$^+$ RNA in the cytoplasm. By establishing a cell line with augmented expression of bPRL mRNA, we can now analyze the influence of undermethylation on a specific mRNA in both nuclear and cytoplasmic compartments.

Pulse-labeling experiments were conducted in which the level of newly transcribed bPRL mRNA in the cytoplasm of NPC-treated cells was measured and compared with that in control cells. Cells were labeled at low level in nonselective medium containing low levels of [^{14}C]uridine for 20 h to establish steady-state labeling of the RNA prior to pulse-labeling. At various times after the addition of high levels of [^{3}H]uridine (0, 5, 15, and 30 min), the level of incorporation of this radionuclide into bPRL mRNA was measured and amounts were standardized to the [^{14}C]uridine present in the RNA. No radioactive RNA was detected when mRNA was hybridized to the control mRNA-sense M13 clone. However, levels of ^{3}H-labeled RNA which increased with time were observed when the mRNA was hybridized to M13 DNA containing the complementary sequence. These levels were normalized based on the amount of ^{14}C-labeled bPRL RNA recovered. bPRL mRNA did not accumulate to the same level in the cytoplasm of cells treated with 10 μM NPC as in control cells (Fig. 5). After pulse-labeling for 30 min, the difference between the levels of newly synthesized transcripts present in the cytoplasm of NPC-treated cells and control cells was statistically significant ($P < 0.05$, Student's *t* test).

Undermethylation influences bPRL nuclear precursor levels. To determine whether the decreased amount of mRNA in the cytoplasm was due to an alteration in the processing of

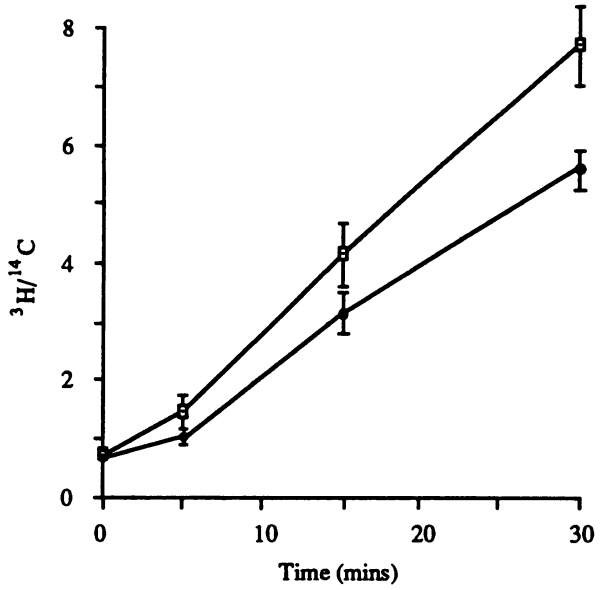

FIG. 5. Time course of newly transcribed bPRL mRNA at 0, 15, and 30 min after pulse-labeling in control cells and cells treated with NPC. CHO 20-16 cells expressing high levels of bPRL were prelabeled with 180 nM [^{14}C]uridine (450 to 540 mCi/mmol) for 20 h, treated with no (□) or 10 μM (●) NPC for 4 h, and labeled with 4 μM [^{3}H]uridine (50 Ci/mmol) without removing the NPC. At various times after the addition of [^{3}H]uridine, cells were harvested and cytoplasmic poly(A)$^+$ RNA was prepared. Specific bPRL transcripts were hybrid selected on nitrocellulose filters. The ratio of [^{3}H]uridine to [^{14}C]uridine in the cytoplasmic bPRL poly(A)$^+$ RNA was calculated, and the results are expressed as the mean ± standard error of five experiments.

the bPRL RNA precursor, we analyzed the nuclear steady-state levels of this precursor RNA. The augmented expression of bPRLMINI in CHO 20-16 cells established a model system for this analysis: there is a high level of nuclear precursor present, the modified gene requires only one splicing event to remove the single intron, and m^{6}A has been detected within this intron-specific region.

Total nuclear RNA was prepared from cells either treated with 10 μM NPC for 8 h or untreated and subjected to quantitative S1 nuclease analysis designed to discriminate between mature-form RNA present in the nucleus and precursor RNA molecules. RNA was hybridized to a 5′-end-labeled cDNA fragment (Fig. 6). A protected fragment corresponding to the precursor molecule of 719 bases is distinguished from that corresponding to the mature-form RNA of 277 bases (Fig. 6). Autoradiograms of various intensities were prepared for analysis by laser scanning densitometry (LKB Instruments, Inc.). When the precursor band is normalized against the mature-form band, the NPC-treated cells contained four- to sixfold more precursor in the nucleus than did control cells.

DISCUSSION

In this study, we have determined the presence of m^{6}A in an intron-specific region of a bPRL precursor RNA. The detection of m^{6}A modification in this segment may signal an important role in the nuclear preparation of mRNAs. Furthermore, as this region of RNA will be lost during processing, m^{6}A at specific locations in precursor RNA is not conserved during maturation. Previous investigators exam-

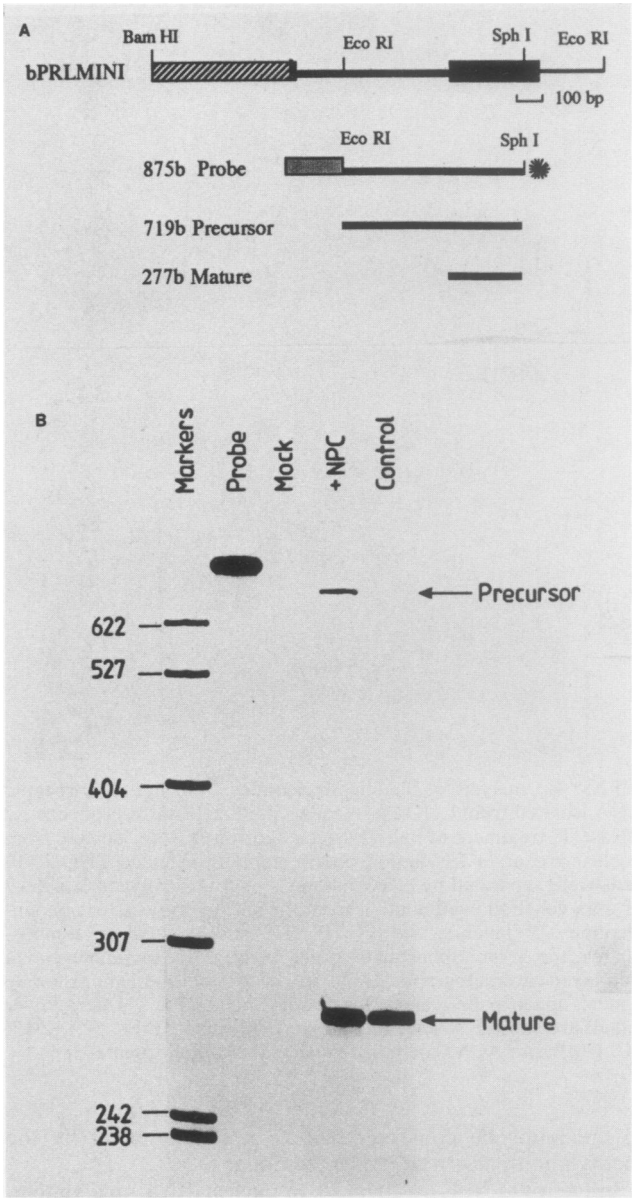

FIG. 6. Quantitative S1 nuclease mapping of nuclear bPRL precursor and mature-form RNA in cells treated with NPC. (A) Structure of bPRLMINI is the same as in Fig. 1. Structure of the 5′-end-labeled DNA fragment used as a probe in this experiment. The probe extends from the *Sph*I site in the final exon (■) through to the *Eco*RI site of the intron (▬) and contains the cloning vector sequence (▦). The predicted protected fragments are shown for the precursor (719 bases) and the mature-form (277 bases) RNAs. (B) Autoradiogram of a gel showing the DNA fragments protected by 5 μg of total nuclear RNA isolated from cells treated with 10 μM NPC for 8 h (lane +NPC) and untreated cells (lane Control). Other lanes display are as follows: Markers, end-labeled *Hpa*II-cut pBR322 with sizes indicated; Probe, 10% of input amount of probe not treated with S1 nuclease; Mock, probe hybridized in the absence of RNA and digested with S1 nuclease. This experiment was repeated twice, and autoradiograms of various intensities were subjected to laser-scanning densitometry.

ining viral RNAs have shown m^{6}A in early and late transcripts of simian virus 40 (7), genomic RNA of Rous sarcoma virus (3), adenovirus type 2 nuclear and cytoplasmic RNA (9), and influenza virus mRNAs (21). The detailed kinetic studies of m^{6}A in adenovirus nuclear and cytoplasmic RNAs demonstrated m^{6}A conservation during mRNA formation (9, 33). When the content of m^{6}A in heterogeneous nuclear RNA and mRNA of HeLa cells was analyzed, the poly(A)$^+$ cytoplasmic RNA was found to contain threefold higher levels of m^{6}A per RNA molecule than was heterogeneous nuclear RNA larger than 28S. However, when nuclear poly(A)$^+$ RNA was examined, levels similar to those in cytoplasmic poly(A)$^+$ RNA were recorded, indicating that m^{6}A was most probably not present in intron sequences (19). One explanation is that each RNA molecule has a characteristic level of m^{6}A, specific for that mRNA, such that following m^{6}A loss due to intron excision, other sites may be modified to maintain this level. These individual events would be obscured by an examination of mixed mRNAs. This possibility is consistent with the nonstoichiometric nature of the modification at specific locations within a given mRNA and the variation in m^{6}A levels between mRNAs. For example, globin mRNA is reported to be devoid of m^{6}A (25), whereas dhfr mRNA is heavily modified (28).

Both in vivo and in vitro analyses were used to detect the presence of m^{6}A in an intron-specific region of the bPRL precursor RNA. To examine the in vivo occurrence of m^{6}A, elevated levels of precursor were obtained by overexpression of the bPRLMINI gene in cells transfected with the expression vector pSV2dhfr CMVPRLMINI (Fig. 1B) and treatment with MTX to force gene amplification. This strategy was required to obtain adequate levels of both nuclear precursor and mature cytoplasmic prolactin mRNA. The intron-specific region was hybrid selected from nuclear RNA and specific T_1 oligonucleotides analyzed for base composition analysis. Indeed, the results showed that the 9-base oligonucleotides contained m^{6}A residues. Within the selected intron region, there exist two 9-base oligonucleotides, only one of which possesses an m^{6}A consensus sequence. As reported previously, the m^{6}A methyltransferase shows remarkable sequence specificity, such that m^{6}A occurs only at two consensus sites, namely, Am^{6}AC and Gm^{6}AC (11, 18, 32, 39). Comparison of m^{6}A and A levels in the 9-base oligonucleotides revealed that approximately 24% of adenosine residues in the 9-base oligonucleotide containing the consensus sequence exist as m^{6}A. Therefore, methylation at this site is nonstoichiometric. Furthermore, the methyl addition found in both the 13- and 39-base oligonucleotides is nonstoichiometric and corresponds to that reported for bPRL mRNA (22) and viral RNAs (18, 21). One of the alternatives which may give rise to this nonstoichiometry is that the methyltransferase is influenced by the sequence context in which the consensus motif is located. On the other hand, if a demethylase exists and all sites are modified, certain sequences may be more refactory to its actions, resulting in varying levels of methylation.

The limited distribution of m^{6}A in only a subset of intron-containing consensus sequences was predicted by analysis of the methylation of the same sequences with an in vitro methylating system capable of forming m^{6}A at specific adenosine residues in synthetic transcripts (22). This system, which was shown previously to faithfully reflect the m^{6}A methylation pattern in mature mRNA, has now been shown to methylate adenosine residues in intron sequences identical to those observed in the CHO cells that express the minigene construct. Analysis of this intron-specific sequence

TABLE 2. Local base composition surrounding putative m^{6}A consensus sites in the intron-specific region of RNA[a]

No. of bases in T_1 oligonucleotide	Consensus site region	Relative intensity of *methyl*-^{3}H-labeled oligonucleotides
9	AGA GAC UUU	100
13	UUG AAC UAU	38
39 (i)	UAA AAC UCA	20
39 (ii)	AAU AAC UCA	
12	AAG AAC ACA	19
7	AAG AAC AAA	8
6	AAG AAC CUG	6
31	ACU AAC AUA	<5
18	CAA AAC CAA	<5
30	UCA AAC CAC	<5
4	CUA GAC CGG	ND[b]

[a] Laser-scanning densitometry was used to record relative intensities of the various T_1 oligonucleotide bands shown in Fig. 3.
[b] ND, Not detected.

demonstrated that virtually all the [^{3}H]methyl groups occurred as m^{6}A and that the bulk of these modifications were targeted to a single 9-base oligonucleotide. To a much lesser extent, in vitro modification occurred at other consensus sites contained within the 12-, 13-, and 39-base oligonucleotides.

Closer study of the consensus site context which serve as substrates for this methylation reaction may assist in establishing a priority of flanking bases which influence methyltransferase activity. Table 2 lists the local base composition of the consensus sites contained in the RNA fragment of this study. By using laser scanning densitometry, the intensity of each of the methylated oligonucleotides relative to the 9-base oligonucleotide (Fig. 3) has been ascertained. It can be seen that earlier observations of a PuGACU sequence being more frequently methylated are confirmed (18, 32). By using this small set of consensus sites, a hierarchy for the methylation sites is predicted. In the sequence Pu(G/A)ACN, the presence of a cytidine residue at N negatively influences the methyltransferase activity, whereas adenosine and uridine at this position show a positive effect. It is also possible that the presence of cytidine at the N position overrides the strong effect of the G in the consensus core region GAC, as suggested by the absence of signal in the 4-base oligonucleotide (with consensus site PuGACC).

Therefore, we have detected the presence of m^{6}A in an intron-specific region of eucaryotic RNA precursor molecule. Moreover, the use of an in vitro methylation system has predicted correctly the presence of this modification in both the intron region of the precursor and the mature mRNA (22). With the aid of this system, it should now be possible to systematically analyze sequences that are important for methylation of adenosine residues.

By using the methylation inhibitor NPC, the effect of undermethylation on the biogenesis of bPRL RNA can be investigated. NPC reduces m^{6}A and 2′-*O*-methylnucleoside levels in cellular cytoplasmic mRNA fractions (26), and we have now shown that nuclear RNA also has reduced m^{6}A levels with inhibitor treatment. When CHO 20-16 cells were exposed to low levels of NPC, newly synthesized bPRL mRNA accumulated to a significantly lower level in the cytoplasm of the inhibitor-treated cells than in control cells. In addition, a four- to sixfold increase in steady-state nuclear levels of the precursor relative to mature-form mRNA was observed in NPC-treated cells. As with most inhibitor-based studies, consideration must be given to pleiotropic effects

and to lack of specificity of this methylation inhibitor. Studies have shown that the level of NPC treatment used here does not influence RNA synthesis in HT-29 carcinoma cells (14), and both RNA and protein syntheses are not affected in CHO cells (26). Effects on other cellular processes cannot be ruled out completely. Nevertheless, these results clearly indicate that conditions which give rise to undermethylated mRNA sequences are also accompanied by nuclear accumulation of precursor RNA. The increase in steady-state bPRL precursor RNA levels may be due to an alteration in the efficiency of splicing or polyadenylation or both. In bPRL mRNA, the major site of the methylation is at the 3′ end, between the consensus hexanucleotide and the site of cleavage and polyadenylation (22). The influence of undermethylation on the accuracy and efficiency of polyadenylation has not been determined. Our current analysis is unable to define the exact mechanism by which undermethylation increases precursor levels. However, the demonstration that only a unique set of potential methylation sites are utilized within the intron sequence strengthens the hypothesis that m^6A may play a role in nuclear processing of bPRL mRNA.

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